Neonatal encephalopathy: pre-clinical studies in neuroprotection

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Abstract
Neonatal encephalopathy resulting from HI (hypoxia–ischaemia) continues to be a significant cause of mortality and morbidity in infants and children, affecting 1–2/1000 live term births and up to 60 % of pre-term births. In order to understand the pathophysiology of this insult, as well as design therapeutic interventions, it is important to establish a relevant animal model for pre-clinical studies. One of the most frequently used models of HI-induced brain damage in immature animals is the unilateral carotid ligation/hypoxia model, initially developed in our laboratory more than 30 years ago. The original model employed the postnatal day 7 rat, whose brain is representative of that of a late gestation, pre-term [32–36 weeks GA (gestational age)] human infant. We, and others, have employed this model to characterize the pathophysiological, biochemical/energetic and neuropathological events following HI, as well as the determination of the unique characteristics of the immature brain that define its vulnerability to, and outcome from, HI. In defining the cascade of events following HI, it has become possible to identify potential targets for intervention and neuroprotection. Currently, the only available therapeutic intervention for neonatal encephalopathy in the term asphyxiated infant is therapeutic hypothermia, although this must be initiated within 6 h of birth and is at best partially effective in moderately injured infants. Ongoing pre-clinical studies are necessary to determine the basis for the partial protection afforded by hypothermia as well as the design of adjunct therapies to improve the outcome. The present review highlights the importance of using a well-characterized and relevant animal model to continue to pursue translational research in neuroprotection for the infant brain.

Introduction
Perinatal asphyxial brain damage is a major cause of acute mortality and chronic neurological morbidity in infants and children. The resulting HIE (hypoxic–ischaemic encephalopathy) affects 2–4/1000 full term births [1,2]. Between 20 and 50 % of asphyxiated newborns with HIE die within the newborn period, and up to 25 % of the survivors will exhibit permanent neuropsychological handicaps, including mental retardation, cerebral palsy, epilepsy or learning disability. Care of the fetus and newborn infant at risk of HIE is a high priority in current healthcare, and an understanding of the pathophysiology of perinatal HIE is essential to the design of neuroprotective strategies needed to both slow the evolution of the damage and promote repair to support normal development. A tremendous amount of progress has been made in the development of MRI neuroimaging and MRS (magnetic resonance spectroscopy) analysis of the newborn brain which has shed light on structural and metabolic aspects of the evolution of HIE (reviewed in [3]). However, the utilization of relevant pre-clinical animal models continues to be essential to the development of neuroprotective studies. The present review discusses the essential components in the development of relevant animal models, with specific discussion of the unilateral carotid ligation/hypoxia model of HI (hypoxia–ischaemia) in immature rats [4]. This model has been used in our laboratory and others to pursue a variety of translational studies in neuroprotection.

Key words: cerebral energy failure, hypoxia–ischaemia, mast cell, perinatal asphyxia.
Abbreviations: CBF, cerebral blood flow; EEG, electroencephalogram; GA, gestational age; HI, hypoxia–ischaemia; HIE, hypoxic–ischaemic encephalopathy; MRS, magnetic resonance spectroscopy; P, postnatal day.
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A pre-clinical model for translational studies in neonatal encephalopathy
Successful translational research requires the use of model systems that faithfully reproduce the key components of HI/stroke pathophysiology, as discussed relative to studies of acute ischaemic stroke in adult models [5,6]. Although not specifically reviewed to date, it is clear that such requirements should apply to studies in neonatal encephalopathy as well. Commonly used experimental models for the study of neonatal encephalopathy in both pre-term and term infants involve induction of hypoxic–ischaemic, focal ischaemic or excitotoxic lesions, with or without addition of inflammation, in newborn rodents, piglets, rabbits and baboons, as well as fetal sheep [4,7–9]. The choice of which model to use depends on the particular paradigm under study; however, a thorough discussion of all of the existing models is well beyond the scope of the present review. Rather, the discussion focuses on the study of hypoxic–ischaemic injury due to birth asphyxia in the late gestation/term infant. HI of sufficient duration to cause a reduction in CBF (cerebral blood flow) to the fetal brain produces a rapid

