Late Measures of Brain Injury After Neonatal Hypoxia–Ischemia in Mice

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Background and Purpose—This work was undertaken to determine to what degree long-term neurofunctional outcome of neonatal hypoxic–ischemic (HI) brain injury in mice correlates with anatomical extent of cerebral damage assessed by magnetic resonance imaging (MRI) and histopathology.

Methods—On postnatal day 7, mice were subjected to HI. At 7 to 9 weeks after HI neurofunctional outcome was assessed by water-maze, rota-rod, and open-field test performance, followed by cerebral MRI and histopathology evaluation.

Results—At 10 weeks after HI, MRI revealed ipsilateral brain atrophy alone or with porencephalic cyst formation and contralateral ventriculomegaly. Adult HI-affected mice, especially those that developed a porencephalic cyst, demonstrated significant neurofunctional deficit compared with age-matched naive mice. HI-affected mice with ipsilateral cerebral atrophy but without porencephaly demonstrated no or an intermediate level of neurofunctional deficit. Neurobehavioral assessment of mice subjected to HI insult revealed a strong correlation between degree of brain injury and functional neurohandicap.

Conclusions—This is the first study to demonstrate that long-term neurofunctional outcome in mice after a neonatal HI correlates tightly with anatomical pattern/extent of cerebral damage, defined by MRI and histopathology. (Stroke. 2004; 35:2183-2188.)

Key Words: hypoxia ■ ischemia ■ animals, newborn ■ mice

In rodent models of perinatal hypoxic–ischemic encephalopathy (HIE), the predominant adverse outcome measure is based on histopathological assessment of neuronal cell loss.1–3 Given the great plasticity of the developing brain, functional measures of injury are likely to be more clinically relevant. Although neurofunctional deficit after an HI insult in rats has been reported,4,5 in mice neurofunctional studies have been applied in an adult mouse stroke model and were limited to assessment of anatomical/pathological changes as they correlate with neuroanatomical and neurofunctional outcomes of HIE in mice. The goal of presented study was to describe long-term neuroanatomical/pathological changes as they correlate with neurofunctional deficit in adult mice subjected to HI at a very early (neonatal) developmental stage.

Materials and Methods

Murine Model of HIE

Three-day-old (P3) C57/BL6J mice of both genders were purchased from Jackson Laboratories (Bar Harbor, Maine) with their birth mothers. All research was conducted according to a protocol approved by the Columbia University Animal Care and Use Committee (IACUC).

On P-7, the right common carotid artery was ligated under isoflurane anesthesia. After 2 hours of recovery, mice were subjected to 8% O2 balanced with N2 for 20 minutes at 37°C. After hypoxic exposure, pups that have formed an experimental group were returned to their dams. Because it has been shown that carotid artery ligation without hypoxia (sham) produced neither brain damage9 nor functional impairments in rodents,10,11 control animals consisted of age- and strain-matched naive (no HI) mice.

Neurofunctional outcome was assessed in adult mice at 7 to 9 weeks after HI insult.

In rodents, the sensorimotor cortex, the striatum, and the hippocampus are predominantly damaged after HI insult. Therefore, 3 corresponding behavioral tests were chosen; water-maze test to assess spatial learning and memory for function of the hippocampus,15 rota-rod test for a motor cortical dysfunction,14 and open-field paradigm served as a screening behavioral test for cerebral structural integrity.12,13

Morris water-maze was performed according to a protocol described previously.16 Rota-rod test was performed in mice at 8 weeks of age subjected to HI in the neonatal period, and their naive counterparts were given 2 attempts (5 minutes each) daily for 3 consecutive days of training. During the training period, the rota-rod was set on an accelerating mode (from 4 to 20 rpm over 5 minutes), and this rotational speed was increased by 5 rpm each day. On the third day of training, the rotational speed had reached 30 rpm, representing the speed used for the subsequent day’s challenge. During this third day of probative trial, mice were given 2 attempts, and the summed duration of on rod-holding was recorded.
Open-field test was performed at 7 weeks after HI insult, and mouse behavior was monitored in the open-field apparatus consisting of a plastic chamber (43.2×43.2×30.5 cm), which was criss-crossed with infrared light beams spaced 1.25 cm apart (Med Associates) to record the location and the traveled path length. Each mouse was tested for 60 minutes, during which multiple behavioral variables were recorded by computer.

