Selective Losses of Brainstem Catecholamine Neurons After Hypoxia-Ischemia in the Immature Rat Pup

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ABSTRACT: Hypoxic-ischemic (HI) injury in the preterm neonate incurs numerous functional deficits, however little is known about the neurochemically-defined brain nuclei that may underpin them. Key candidates are the brainstem catecholamine neurons. Using an immature animal model, the postnatal day (P)-3 (P3) rat pup, we investigated the effects of HI on brainstem catecholamine neurons in the locus coeruleus, nucleus tractus solitarius (NTS), and ventrolateral medulla (VLM). On P21, we found that prior P3 HI significantly reduced numbers of catecholaminergic neurons in the locus coeruleus, NTS, and VLM. Only locus coeruleus A6, NTS A2, and VLM A1 noradrenergic neurons, but not NTS C2 and VLM C1 adrenergic neurons, were lost. There was also an associated reduction in dopamine-beta-hydroxylase-positive immunolabeling in the forebrain. These findings suggest neonatal HI can affect specific neurochemically-defined neuronal populations in the brainstem and that noradrenergic neurons are particularly vulnerable to HI injury. (*Pediatr Res* 63: 364–369, 2008)

Prematurity and hypoxia-ischemia (HI) in newborn infants are critical risk factors contributing to perinatal mortality and neurologic morbidity. Perinatal HI in the preterm neonate can lead to long-term brain injury and a broad range of neurologic deficits that include motor disabilities, autonomic dysfunction, epilepsy, memory, and attention disorders (1,2). Premature neonates are particularly susceptible to white matter injury in the brain. Neuronal injury in the brain also ensues and damage to certain areas of the brain may be associated with neurologic impairments in adults or children who have experienced HI as a neonate. However, very little is known about how perinatal HI affects neurochemically-defined groups of neurons in the immature preterm brain. It is therefore difficult to ascertain which specific brain nuclei and neuronal networks might be responsible for, or at least contribute to, the long-term functional deficits observed and how different neurons vary in their susceptibility to HI injury.

Injury to the brainstem after HI may be a critical contributor to autonomic and cardiorespiratory dysfunction in premature infants and neonatal animals (3–5). The brainstem consists of well-defined neurochemical groups of neurons, in particular the catecholamine neurons, serving as critical integratory sites in the brain. Distinct populations of brainstem catecholamine neurons reside in the locus coeruleus (A6 noradrenergic neurons), nucleus tractus solitarius (NTS) (A2 noradrenergic and C2 adrenergic neurons), and ventrolateral medulla (VLM) (A1 noradrenergic and C1 adrenergic neurons). These neuronal populations constitute the major sources of noradrenergic and adrenergic networks in the brain. The NTS and VLM catecholamine neurons, located in the medulla oblongata, have major roles in cardiovascular and respiratory control mechanisms, gastrointestinal, vasomotor tone, and neuroendocrine regulation (6,7). The locus coeruleus A6 noradrenergic neurons in the pontine tegmentum have significant roles in central processing of attention, arousal, learning, cognitive behaviors, and have also been implicated in respiratory control (8,9). Thus, the brainstem catecholamine neurons represent key candidates that might contribute to numerous HI-induced functional deficits.

Brainstem injury occurs in the dorsal medulla and pontine structures in neonates with HI encephalopathy (4,10–13). However, the effects of perinatal HI on specific neurochemical populations in the brainstem, such as the locus coeruleus, NTS and VLM catecholamine neurons, have not been investigated. In the adult rat, the noradrenaline concentration in the locus coeruleus and ipsilateral cortex is reduced after middle cerebral artery occlusion (14,15). Although the brainstem catecholamine populations are the major sources of noradrenaline in the brain, it is not known if these findings reflect losses of brainstem noradrenergic neurons. Necrotic and apoptotic markers have been demonstrated in the brainstem of postnatal day (P)-7 (P7) rat pups subjected to HI (16) but others, using prenatal hypoxia models, have found conflicting effects (17–19). The different findings may reflect the type of hypoxia

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Abbreviations: ACTH, adrenocorticotropin hormone; D β H, dopamine-beta-hydroxylase; HI, hypoxia-ischemia; HPA, hypothalamic-pituitary-adrenal; MBP, myelin basic protein; NTS, nucleus tractus solitarius; P, postnatal day; PNMT, phenyl-N-methyl transferase; TH, tyrosine hydroxylase; VLM, ventrolateral medulla

and/or ischemia model, species differences, the region of the brainstem investigated, or the developmental stage at which the perturbation was applied.

In the present study, we hypothesized that HI in the immature preterm brain can affect brainstem catecholamine neurons. We examined whether HI in P3 rat pups can alter numbers of brainstem noradrenergic and adrenergic neurons in the locus coeruleus, NTS, and VLM 18 d after HI. We also investigated whether there are changes in forebrain catecholamine fibers after P3 HI.

