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Long-Term Functional and Protective Actions of Preconditioning With Hypoxia, Cobalt Chloride, and Desferrioxamine Against Hypoxic-Ischemic Injury in Neonatal Rats

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ABSTRACT: Preconditioning with hypoxia and hypoxia-mimetic compounds cobalt chloride ($CoCl_2$) and desferrioxamine (DFX) protects against hypoxic-ischemic (HI) injury in neonatal rat brain. We examined long-term functional and protective actions of preconditioning induced by hypoxia, $CoCl_2$ and DFX in a neonatal rat model of HI. Postnatal day six rat pups were exposed to preconditioning with hypoxia (8% oxygen) or injections of $CoCl_2$, DFX or saline vehicle and 24 h later rats underwent HI or sham surgery. Behavioral tests were performed and at the conclusion of experiments, brains removed for morphologic analyses. HI resulted in a large unilateral lesion in the ipsilateral hemisphere compared with sham control rats. All preconditioning treatments significantly reduced the total lesion volume. Behavioral deficits were observed in HI rats compared with sham controls. The reduction in forelimb grasping strength in HI rats was attenuated by preconditioning with hypoxia, $CoCl_2$ and DFX. HI increased the number of foot faults in a grid-walking test and resulted in forelimb asymmetry in the cylinder test. Only preconditioning with hypoxia reversed all three functional deficits after HI. These findings indicate that preconditioning, especially when induced by hypoxia, has the potential to minimize the morphologic and functional effects of neonatal HI injury. **(*Pediatr Res* 63: 620–624, 2008)**

Brain injury in humans, which occurs as a result of an hypoxic-ischemic (HI) episode during the perinatal period, is a major cause of mortality and long-term disability (1). Although there is now a greater understanding of the mechanisms involved in cell death processes associated with HI injury (2), there are still no beneficial therapeutic interventions. Several treatments, which have long-term protective actions in animal models of HI, include brain-derived neurotrophic factor, corticosteroids, glutamate receptor antagonists, and hypothermia (3,4). For a long time, mild stress has been known to "precondition" and protect against a subsequent brain insult (5). Treatments providing protection against subsequent injuries include mild ischemia (6), hyperthermia (7), bacterial lipopolysaccharide (8), and hypoxia (9,10).

Tolerance produced by a mild preconditioning episode of hypoxia has been well investigated, and recent studies have indicated that hypoxia-inducible genes may contribute to tolerance in the rat brain (9–11). Tissue hypoxia can stimulate the expression of many genes involved in adaptive processes such as erythropoiesis, angiogenesis, glucose transport, and anaerobic glycolysis (12). One vital element involved in regulating expression of hypoxia-responsive genes is the transcription factor, hypoxia-inducible factor-1 (HIF-1) (12). In the brain, HIF-1 has been found to have complex roles in brain injury and protective processes, which may relate to insult severity (12). Following exposure to hypoxia or hypoxia-mimetic compounds, such as cobalt chloride ($CoCl_2$) or desferrioxamine (DFX), HIF-1α mRNA and protein expression are increased (13). Interestingly, preconditioning with hypoxia, $CoCl_2$ and DFX can prevent against a subsequent HI injury and the protection appears to directly relate to HIF-1α protein expression (9). These preconditioning treatments do not cause neuronal injury (9), but have been shown to induce changes in gene expression and intracellular signaling pathways (9–11,14). Those genes affected include HIF-1 (9) and HIF-1 target genes: glucose transporters, glycolytic enzymes (10), erythropoietin and vascular endothelial growth factor (VEGF) (11). Numerous experimental studies have highlighted the need to examine long-term functional improvements in addition to histologic outcomes after HI injury in the neonate (3,15,16). Therefore, we have investigated whether preconditioning with hypoxia (HP), DFX and $CoCl_2$ has long lasting cytoprotective actions and thus improves both morphologic and behavioral endpoints 5 wk after an HI insult performed in postnatal day 7 rat pups.

METHODS

All animal work conducted in this study was approved by the Howard Florey Institute Animal Ethics and Experimentation Committee and performed in accordance with the guidelines of the National Health and Medical Research Council (Australia). Sprague–Dawley rat pups were obtained from Animal Resources Centre (Perth, WA) and were housed under standard housing conditions in the Howard Florey Institute animal facility throughout experiments.