Neuroimaging Study
At age 11 weeks, mice were subjected to a T2-weighed magnetic resonance imaging (MRI) study. MRI was performed on a Bruker AVANCE 400WB spectrometer (Bruker NMR Inc) with an 89-mm vertical bore magnet of 9.4 T (Oxford Instruments Ltd) using a 30-mm internal diameter birdcage radio frequency probe and a shielded gradient system of 100 G/cm. During the imaging experiment, the mouse was anesthetized with isoflurane/air gas (1.5 volume % at 2 L/min) via a nose cone. Images were obtained using a 2-dimensional multislice spin echo (SE) sequence with the following parameters: field of vision = 30 mm, acquisition matrix = 256×256, slice thickness = 1 mm, time of repetition (TR)/time of echo (TE) = 2000/45 ms, and number of averages = 2. Twelve coronal slices were acquired, covering the entire brain. The damaged cerebral region on MRI image appeared bright compared with dark normal brain tissue. All 12 MRI images from each mouse were analyzed using a computer-assisted image analysis program (NIH Image Analysis). The volume of remaining brain tissue (dark on MRI image) was expressed as a percentage of total cerebral volume (dark plus bright areas on MRI).

Anatomical Assessment of Cerebral Injury
On completion of neurobehavioral testing and MRI study (10 weeks after HIE), brains were taken and fixed in 10% formaldehyde at 4°C for 36 hours, and then mounted in paraffin. Standardized 10-μm coronal sections were obtained and the degree of cerebral atrophy was quantified as described previously. In each cerebral section, measurements were taken of the hippocampus, the nonhippocampus (hemisphere — hippocampus=cortex and striatum), and thalamic areas. The latter included hypothalamus, because discrimination of these 2 areas is unclear at low magnification used for volumetric measurements. The ratio of the ipsilateral remaining cerebral tissue volume (cortex and striatum, hippocampus, or thalamus) to the volume of corresponding contralateral cerebral structures was expressed as a percentage.

Statistical Analysis
Data are means±SEM. ANOVA test was used for comparative analysis of brain volumes and neurofunctional performance. Linear regression analysis was performed to determine correlation between degree of cerebral atrophy and neurobehavioral performance. Data were considered significantly different if P≤0.05 between groups.

Results
Of the 15 neonatal mice subjected to HI, 13 animals survived until euthanization (age 11 weeks). One mouse was found dead in the cage at 2 days after the HI insult and another was cannibalized by its mother within the first day after the HI insult. All 7 naïve control animals survived until euthanization. Four randomly chosen naïve adult mice underwent brain MRI and histopathology for comparative analysis (Figure 1A).

Figure 1. T2-weighted cerebral MRI and corresponding Nissl-stained brain sections. A, Naïve. B, C, and G, Mice with ipsilateral hemisphere atrophy. D, E, F, and H, Mice with development of porencephalic cyst, thinning of neocortex, and ventriculomegaly in the contralateral hemisphere.
Histopathological and Neuroimaging Outcomes of HIE in Neonatal Mice

Brain neuroimages and histopathology revealed that mice subjected to HI had different degrees of cerebral injury (Figure 1B through 1F). MRI of the brains demonstrated 2 major patterns of cerebral injury: (1) mild atrophy of the brain ipsilateral to the side of carotid artery ligation without development of a porencephalic cyst and an intact contralateral side (7 animals, Figure 1B, 1C, and 1G); and (2) severe cerebral atrophy with formation of a large porencephalic cyst, loss of the ipsilateral cortex, and contralateral hemispheric changes, which consisted of ventriculomegaly and thinning of the cortex (6 animals, Figure 1D through 1F and 1H). In 2 mice, the porencephalic cyst extended to the contralateral hemisphere and fused with the contralateral ventricle into one large cystic lesion (Figure 1E, 1F, and 1H). Although porencephalic cysts were identifiable in histopathological specimens obtained postmortem, the size of these cystic lesions was much smaller than that expected given the size of the same lesions seen in vivo on MRI (Figure 1E and 1F). In 2 mice subjected to HI, MRI showed very subtle changes in the ipsilateral cortical region with minimal atrophy (Figure 1B and 1G). In these mice, histology demonstrated focal areas of a gliosis with increased number of glial cells and reduced number of neurons in the affected area (Figure 2A through 2D). Planimetric analysis of the histological sections of the brains demonstrated significant reduction in volume of the injured hemisphere and especially the hippocampus in HI-affected compared with naïve mice (Figure 2E and 2F). MRI-based assessment of cerebral tissue loss revealed significant difference between HI mice with porencephaly compared with HI mice without cystic lesion and naïve animals (Figure 2G). When histological specimens of HI mice were stratified by the presence or absence of cyst, significant atrophy of the ipsilateral hemisphere, thalamus, and hippocampus was found in those mice that had developed porencephalic cysts (Figure 2H, 2I, 2J). Linear regression analysis demonstrated a very strong correlation ($r=0.91$ for