reduction in both oxygen and glucose delivery, resulting in a depletion of cerebral high-energy phosphates, or primary energy failure, in vulnerable brain regions. Since the onset and extent of energy failure is a function of the maturational state of the brain, including metabolic rate and number of excitatory/glutamatergic neuronal synapses, the resulting pattern of brain injury will depend on the gestational age of the infant and the severity of the hypoxic–ischaemic insult. Although post-asphyxial resuscitation and reperfusion initially promotes recovery of the energy state, studies employing ^{31}P-MRS in newborn infants and experimental animal models demonstrate a biphasic pattern of energy failure, including a secondary energy failure usually occurring 6–15 h later [10,11]. The extent of the secondary energy failure has been shown to correlate with a poor neurological outcome in children at 1 and 4 years of age [12]. Thus a translationally relevant pre-clinical model should (i) replicate a transient hypoxic–ischaemic insult to a newborn animal whose extent of cerebral maturation is comparable with that of human infants at different gestational ages; (ii) replicate both a primary and secondary energy failure; (iii) result in neuropathological changes that reflect the severity of injury; and (iv) be amenable to long-term assessment.

One of the most commonly used pre-clinical models that includes these components is the immature rat model of unilateral hypoxic–ischaemic brain damage, originally developed in our laboratory [4,13,14], and also subsequently adapted to the immature mouse [15]. The methodology consists of a permanent unilateral common carotid artery ligation followed by a period of systemic hypoxia produced by inhalation of 8 % oxygen/balance nitrogen for various periods of time at a controlled temperature. The ligation of one carotid artery in itself does not affect CBF to either hemisphere, because of the Circle of Willis in the vasculature of the rat brain. However, during the hypoxic exposure, the rats become hypotensive, with a fall in mean blood pressure of 25–30 %, with a corresponding fall in CBF in the hemisphere ipsilateral to the ligation of 40–60 % of control [16]. CBF returns to normal immediately upon return to normoxia and reperfusion [17]. The brain damage that results is normally confined to the ipsilateral hemisphere and can range from mild selective neuronal necrosis to severe infarction, depending largely on the duration of the hypoxic–ischaemic interval, although there is animal-to-animal variability in the model.

Cerebral maturation in the immature rat and relation to gestational age

The immature rat model of unilateral HI, as originally described, utilized the P (postnatal day) 7 rat pup [4], and this is the age that has been most consistently studied by a variety of investigators. In the original description of this animal model, the brain of the P7 rat was roughly equated with that of a 32–36-week GA (gestational age) human infant, primarily on the basis of the degree of maturation of the germinal zone and the extent of cortical layering [4]. A number of investigators have utilized this model in postnatal animals of all ages, and the question of the correspondence between the maturation of the rat brain and the human brain is relevant to the translational value of these studies. It has been suggested that the total number of synapses in the striate cortex of the human infant at term is approximately 62 % of the adult value and that this value is similar to the P14 rat [18–20]. Patterns of cerebral lesions following hypoxic–ischaemic insults to premature and term infants, reflect dynamic and selective vulnerabilities of the newborn brain. Ischaemic/excitotoxic injury to the pre-term brain (24–32 weeks GA) is primarily in the form of PVL (periventricular leukomalacia) or DWMI (diffuse white matter injury), due to the heightened vulnerability of the early lineage oligodendrocytes, and in the rat this corresponds to P3–P6, with reduced vulnerability by P10 [7,21–23]. In contrast, a hypoxic–ischaemic injury to the term infant brain has a greater impact on deep grey nuclei such as the basal ganglia and thalamus, with little to no involvement of the white matter tracts [21]. The pattern of injury in the P7 rat brain following HI is characterized by white matter necrosis originating from myelogenic foci with a columnar pattern of necrosis in the cortex, and less involvement of the hippocampus [4], suggesting a closer correlation with a late gestation pre-term brain still within the window of vulnerability of the white matter. By P13, a hypoxic–ischaemic insult produces a laminar pattern of necrosis in the cortex similar to that seen in the adult brain, and a greatly increased vulnerability of the hippocampus [24]. Another relevant and useful parameter to consider is the developmental pattern of the electrical activity of the cerebral cortex. Romjin et al. [20] compared reports of EEG (electroencephalogram) patterns from premature and full-term human infants with several studies in the developing rat, and concluded that, before P10, the EEG pattern was more similar to that of the premature infant, with a rapid maturity of the EEG between P10 and P13, thus equating P12–P13 rat pups with full-term infants. More recently, Tucker et al. [25] studied aEEG (amplitude-integrated electroencephalography) and the longest IBI (interburst interval) daily in postnatal rat pups from P1 to P21 and concluded that at P7 the pattern reflects what is seen at 30–32 weeks GA and that, similar to the analysis of Romjin et al. [20], the P10–P12 rat is more comparable with the 40–42-week GA human infant.

