

Brief Update on Animal Models of Hypoxic-Ischemic Encephalopathy and Neonatal Stroke

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Abstract

The discovery of safe and effective therapies for perinatal hypoxia ischemia (HI) and stroke remains an unmet goal of neonatal-perinatal medicine. Because of the many developmental and functional differences between the neonatal brain and the adult brain, the ability to extrapolate adult data to the neonatal condition is very limited. For this reason, it is incumbent on scientists in the field of neonatal brain injury to address the questions of therapeutic efficacy of an array of potential therapies in a developmentally appropriate model. Toward that end, a number of new models of neonatal HI and stroke have been introduced recently. Additionally, some of the established models have been adapted to different species and different ages, giving scientists a greater choice of models for the study of neonatal HI and stroke. Many of these models are now also being used for functional and behavioral testing, an absolute necessity for preclinical therapeutic trials. This review focuses primarily on the newly developed models, recent adaptations to established models, and the studies of functional outcome that have been published since 2000.

Key Words: asphyxial brain injury; excitotoxicity; mice pups; rabbits; rat pups

Introduction

The use of animal models for the study of prenatal/neonatal hypoxia-ischemia (HI¹) and stroke has been reviewed in detail periodically (Ashwal and Pearce 2001; Hagberg et al. 1997, 2002b; Roohey et al. 1997; Vannucci et al. 1999; Yager 2004). These extensive reviews have generated information regarding the variety, frequency of use, and relative appropriateness of both large and small animal models to the study of brain injury in the perinatal period. Other reviews have focused on the numerous molecular and biochemical mechanisms of injury that have been studied in the neonatal rodent model (Vexler and Fer-

riero 2001). Recent reviews have specifically discussed the challenge of providing therapies that are both efficacious and safe for the developing brain (Yager 2004), models of white matter injury (Hagberg et al. 2002a), and the experimental background for the use of hypothermia to treat neonatal asphyxial brain injury (Thoresen 1999, 2000). Rather than attempting to duplicate these efforts, this article primarily focuses on the literature published since 2000. The discussion includes (1) recently developed models of neonatal HI and stroke, and (2) recent adaptations of established models that have provided new insights into the neurobiology and neuropathology of perinatal brain injury. The text also focuses on (3) the increased number of studies providing long-term neurobehavioral and neuropathology outcome data in animal models of neonatal HI and stroke, as well as (4) the successful translation of hypothermia research into a clinical research tool, and the potential for hypothermia to serve as a key component of combination therapy for asphyxial brain injury.

Classic studies that established neurodevelopmental parallels across species (Clancy et al. 2001; Dobbing and Sands 1979) have provided the foundation for the use of animal models to study neonatal brain injury. While Dobbing and Sands used rates of brain growth to make cross-species comparisons, recent multivariate analysis of aspects of both histological and functional maturity of multiple brain regions and neural systems has provided an extremely useful tool for comparing the prenatal and early postnatal brain development of eight widely used mammalian species with that of the human. Using these data, we now have compelling evidence that the most widely used model of neonatal asphyxial brain injury, the 7-day-old rat, in many ways has brain maturity equivalent to that of an early third trimester human fetus (Clancy et al. 2001). Based on the large portion of current work on neonatal HI and stroke that utilizes neonatal rodent models, and because the focus of this paper is on this body of work, these intraspecies developmental comparisons are referred to extensively below.

Recently Developed Models for the Study of Neonatal HI and Stroke

Models of perinatal and neonatal HI continue to be developed to match our understanding of the various pathologies resulting in neonatal HI brain injury. One of the greatest deficits in neonatal HI research has been that very few studies have actually described true intrauterine HI. Until re-

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¹Abbreviations used in this article: BDNF, brain-derived neurotrophic factor; HI, hypoxia-ischemia; PVL, periventricular leukomalacia.

cently, fetal sheep exposed to maternal hypoxemia (Gleason et al. 1990; Harris et al. 2001) or to umbilical cord occlusion (Gonzalez et al. 2005; Lotgering et al. 2004) were the most widely utilized models of intrauterine HI. Despite the abundance of neurophysiological data obtained from these studies, the model has major disadvantages. Pregnant ewes are large and very expensive. If they survive, the affected lambs show little clinical evidence of brain injury, which has resulted in the absence of follow-up neurobehavioral testing. A recently described model of perinatal brain injury—global HI in the near-term fetal rabbit—avoids most of the problems associated with the fetal lamb model. This new model very nicely mimics acute placental insufficiency in humans, and the surviving newborn rabbits that display persistent hypertonia and motor deficits provide a striking phenotype of cerebral palsy (Derrick et al. 2004). Biochemical studies of the model have already been published (Derrick et al. 2001; Tan et al. 2001), and imaging studies are currently under way (S. Tan, Northwestern University, Evanston, IL, personal communication, 2005).