Preconditioning treatments. Male and female Sprague–Dawley rat pups (postnatal day 6 (p6)) were randomly exposed to preconditioning treatments (typically two to three different preconditioning treatments were used per litter). Hypoxic preconditioning (HP) was performed as described previously. Briefly, pups (n = 19) were exposed to an 8% O_2/92% N_2 humidified

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Abbreviations: $CoCl_2$, cobalt chloride; **DFX,** desferrioxamine; **HIF-1,** hypoxia-inducible factor-1; **HI,** hypoxia-ischemia; **HP,** hypoxic preconditioning; **p,** postnatal day

atmosphere in 1 L chambers, which were partially submerged in a water bath maintained at 37°C for 3 h. Control rats (no preconditioning) were maintained at 37°C for 3 h in normoxic conditions ($n = 8$). Preconditioning with the hypoxia-mimetic compounds DFX and $CoCl_2$ was performed as follows. Rat pups received a single s.c. injection on p6 (up to a maximum volume of 0.1 mL) of one of the following treatments: DFX (200 mg/kg; $n = 16$) or $CoCl_2$ (60 mg/kg; $n = 16$). Vehicle control rats were injected with a single s.c. injection of saline (0.9% sterile saline, up to a maximum volume of 0.1 mL) vehicle solution ($n = 13$). Both "nonpreconditioned" control groups (normoxia and vehicle injection) did not differ statistically; therefore, all data for these two groups were combined into the HI alone group ($n = 21$). Following preconditioning treatments, all pups were returned to their dam.

Animal surgery. Twenty-four hours after control or preconditioning treatments, pups were anesthetized with 1.5% isoflurane in 30%O_2/70% N_2 mixture and underwent unilateral HI as described previously (17). The right common carotid artery was exposed through a ventral midline neck incision and permanently occluded by electrocoagulation using a cautery device. The wound was sutured and rats were returned to their mother for 1.5–2 h. Sham control rats ($n = 7$) underwent the identical procedure, without carotid artery occlusion. Pups were then placed in an 8% O_2/92% N_2 humidified chamber at 37°C for 2.5 h. This combined procedure results in select neuronal damage or infarction in the hemisphere ipsilateral to the carotid occlusion, whereas hypoxia alone (contralateral hemisphere) does not produce any significant brain injury (17–19). Following the HI or sham surgery procedure, all pups were returned to their dam and kept under standard housing conditions for the remainder of the study. On p21, pups were weaned and separated out into same-sex cages. Pups were handled every 2–3 d and exposed to behavioral apparatus on the day before testing.

Behavioral testing. Sensorimotor tests were videotaped and behaviors were assessed by an experimenter blinded to the treatment rats had received. All behavioral testing was conducted during the second half of the light portion of a 12 h light/12 h dark cycle. All of the sensorimotor tests were conducted on the same day (p42), whereas locomotor testing was performed on p43 and p44.

Spontaneous locomotor activity. Spontaneous locomotor activity was monitored on p43 and p44, using the Truscan system (Coulbourn Instruments, Allentown, USA). Individual rats were placed in locomotor cells and monitored for a single session of 15 min on each day. Locomotor activity was monitored in horizontal and vertical planes and time spent moving was also measured. Only data from p44 was collected and analyzed.

Grip traction test. A modified grip traction test was used to measure forelimb muscle strength (20). The ability of each animal to hang on to a horizontal rope (plastic tube – 0.6 cm diameter placed horizontally 50 cm above table) by the forelimbs was monitored and the time to falling was noted. If a rat had not fallen within 60 s, this was recorded as the maximum time to falling.

Grid-walking test. To measure fore and hind limb deficits, the grid walking test was used (20). Rats were placed on a horizontal grid (40 × 40 cm, squares – 2 × 2 cm, wire – 0.4 cm diameter). When rats misplaced a limb between the grid squares, a foot fault was counted. The number of foot faults made in 2 min and the total time spent moving were noted. Results are expressed as number of faults/time spent moving (min).

Cylinder test. The cylinder test was used to assess forelimb asymmetry (21). Forelimb bias was analyzed by measuring exploratory (rearing) behaviors of rats placed in a 4 L transparent cylinder. A mirror was placed behind the cylinder to allow analysis of forelimb movements when rats were facing away from the video camera. The number of forelimb placements on the cylinder wall (ipsilateral, contralateral, "combined"—both paws used simultaneously) made within a 2-min period were counted. Results are expressed as an Asymmetry Score which is calculated as described previously (22). Briefly, the number of ipsilateral touches of the wall plus half the number of "combined" movements is then divided by the total number of forelimb movements (ipsilateral, contralateral, and combined). This gives an asymmetry score—whereby, if there is no asymmetry, the score is 0.5 and an increase in use of the ipsilateral forelimb is indicated by a score (<0.5).