Furthermore, studies from our laboratory [26] compared EEG patterns in the P7 and P12 rat showing the clear development of a more continuous pattern at the latter age with clearly identifiable electrographic seizures during HI.

Biphasic pattern of cerebral energy failure: importance of secondary energy failure to damage

The mammalian brain depends on a continual supply of both oxygen and glucose to meet its energetic demand. Hypoxia

and HI interrupt this supply and the brain is forced to switch to anaerobic glycolysis for energy generation, leading to an increase in cerebral glucose utilization reflecting the increased glycolytic demand [27]. However, the limitation on cerebral glucose utilization in the neonatal brain imposed by the low capacity for glucose transport prevents adequate generation of ATP in the hypoxic–ischaemic hemisphere, leading to energy depletion. This primary energy failure sets off a cascade of events that have been characterized in adult as well as neonatal stroke models [28,29]. In the neonatal brain, the pattern of brain injury that results from this cascade of events will depend on the timing of the initial hypoxic–ischaemic insult, i.e. the maturational state of the brain, as well as the severity. Although there is a partial restoration of cerebral energy stores in the early period of reperfusion, numerous studies in both asphyxiated infants and pre-clinical animal models, i.e. newborn piglet, have demonstrated the occurrence of a secondary depletion of cerebral high-energy compounds, or secondary energy failure, during the subsequent hours to days, as being indicative of lasting brain injury [10,11]. Clinically, the severity of the second energy failure as determined with ^{31}P-MRS has been shown to correlate with adverse neurological outcomes in children at 1 and 4 years of follow-up [12]. Such secondary depletions of high-energy phosphate compounds have been observed in the immature rat HI model [30–33] and correlate temporally with both histological evidence of brain damage and loss of the neuronal markers MAP-2 (microtubule-associated protein 2) and SNAP-25 (25 kDa synaptosome-associated protein) at both early (6–18 h) and late (24–48 h) periods of reperfusion [33]. Thus the immature rat model accurately replicates the cerebral metabolic changes consistent with hypoxic–ischaemic injury in the newborn. In addition, any experimental intervention that can be shown to prevent the occurrence of this secondary energy failure is consistent with long-term neuroprotection in this and other animal models [34,35]. One such intervention that has now been translated into standard of care for HIE clinically is therapeutic hypothermia.