Figure 2. A. Nissl-stained brain section ($\times 1$). B. Area of cortical gliosis ($\times 10$). C and D, Magnified ($\times 20$) ipsilateral area of cortical gliosis (D), and corresponding contralateral area (C). E and F, Histopathology-assessed brain tissue loss in the ipsilateral cortex and striatum (hemisphere – hippocampus) and hippocampus in HIE (n=13) and naïve (n=4) adult mice. G through J, MRI (G) and histopathological (H–J) assessment of cerebral tissue loss in HI–cyst (n=6) and no-cyst (n=7) mice and in naïve (n=4) animals. Data shown as mean±SEM, studied groups and structures of the brain, $P$ values are indicated.
cortex and striatum and $r=0.93$ for hippocampus) between MRI- and histopathology-assessed degrees of cerebral damage (Figure 3A and 3B).

Thus, the anatomical pattern of cerebral injury assessed by MRI and histopathology in adult mice subjected to an HI insult as neonates could be divided in 2 categories: mice with severe cerebral atrophy and porencephalic cyst formation (cyst mice) and mice with mild/moderate cerebral atrophy, but without a cystic lesion (no-cyst mice).

**Neurofunctional Outcome in Adult Mice With HIE**

**Morris Water-Maze Test**

Analysis of summated latencies of training period showed a significant delay in locating the platform in HI cyst mice compared not only to naïve animals but also to HI–no cyst animals, which also demonstrated poorer spatial orientation compared with naïve mice (Figure 3C). During the trial in which the flag (the major navigational cue) had been removed, only cyst mice became spatially disoriented (Figure 4A). Testing of navigational memory demonstrated that both HI–cyst and no-cyst mice spent significantly less of the allotted time searching for platform in the “landing quadrant” compared with naïve animals (Figure 4B). The anatomical extent of brain damage assessed by both MRI and neuropathology significantly correlated with degree of navigational memory deficit (Figures 4C, 4D, and 6A).

**Rota-Rod and Open Field Test**

In comparison with naïve animals, only HI–cyst mice held on to the rota-rod for a significantly shorter time when the rod was rotated at a steady-rate of 30 rotations per minute. In contrast, HI–no cyst mice were able to hold on to the rota-rod as long as naïve animals (Figure 5A).

Open-field test demonstrated that exploratory behavior of HI–mice was not significantly different compared with naïve mice (data not shown). However, when HI mice with and without porencephaly were compared separately with naïve animals, only HI–cyst mice exhibited significantly reduced ambulatory velocity, reduced number of ambulatory episodes, and increased ambulatory distance compared with either naïve or no-cyst HI mice (Figure 5B to 5D). Linear regression analysis revealed that only ambulatory velocity correlated significantly with degree of the MRI-defined cerebral tissue loss and ipsilateral thalamic or hippocampus atrophy assessed by brain histopathology (Figure 6B to 6D).

**Discussion**

In the first study of cerebral MRI assessment of neonatal mice subjected to HI, Aden et al described an early evolution of neuroanatomical changes. According to this report, the damaged area of the brain reaches a maximal extent at 3 to 6 hours after HI. All MRI-assessed cerebral changes were found within the affected hemisphere at all time points studied. The delayed MRI and histopathological study used in the present work extended the observational strategy to the later stage of murine HIE and highlighted another very important aspect of HIE progression in severely affected mice—porencephalic cyst formation with involvement of the contralateral hemisphere. In the
rat model of HIE, the contralateral hemisphere was found to be intact and served as a control in short- and long-term histopathological assessment of brain damage. In contrast, Andine et al. found decreased weight of the hemisphere contralateral to the injured side in rats. Jansen et al., however, demonstrated histologically assessed hypertrophy of the contralateral hemisphere in adult rats after a perinatal HI insult. Sample assessment-related methodological differences may explain the conflicting literature related to the appearance of the contralateral hemisphere.

The advantage of the delayed MRI assessment is that it allows to identify and measure the size of porencephalic cysts, fluid-filled lesions of the brain, which likely dissipate during histological sectioning. The importance of identifying these porencephalic cysts cannot be underestimated for 2 reasons. First, similar cysts are seen in humans after neonatal HI. Second, the data shown here demonstrate that the presence or absence of cyst formation was strongly associated with the degree of cerebral tissue loss, which significantly correlated with severity of neurofunctional handicap. Similarly, Bona et al demonstrated strong correlation between degree of the brain damage and long-term sensorimotor deficit in combined analysis of control and HI-subjected rats.