Morphologic staining. After behavioral testing (on p45), all rats were anesthetized with pentobarbitone sodium (100 mg/kg, i.p), perfused with PBS and brains removed and frozen using isopentane. Brains were subsequently sectioned using a cryostat and 20 μm sections collected on gelatin-coated slides stored frozen at −80°C until use. Nissl stained (cresyl violet) sections were examined under a light microscope (Leica DMR). Images were quantified using MCID™ Analysis (Imaging Research) imaging system to measure left and right hemispheric volume and to calculate lesion volume. Volume was calculated by measuring the area of left and right hemispheres and multiplying by the distance between sections. On average, 18–20 sections per brain were used to calculate lesion volumes. In a similar manner, the volumes of left and right striatum and cortex were measured. All procedures related to lesion volume analysis were performed by an experimenter blinded to the animal treatments. Data are expressed as volume of injured area (mm^3; volume of left hemisphere − volume of right hemisphere).

Statistics. Statistical analyses were performed using one-way ANOVA, with multiple intergroup comparisons made using Newman-Keuls *post hoc* test. All data are represented as the mean ± SEM. A probability value of less than 0.05 was considered statistically significant.

RESULTS

Similar to our previous findings and those of others using the Rice-Vannucci model of neonatal HI injury, in the present study we observed unilateral brain lesions (10,17,23), which were accompanied by behavioral deficits. There were large lesions affecting the ipsilateral (right) hemisphere in HI rats, including damage to cortical and striatal regions (Fig. 1). Damage in the ipsilateral cortex after HI was reflected by shrinkage of the whole cortex. All cortical layers exhibited damage and in agreement with previous authors, we observed columnar cortical cell loss after HI (Fig. 1*B*). In the striatum, damage occurred throughout the caudate putamen, predominantly affecting areas close to the lateral ventricle and corpus callosum (Fig. 1*G*). Rats preconditioned with HP, $CoCl_2$ or DFX at 24 h before HI, exhibited a differential reduction in cell loss throughout the ipsilateral hemisphere in the two specific brain regions examined (Fig. 1). The total volume of brain damage at 5 wk after surgery was significantly increased in HI animals (67.8 ± 14.0 mm^3) compared with control rats (1.4 ± 4.7 mm^3) (†$p < 0.05$). A marked reduction in total lesion volume occurred in all groups of rats exposed to preconditioning treatments 24 h before HI surgery. HP, $CoCl_2$ and DFX reduced overall lesion volume by similar amounts: 68%, 68%, and 53%, respectively, compared with rats exposed to HI alone (Fig. 2). Preconditioning with hypoxia and $CoCl_2$ produced appreciable reductions in the extent of cortical damage and only HP significantly reduced striatal damage. There was a trend for $CoCl_2$ to reduce damage in the striatum, but this was not significant and DFX preconditioning had no effect on striatal damage compared with HI alone.

At 5 wk after HI, rats exhibited several sensorimotor behavioral deficits when compared with control rats, which have been previously described (4,20). Rats subjected to HI displayed significantly reduced forelimb muscle strength as assessed using the grip traction test compared with sham control rats. These animals were able to hold on to the rope for 17 ± 3 s, whereas control rats lasted 36 ± 5 s before falling. There was a significant improvement in the forelimb muscle strength in all of the preconditioning groups when compared with HI alone. Rats exposed to preconditioning with HP, $CoCl_2$, and DFX were able to hold on to the rope for 1.8, 1.8, and 2.4 times longer than HI alone group, respectively (Fig. 3*A*).

The number of foot faults made was assessed using the grid walking test and HI rats (13.6 ± 1.4 mistakes/min) made more mistakes compared with control rats (9.5 ± 1.1 mistakes/min). Only HP significantly reduced the number of foot faults made after HI to 8.3 ± 0.9 mistakes/min. $CoCl_2$ and DFX preconditioning did not reduce the number of foot faults made (Fig. 3*B*).