Pre-clinical studies in neuroprotection: therapeutic hypothermia

Therapeutic hypothermia is currently the only post-insult intervention clinically approved to treat neonatal encephalopathy due to HI in term newborns, on the basis of results of multiple randomized clinical trials demonstrating reduced mortality and morbidity in these children [36,37]. Safety studies and clinical trials in the newborn derived from experimental studies in large animal models, demonstrating histopathological neuroprotection with moderate hypothermia [35,38]. The value of the immature rat model of HI in contributing to the experimental support of therapeutic hypothermia is in the ability to both study a larger number of animals as well as demonstrating both short- and long-term protection, including assessment of functional outcome [39]. Subsequently, multiple investigators have studied post-HI hypothermia, with and without combination therapy, in the rodent model using both rats and mice [40,41]. The results of these studies, however, have been inconsistent and have not always been able to demonstrate a significant degree of neuroprotection. Possible confounding factors might include variation in the experimental paradigm, i.e. duration of HI/severity of insult, duration between end of HI and start of hypothermia (0–6 h), degree and duration of hypothermia (3–24 h), and of course different combination therapies. In addition, all of these studies have employed the P7 model, which may actually represent a developmental stage (32–36 weeks GA) less relevant to the full-term newborn population eligible for cooling. Current studies in our laboratory have focused on the term-equivalent P10 rat and have shown significant short- and long-term protection when cooled pups achieve a rectal temperature of $\leqslant 32\,^{\circ}$C for 4 h immediately following a moderate–severe HI insult [42]. Many more pre-clinical studies are needed to further define aspects of hypothermic neuroprotection including long-term analysis of both sensorimotor and cognitive function, as well as efficacious and synergistic pharmacological interventions.

Pharmacological interventions for neuroprotection: mast cell stabilization

HI of sufficient severity to deplete cerebral energy reserves activates multiple pathways leading to ongoing cell death, including excitotoxicity, oxidative stress, apoptosis and post-ischaemic inflammation [43,44]. As information about the involvement of these pathways has evolved, they have become specific targets for therapeutic intervention, either alone in the treatment of pre-term brain injury, or more recently in combination with therapeutic hypothermia in the term infant [45]. Initially, studies focused on inflammatory events have targeted microglial activation as being pivotal in the inflammatory response; however, more recently the role(s) of other pro-inflammatory cells from the circulation (e.g. neutrophils and T-cells) have also received attention. Recent studies from several laboratories, including ours, highlighted a novel cell in the inflammatory response to HI in both the adult and the neonatal brain: the mast cell [46–49]. Relatively small numbers of mast cells are resident in the CNS (central nervous system) of several species, including humans and rats, where they are found primarily in association with cerebral blood vessels, pia and meninges [50–52]; their numbers are highest during cerebral development [52]. Mast cells contain pre-formed granules of several pro-inflammatory mediators, including TNFα (tumour necrosis factor α), hence they are well-equipped to be the first responders to an ischaemic or traumatic insult to the brain [47,49]. Using the P7 rat model of HI, studies from our laboratory demonstrated that inhibition of the early mast cell response using the mast cell stabilizer disodium chromoglycate (cromolyn) produced a significant and lasting degree of neuroprotection [46,47]. As cromolyn is an FDA (U.S. Food and Drug Administration)-approved drug, which has been used in infants and children primarily for the treatment of pulmonary disease, it may have

a new and valuable clinical application in the protection from HIE. If this intervention is effective clinically in pre-term brain damage, which is known to have a large inflammatory component [53], it could provide a clinically safe therapeutic intervention in a population not eligible for hypothermia. Current and future studies will determine the extent to which early mast cell stabilization will synergize with therapeutic hypothermia for lasting neuroprotection in the asphyxiated term newborn.

Summary

Despite great advances in obstetric and neonatal care, asphyxial insults still occur in the perinatal period, and will result in variable degrees of permanent brain damage in the survivors depending on the maturational state of the brain, location of the insult and possible intervention of hypothermia. Studies in pre-clinical animal models have not only contributed to our growing understanding of the pathophysiology of perinatal brain injury leading to neonatal encephalopathy, but have also served an essential purpose in evaluating potential neuroprotective strategies. Ongoing studies are needed to continue the search for single and combination therapies, and rigor in the application of the pre-clinical animal models is of vital importance.

Funding

This work was supported by the National Institutes of Health (NIH)/National Institute of Neurological Disorders and Stroke (NINDS) [grant numbers R211NS083425 and R03NS071296], and the Fondation Leducq [Transatlantic Network Grant number 10CVD01] (to S.J.V.).

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Received 23 October 2013
doi:10.1042/BST20130247