In children, long-term neurofunctional outcome correlates with neuroanatomical type of HI injury. Intellectual function is often relatively preserved in those infants with late development of choreoathetosis, the neurological consequence of perinatal HI if basal ganglia and thalamus (not cortex) are involved. In our study, we found that no-cyst mice had most of the cortical tissue spared in the ipsilateral hemisphere, with normal appearance of the contralateral side. These mice demonstrated significantly better neurobehavioral performance compared with those animals that lost their ipsilateral cortex and developed porencephaly. In children after focal perinatal stroke, cognitive and motor development is mildly affected. However, the presence of significant cognitive deficit in apparent unilateral focal brain injury is highly suggestive of contralateral hemispheric involvement.

In our study, despite identical experimental conditions, not all animals subjected to HI exhibited MRI and histopathology-based findings of extensive cerebral injury. In the only other available dataset describing MRI-assessed injury in neonatal mice after HI, 4 out of 18 mice did not develop MRI positive findings. These 4 mice exhibited normal sensorimotor function assessed by the walking beam test 3 weeks later. The results of delayed sensorimotor assessment shown here are similar; only severely HI-affected mice demonstrated significant deficit when tested by rota-rod and in open-field. In rats subjected to HI, some authors found no long-term sensorimotor deficit assessed by the rota-rod test. In contrast, Jansen et al reported significant impairment of sensorimotor neurofunction in rats assessed by the rota-rod test at 6 to 9 weeks after HI. These conflicting data reported by different authors who using the same model might be explained by differing degrees of brain injury in the cohort of HI-affected rats. A very well-designed study of neuropathological and functional outcomes of neonatal HIE in adult rats revealed that only 11 of 23 (47%) of animals developed severe brain damage with significant tissue loss and porencephaly. These data are consistent with our findings in mice, wherein 6 of 13 (46%) developed extensive brain injury and porencephaly. These authors did not find significant sensorimotor dysfunction based on rota-rod test performance in the selected cohort of 18 rats subjected to HI. In that cohort, however, 39% of animals had mild cerebral damage without cystic lesions.
Similar to the results of the rota-rod test, only severely HI-affected mice demonstrated significantly different behavior in an open-field test in comparison with naïve animals. An abnormal hyperactive exploratory behavior in an open-field after early postnatal anoxia insult has been observed in rats.28,29 Baldini et al27 did not find significant differences in spontaneous and apomorphine-induced behavior in open-field between HI-subjected and control rats tested at 96 days of life. It is important to mention, as in the present study, in Baldini’s experiments 11 of 19 HI-subjected rats developed severe brain injury with significant tissue loss. Another 8 HI rats developed mild ipsilateral hemispheric atrophy with more prominent neuronal loss in the hippocampus. Only 8 out of 19 HI-subjected rats were tested in the open-field paradigm and the degree of cerebral damage in those tested animals was not specified.27 Thus, in different species, rat and mouse, this model of HI brain injury results in various extent of cerebral damage, which ultimately predicts the degree of neurofunctional deficit as it shown in current study.

In our experiments the water-maze test appears to be superior to the rota-rod or the open-field test for assessment of long-term neurofunctional handicap. Only the water-maze test is sensitive enough to demonstrate significant impairment in navigational memory and spatial orientation in not only severely affected HI–cyst mice, but also those HI–no-cyst animals. In addition to our previous observation,16 current data highlight a strong correlation between spatial orientation and memory deficit and neuroanatomical extent of cerebral damage. This result is in agreement with a report by Wagner et al13 who found a strong correlation between spatial memory and hippocampus volume in HI-subjected rats. Thus, our data on mice and previously reported results on rats suggest that the water-maze paradigm can be considered not only species-independent (rat, mouse) for evaluation of HI-induced neurofunctional deficit but also very sensitive for detection of moderate injury. At contrast, the rota-rod and open-field tests have limited sensitivity for detecting moderate degrees of brain damage.

Our major finding is that long-term neurofunctional outcome is strongly associated with the anatomical extent and pattern of the cerebral damage quantified by delayed neuroimaging and histopathology. Given that a mouse model of HIE may serve as a valuable guide for functional definition of neuroprotection or harm in the experimental setting.

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