The cylinder test is used to assess forelimb asymmetry in models of unilateral brain injury (21,22). Control rats had an

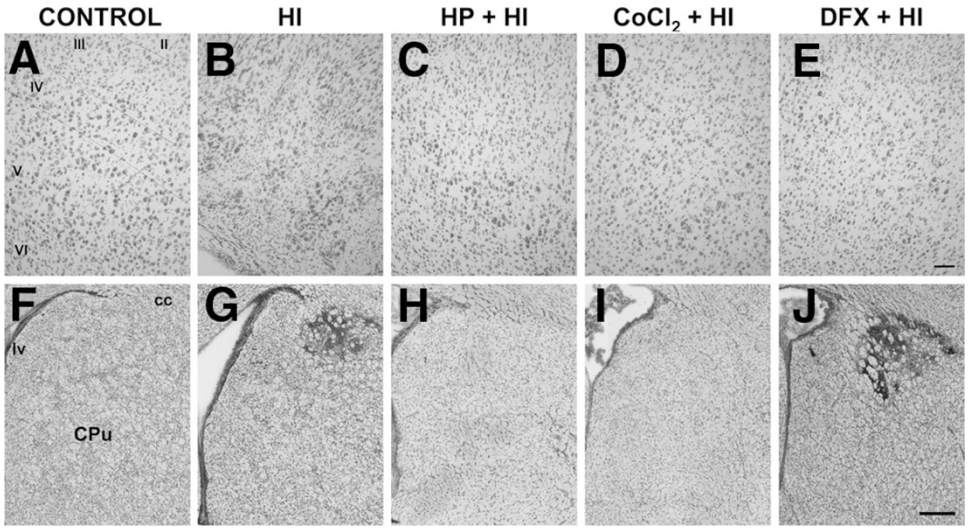

Figure 1. Photomicrographs demonstrating the protective effects of preconditioning with hypoxia (HP), $CoCl_2$, and DFX at 5 wk after HI. On postnatal day 6 (p6), rats were preconditioned with HP, $CoCl_2$, or DFX and on p7, subjected to an HI insult. Brains were collected at 5 wk after HI and coronal brain sections were stained with cresyl violet to illustrate damage to cortex (*A–E*) and striatum (*F–J*). HI resulted in damage to the cortex (*B*) throughout cortical layers (II–VI) compared with control (*A*). HP, $CoCl_2$, and DFX preconditioning treatments reduced the extent of cortical damage after HI (*C–E*). In the striatum, HI (*G*) produced damage throughout caudate putamen (CPu), predominantly affecting areas adjacent to the lateral ventricle (lv) and corpus callosum (cc) compared with control (*F*). HP and $CoCl_2$ preconditioning (*H*, *I*) appeared to improve cell survival in striatum, whereas DFX had no effect (*J*). Scale bar = 100 μm (*A–E*); 500 μm (*F–J*).

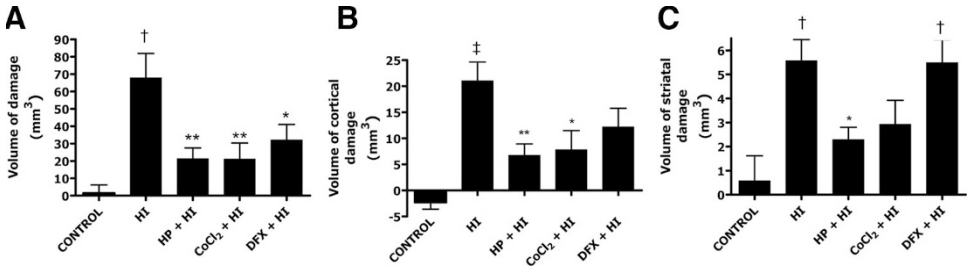

Figure 2. Quantification of brain damage at 5 wk after HI injury. (*A*) HI ($n = 21$) significantly increased the total volume of brain damage compared with sham control rats ($n = 7$). Preconditioning with hypoxia ($n = 19$), $CoCl_2$ ($n = 16$), and DFX ($n = 16$) significantly reduced the total amount of brain damage after HI (†$p < 0.05$, compared with control rats; *$p < 0.05$, **$p < 0.01$, compared with vehicle treated HI rats). HI significantly increased the volume of (*B*) cortical and (*C*) striatal brain damage compared with control rats (†$p < 0.05$, ‡$p < 0.01$). (*B*) HP and $CoCl_2$ reduced the amount of cortical injury (*$p < 0.05$), while only (*C*) HP reduced the amount of striatal injury compared with vehicle treated HI rats. Data are shown as the mean ± SEM.