Other rodent models reported in the late 1990s will allow more detailed studies of the role of ischemia in the development of white matter injury and the role of reperfusion in the overall contribution to injury. Bilateral carotid occlusion in the 5-day-old rat, without accompanying hypoxia, causes preferential white matter injury (Uehara et al. 1999) with only scattered neuronal injury within the cortex. Similar results have been found with bilateral carotid occlusion as early as postnatal day 1 (Cai et al. 2001). The selectivity of this model appears to be due to the reduced degree of cerebral blood flow reduction caused by bilateral carotid occlusion rather than by unilateral carotid occlusion plus hypoxia (Vannucci et al. 1988). This model holds a great deal of promise for the study of mild to moderate handicap that is associated with ventriculomegaly but minimal other detectable neuropathology. One follow-up study using this model has been published, and the results are cautionary for neonatal caretakers. The respiratory stimulant doxapram appears to exacerbate injury in the setting of the moderate ischemia caused by bilateral carotid artery occlusion (Uehara et al. 2000). Many similar studies could be performed to determine the effect of drugs on the development of periventricular leukomalacia (PVL¹) if additional studies validate bilateral carotid occlusion as a good model for PVL.

Several models are currently being used for investigation of neonatal stroke (Derugin et al. 1998; Renolleau et al. 1998; Wen et al. 2004a). Each of these models allows a different degree of reperfusion after the period of ischemia. These models are currently being used to determine the role of reperfusion in caspase activation after stroke (Manabat et al. 2003) and the therapeutic potential of caspase inhibition in neonatal stroke (Joly et al. 2004). In the model of permanent middle cerebral artery occlusion, the effect of stroke on the erythropoietin system and the potential use of erythropoietin for neuroprotection after neonatal stroke is being tested (Sola et al. 2005; Wen et al. 2004b). With the rec-

ognition of neonatal stroke as a major contributor to the total burden of neonatal brain injury (Lynch and Nelson 2001; Lynch et al. 2002; Nelson and Lynch 2004), these models provide a valuable tool for investigation of this clinical problem.

Recent Adaptations of Established Models of Neonatal HI and Stroke

Most biochemical and pathological data in neonatal HI have been derived from highly utilized rodent models. With the initial description of the adaptation of the Levine model of HI to the postnatal day 7 rat in 1981 (Rice et al. 1981), Vannucci and colleagues provided investigators with one of the most robust and productive models of brain injury ever described. The original authors, as well as many others, have provided reviews of this and related models of neonatal HI and stroke, and the reader is referred to this abundant literature (Ashwal and Pearce 2001; Hagberg et al. 2002b; Roth and D'Sa 2001; Tuor et al. 1996; Vannucci and Vannucci 1997; Vannucci et al. 1999; Vexler and Ferriero 2001; Yager 2004). Several of the more recent adaptations of this model are mentioned below.

With the continuing increase in the number of available transgenic mice strains, it was natural to adapt the Vannucci model to the mouse (Ditelberg et al. 1996). In the process of developing the murine model, several important modifications were required. These alterations include basic changes in technique (e.g., a surgical microscope for the carotid ligation in the much smaller postnatal day 7 mouse) and significant titration of the degree of hypoxic exposure. Whereas most investigators use at least 90 min of exposure to $F_1O_2 = 0.08$ to produce a moderate to severe injury neonatal rat (Rice et al. 1981; Towfighi et al. 1991), the length of hypoxia used to produce injury in the neonatal mouse is significantly less (40-70 min in most studies) (Ferriero et al. 1996; Fullerton et al. 1998; Graham et al. 2004; Hagberg et al. 2004; Xu et al. 2001).

Recognition that there are marked strain differences in susceptibility to HI injury has increased the complexity of studies of neonatal HI in the mouse model. In one of the most important studies to date using the neonatal mouse model, Sheldon and coworkers demonstrated that susceptibility to and severity of injury after neonatal HI is highly strain dependent (Sheldon et al. 1998). Marked differences between strains were seen, including a four-fold increase in the percentage of CD1 mice being injured after 30 min of hypoxia compared with 129Sv mice. Differences were also seen between strains in HI-induced mortality and median histopathological injury score. Because of these marked strain differences, in studies in which no wild-type littermates are available, careful attention must be paid to the selection of wild-type controls.