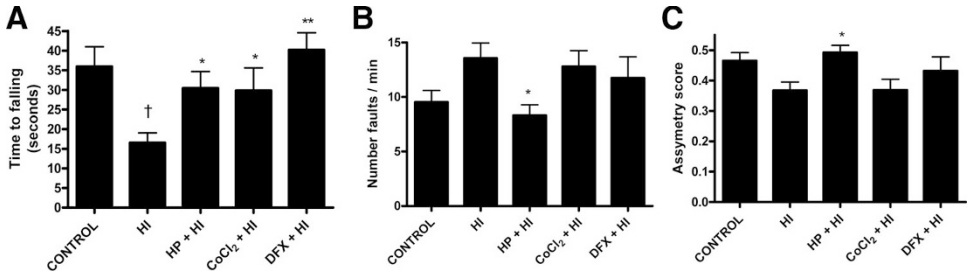

Figure 3. Preconditioning improves behavioral deficits up to 5 wk after HI. (*A*) HI ($n = 21$) reduces forelimb muscle strength compared with control (†$p < 0.05$), as measured by the grip traction test. Preconditioning with hypoxia ($n = 19$), $CoCl_2$ ($n = 16$) and DFX ($n = 16$) significantly improved forelimb muscle strength (*$p < 0.05$, **$p < 0.01$) compared with HI treatment alone. (*B*) There was an increase in the number of foot faults made in the grid-walking task in HI rats compared with control. HP significantly reduced the number of foot faults made (*$p < 0.05$), compared with HI treatment alone. (*C*) There is a forelimb asymmetry observed in HI rats compared with controls, and this is reversed by HP (*$p < 0.05$).

asymmetry score of 0.46 ± 0.03 indicating that there was no limb preference (~50% use of left and right forelimbs) for exploring the cylinder. HI rats had an asymmetry score of 0.36 ± 0.03, indicating that they prefer using the nonaffected (ipsilateral) forelimb when exploring the cylinder. Preconditioning with hypoxia prevented the ipsilateral forelimb preference observed after HI, with rats having an asymmetry score of 0.49 ± 0.02. $CoCl_2$ and DFX failed to exert significant effects on forelimb asymmetry after HI (Fig. 3*C*).

Locomotor activity has previously been shown to be altered at various times after neonatal HI injury (24,25). We did not observe any significant changes in locomotor activity (hori-

zontal and vertical movements or time spent moving) with HI treatment compared with control or between any of the preconditioning treatments at 5 wk after HI (data not shown).

DISCUSSION

The present study was undertaken to determine the effectiveness of various preconditioning treatments on functional outcomes after HI injury in rats in addition to morphologic outcomes. We have shown that preconditioning with HP, DFX, and $CoCl_2$ can improve some long-term functional deficits and also reduce the total lesion size after HI. HP and $CoCl_2$ preconditioning were able to reduce the extent of cortical injury and only HP reduced striatal damage. Although many treatments have been tested in the HI model at short term intervals, only a few have been examined and found to yield long-term functional outcomes (3,16,26,27). Using preconditioning with hypoxia and two hypoxia-mimetic compounds, $CoCl_2$ and DFX, we observed long-term protective actions of these treatment strategies. One possible mechanism involved in the neuroprotection described in the present study is likely to be the induction of the transcription factor HIF-1, its many target genes and adaptive processes (12).

Previous studies have found that HI causes a number of functional deficits including slowing of various developmental milestones (eye opening, righting reflex), locomotor activity and sensorimotor and memory deficits (24,25,28,29). The present study used three different functional indices to assess behavioral deficits after HI and of these, the grip traction test which is used to assess forelimb muscle strength, appeared to be the most useful index relating to total lesion volume. The grip traction test has previously been used to test forelimb placing and muscle strength after injury (20,30). Bona *et al.* (20) used the grip traction test 5 wk after rats underwent HI on p7 and observed a deficit in forelimb muscle strength, which was accompanied by a large ipsilateral lesion. We observed a similar deficit in the grip traction test after HI, which was reversed by all of the preconditioning treatments used and this functional improvement was accompanied by a reduction in total lesion volume.

The cylinder test has been widely used in various adult and neonatal models of unilateral brain injury to assess forelimb asymmetry (21,22), but in our hands, a mild deficit was observed at 5 wk after HI compared with sham control rats. Additionally, we found a small increase in the number of foot faults made in the grid-walking test. Compared with previous studies employing the HI model to examine behavioral deficits, we seem to have used a slightly milder injury (~35% of the ipsilateral hemisphere is damaged, compared with 50–60% in other studies (15)), which may explain why not all of the behavioral deficits were different in HI animals compared with controls. Indeed, in adult rodents, small lesions produce mild deficits in sensorimotor tasks (forelimb asymmetry, reaching task, grid walking task) compared with a larger lesions (31). Previously, a correlation between the extent of damage in cortex or striatum and forelimb asymmetry has been shown in the rat HI model (32). Indeed, lesions to the ventrolateral regions of the caudate putamen can impair initiation of stepping movements, sensorimotor orientation, and skilled motor behavior in rats and to impair motor coordination (33,34). Such movements are required to perform the grid walking and cylinder tests. Nonetheless, we were able to show that preconditioning with hypoxia could improve function in cylinder and grid walking tasks and reduce cortical and striatal damage after HI.

We did not detect any changes in locomotor activity at 5 wk after HI, which is in agreement with the findings of Bona *et al.* (20). Previous studies have reported that HI can cause changes in spontaneous locomotor activity, at different ages after HI (24). HI only appears to result in an increase in locomotor activity in young rats (postweaning ~p21), and returns to normal at a later age (p90). However, when the injury is severe enough, hyperactivity can persist into adulthood (25).

HP has previously been shown to improve functional recovery after HI in the cylinder test and memory test (15), with protection being observed in cortex, striatum, and hippocampus. In the present study, we confirmed that HP could reverse limb asymmetry in the cylinder test and also found that HP can preserve forelimb muscle strength and reduce the number of foot faults made after HI. Thus, the neuroprotective and functional improvements resulting from HP are long lasting, and not simply because of a delay in cell death processes.

The protective effects of preconditioning with HP, $CoCl_2$, and DFX have previously been shown to relate to their ability to increase HIF-1 (9), but could also involve other mechanisms such as their ability to penetrate the blood brain barrier (BBB) or effects on brain vasculature and cerebral blood flow. Although hypoxia reversed all of the functional deficits after HI, preconditioning with $CoCl_2$ and DFX only improved function in the grip traction test. Both hypoxia-mimetics reduced total lesion volume, but only $CoCl_2$ was able to reduce the size of the cortical lesion produced by HI. Cobalt ions have been found to readily cross the BBB in rodents and humans (35,36). In addition, studies have shown that $CoCl_2$ can induce protective effects in the brain and possible neuroprotective mechanisms involve the production of HIF-1 and HIF-1 target genes (erythropoietin, VEGF) and other protective proteins including heme oxygenase-1 and metallothionein (10,13,37). Although we did observe a robust histologic protective effect of $CoCl_2$ preconditioning against total lesion volume and cortical injury in the present study, the grip traction test was the only functional test that was improved by $CoCl_2$ treatment. Recently, $CoCl_2$ was shown to produce *in vivo* brain protection against hypobaric hypoxia (37); however, the present study is the first to indicate that $CoCl_2$ can produce a long-term functional and histologic protection up to 5 wk after HI injury.

Preconditioning with DFX improved forelimb muscle strength and reduced total lesion volume, but did not reduce the other two behavioral deficits measured after HI. When DFX is administered before insults, it appears to produce a mild protective effect against neonatal HI (9). Interestingly, when DFX is given after an injury, when the BBB is open, greater neuroprotection is observed against cerebral ischemia in neonatal (38) and adult rats (39). Brain levels of DFX have been measured in neonatal rats and represent only ~65% of the levels present in the brains of HI-injured animals (40).

More recently, DFX was shown in rats to induce long term functional improvements after middle cerebral artery occlusion (41) and these protective actions are thought to be mediated by HIF-1 and VEGF. In agreement with our own data demonstrating that DFX does not protect the striatum, long-term postinjury administration of DFX was unable to reduce striatal injury after middle cerebral artery occlusion (41). We have demonstrated the long-term neuroprotective properties of treatments, which activate the HIF-1 pathway and further evaluation of the mechanism of action of these agents and related compounds are likely to be incisive.

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