When using conventional transgenic mice, one also encounters the inflexibility of the altered gene expression and the ubiquitous nature of the altered gene expression with

each cell in the body being affected (Ryding et al. 2001). Complete lack of expression or permanent overexpression of a protein throughout the body causes embryonic or perinatal lethality in some cases (Ohshima et al. 1996; Varfolomeev et al. 1998) or produces a phenotype that confounds the interpretation of subsequent experiments (Kuida et al. 1996, 1998). This limitation is being overcome with conditional transgenic strategies that have been applied to developmental (He et al. 2004; Hirasawa et al. 2004) and degenerative neurological disorders (Beglopoulos and Shen 2004; Beglopoulos et al. 2004), but not yet to acute forms of brain injury. Despite these limitations, the development of the murine model of neonatal HI injury has allowed investigators to begin to take advantage of the large number of genetically modified mouse strains to determine the effect of loss or gain of function of an individual protein on immature brain injury (Ferriero et al. 1996; Fullerton et al. 1998; Graham et al. 2004; Hagberg et al. 2004).

Adapting the Vannucci model to the extremely immature rat has also revealed some important developmental differences in injury susceptibility (Sheldon et al. 1996) and has opened new avenues of investigation in neonatal brain injury (McQuillen et al. 2003). These extremely immature (postnatal day 1-2) animals require a longer and more severe degree of hypoxia to produce injury compared with postnatal day 7 rats, and there is a greater degree of damage to the ipsilateral subcortical developing white matter than in older rats (Sheldon et al. 1996). This subcortical damage has been recognized to include death of both oligodendrocyte progenitors and subplate neurons (McQuillen et al. 2003). Premature death of this transient but important population of neurons may well explain the pervasive abnormalities of neurodevelopment seen in the most extremely immature infants, abnormalities that cannot be explained on the basis of white matter injury alone (McQuillen et al. 2003). These investigators are continuing the study of subplate neurons, attempting to determine which signaling pathways cause cell death versus cell survival in these neurons and how defects in neurotrophin receptors affect thalamocortical innervation (DeFreitas et al. 2001; McQuillen et al. 2002).

A large part of our understanding of the unique susceptibility of the immature brain to excitotoxicity is based on nearly two decades of investigation by Johnston and colleagues, who have used the immature rat model of intrastriatal injection of various glutamate receptor agonists (McDonald and Johnston 1990, 1993; McDonald et al. 1988, 1992; Trescher et al. 1994). This model has also been adapted to the immature mouse, with the initial report of the model including a detailed description of the neurodevelopmental effects of excitotoxic injection during the first 10 postnatal days (Marret et al. 1995). The model is now being used to test the effect of excitotoxic injection in genetically and immunologically modified mice (Hennebert et al. 2004; Mesples et al. 2005). Based on the large number of recent publications using this model, it is well accepted and is providing a continuous flow of information regarding the complex role of cytokines (Mesples et al. 2003, 2005),

thrombophilic agents (Hennebert et al. 2005), oxygen-free radicals (Plaisant et al. 2003), and microglial activation (Dommergues et al. 2003) in the development of injury after excitotoxic injection.

Using the murine model, it has been shown that the neurotrophin brain-derived neurotrophic factor (BDNF¹) exacerbates excitotoxic injury when administered at postnatal day 0, protects against excitotoxicity at postnatal day 5, and is without effect on excitotoxic lesions at postnatal day 10 (Husson et al. 2004). These results are extremely important because BDNF is one of very few drugs shown to provide lasting neuroprotection in the rat model of neonatal HI (Almli et al. 2000). If the highly age-dependent effects of BDNF can be translated into human terms (Clancy et al. 2001), it would suggest that drugs could change from being harmful to being useful in as few as 5 wk, and then be without effect in another 5 wk. Such a model, which displays developmentally specific outcomes, is invaluable for investigators to proceed toward testing of other therapies for neonatal HI and stroke.

Long-term Follow-up and Functional Outcome Studies

An increased number of studies now provide long-term neurobehavioral and neuropathology outcome data in animal models of neonatal HI and stroke. Another important criticism of neonatal brain injury research has focused on the paucity of long-term follow-up studies and of behavioral and functional outcome studies. Roohey's extensively referenced review of animal models of neonatal HI brought this issue to the fore. From 1955 to 1997, only 29% of studies tested any outcome at greater than 24 hr after injury, and only 23% of studies included a clinical developmental, functional, or behavioral endpoint (Roohey et al. 1997). No repetition of this analysis has been performed; however, a cursory Medline search indicates that many studies of neonatal HI are now designed to test learning, memory, coordination, and other correlates of cognition, behavior, and motor function at later time points (Balduini et al. 2000; Bona et al. 1997; Ten et al. 2003; Yang et al. 2004). These studies now enable investigators to determine the following: (1) whether the models show late functional sequelae reminiscent of injury to the human newborn; (2) whether potential therapies alter neuropathology, biochemical, and functional outcomes; and (3) whether late neuropathology and functional outcomes correlate with one another. To test potential therapies rigorously before clinical trials, it is imperative to know whether they improve functional neurological outcomes in relevant models of HI and stroke.

Most of the late neurobehavioral and functional outcome studies have been performed in the neonatal rat following HI (Almli et al. 2000; Arteni et al. 2003; Balduini et al. 2000, 2003; Yang et al. 2004); however, studies in the murine and rabbit models are also now being reported. The importance of late functional and pathological testing is

highlighted by the finding that injury that appears to be minimal at 2 wk after a moderate HI insult evolves to delayed infarction and cerebral atrophy no different from injury after a severe insult (Geddes et al. 2001). The functional and neurobehavioral testing performed in neonatal mice following HI (Ten et al. 2003, 2004) has been combined with neuropathology studies to determine how well pathology predicts function following HI in this model. The same is true for the intrauterine ischemia model in rabbits (Derrick et al. 2004). The neonatal rabbit displays marked functional deficits at birth after intrauterine ischemia at 67 to 70% of gestation, and these deficits are accompanied by injury to subcortical motor pathways, including the basal ganglia and thalamus (Derrick et al. 2004). As yet, no similar late neuropathology and functional studies have been published in the other rodent models of HI and stroke.

Animal Models of Neonatal Asphyxia: Valuable for Preclinical Testing of Hypothermia

Hypothermia, which has been tested extensively and is efficacious in animal models, is likely to be useful in combination therapy for neonatal HI and stroke. The rekindling of interest in hypothermia for treatment of neonatal asphyxial brain injury since the mid-1990s is perhaps the best example of collaborative basic and clinical research resulting in progress in the treatment of neonatal brain injury. It is also a therapy that has been tested in almost every model of available neonatal HI. To date, hypothermia is the only treatment that appears to interrupt the initial rapid and overwhelming necrotic/excitotoxic process that occurs after acute severe HI in both large and small animals. Neuroprotection from hypothermia has been demonstrated in the postnatal day 7 (Bona et al. 1998; Mishima et al. 2004; Trescher et al. 1997; Young et al. 1983) and postnatal day 14 rat (Taylor et al. 2002) subjected to unilateral ischemia plus global hypoxia, the near-term fetal sheep exposed to umbilical cord occlusion (Gunn et al. 1997, 1998) and the 1-day- (Thoresen et al. 2001) and 1-wk-old piglet (Agnew et al. 2003) exposed to cardiac arrest.

Investigators are currently trying to determine whether hypothermia also affects apoptotic cell death following HI and whether it can be combined with antiapoptosis therapies for improved efficacy (Adachi et al. 2001; Zhu et al. 2004). Recent evidence that early anticonvulsant therapy combined with delayed hypothermia provides significant improvement in both function and neuropathology following HI is heartening and may be the beginning of effective combination therapies for neonatal HI brain injury (Liu et al. 2004). This evidence has been generated in the relatively immature postnatal day 7 rat and must now be replicated in other larger and more mature animal models that have been used for hypothermia research. After extensive preclinical testing, researchers investigating use of hypothermia in the newborn have diligently seen it through the arduous process

of clinical trials (Azzopardi et al. 2000; Battin et al. 2003; Debillon et al. 2003). Unfortunately, despite some limited positive outcomes (Battin et al. 2001; Compagnoni et al. 2002; Inder et al. 2004), some data currently suggest that hypothermia alone is unlikely to provide adequate therapy for neonatal HI (Gluckman et al. 2005; Jacobs et al. 2003). These recent data from human studies increase the urgency to pursue therapy combination studies with hypothermia. Fortunately, because of the worldwide interest in hypothermia to treat neonatal HI, many well-developed models are available to enable pursuit of this work (Agnew et al. 2003; Bona et al. 1998; Gunn et al. 1997, 1998, 1999; Mishima et al. 2004; Taylor et al. 2002; Thoresen et al. 2001; Trescher et al. 1997; Young et al. 1983).

Conclusion

It is very fortunate that investigators have a great variety of models available for the study of neonatal HI and stroke. Furthermore, this vibrant research community has demonstrated a willingness to develop new models and adapt established models as needed. With the plethora of available models, the importance of choosing the appropriate model in which to study various aspects of HI and stroke injury on brain development, behavior, and functional outcomes cannot be overstated. However, an even more rigorous selection process must be applied to the choice of the appropriate animal model for testing possible treatments for neonatal HI and stroke.

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