MATERIALS AND METHODS

Subjects. Experiments were carried out on Wistar rats. Dams and their pups were housed under standard laboratory conditions in temperature-controlled rooms (22°C) maintained on a 12-h light/dark cycle (lights on 6.00 AM). Food and water were available *ad libitum*. To minimize possible influences of circadian rhythms, experiments were completed between 8.00 AM and 11.00 AM. All the procedures were conducted in accord with ethical approvals and experiment guidelines stipulated by the University of Auckland Animal Ethics Committee. All efforts were made to minimize the number of animals used and any suffering.

HI insult. On P3, pups of mixed sexes were randomly assigned to a control ($n = 10$) or a HI group ($n = 11$). Pups subjected to HI were anesthetized using halothane administered *via* tubing placed over the pup's snout. The right common carotid artery was isolated from surrounding tissue, cauterized, and cut. Pups were then allowed to recover for 30 min at 37°C in 85% $\pm$ 5% humidity then subjected to 30 min of 6% O₂ at 37°C in the humidified chamber. After hypoxia, pups were returned to the dam. The control group did not undergo surgery but were placed in the chamber and breathed room air for 30 min before being returned to the dam. This is an established preterm HI model in the immature rat pup that produces typical behavioral and pathologic features seen in human premature neonates who have experienced a HI event (20–24). The P3 brain development stage is analogous to the preterm human neonate brain at approximately 24–28 wk gestation in terms of number of synapses, neurochemical development, and cortical organization (22,25).

Immunohistochemistry. On P21, animals were killed by pentobarbitone overdose (80 mg/kg, i.p.) and perfused transcardially with 4% formaldehyde (in 0.1 M PBS, pH 7.4). The brain was removed and postfixed in formaldehyde. The brainstem was cryoprotected overnight in 10% sucrose (in 0.1 M PBS, pH 7.4, 4°C) after which serial 50 μ m sections were collected using a sliding microtome. The forebrain was subjected to an increasing percentage of ethanol then embedded in paraffin. Consecutive 6 μ m forebrain sections were collected at 100 μ m intervals onto Menzel Superplus adhesive slides and dried. Sections were then dewaxed using xylene and rehydrated in preparation for immunohistochemistry.

For brainstem immunohistochemistry, two 1-in-5 series of brainstem sections were processed for cytoplasmic antigen detection of the catecholamine synthesis enzymes tyrosine hydroxylase (TH) (1:15,000; Diasorin, Stillwater) and phenyl-*N*-methyl transferase (PNMT) (1:30,000; Diasorin, Stillwater). For forebrain immunohistochemistry, two series of sections were immunolabeled; one for myelin basic protein (MBP) (1:10,000; Chemicon International, CA) and one for the catecholamine marker dopamine-beta-hydroxylase (D β H) (1:5,000; Chemicon International, CA). Each series was incubated for 36 h in the respective primary antibody followed by 2 h incubation in secondary antibody biotinylated antimouse (TH, MBP, and D β H; 1:400, Jackson ImmunoResearch, PA) or biotinylated antirabbit (PNMT; 1:400, Jackson ImmunoResearch, PA). Sections were then immersed in an avidin-biotin-horseradish peroxidase complex solution (Vector Elite Kit) for 2 h. Horseradish peroxidase activity was visualized with diaminobenzidine. To minimize possible variations in immunocytochemistry, sections from the control and experiment groups were processed simultaneously. Sections were mounted on chrome-alum slides, dehydrated in a series of alcohols, cleared in xylene, and coverslipped.

Data analysis. In the medulla oblongata, numbers of TH- and PNMT-positive cells were counted at 250 μ m intervals over 17 sections from 2.5 mm caudal to 1.5 mm rostral to obex along the VLM and NTS cell columns. The VLM and NTS contain partially overlapped populations of noradrenergic cells (the A1 and A2 cell groups) situated in caudal aspects and adrenergic cells (the C1 and C2 cell groups) situated in rostral aspects. Because PNMT only occurs in adrenergic cells, counts of PNMT-immunolabeled cells were VLM C1 or NTS C2 adrenergic cells. The number of noradrenergic A1 or A2 cells was estimated by subtracting the number of PNMT cells in one section

from the number of TH cells in the adjacent section (26). For the pontine locus coeruleus, numbers of TH-positive cells were counted in two sections.

Forebrain sections were immunolabeled to visualize MBP- and D β H-immunoreactivity. The density of MBP- or D β H-immunolabeling was determined in four consecutive sections using phase analysis software (analysis Life Science Research) and the percentage change ipsilateral to the carotid ligation relative to the left hemisphere was calculated. To determine the effect of P3 HI on cerebral hemisphere size, the outlines of the left and right cerebral hemispheres from four consecutive sections (100 μ m intervals) were traced using the software program analysis Life Science Research. In the brainstem, area was determined in the same way in sections from -0.5 to 0.25 mm. The percentage change ipsilateral to ligation relative to the contralateral side was calculated. The effects of P3 on HI were determined by comparing numbers in control and HI animals using an analysis of variance followed by *t* test *post hoc* tests. Data are expressed as mean $\pm$ SEM. Statistical significance was set at $p < 0.05$.

RESULTS

Effects of P3 HI on brain area and myelin. In animals subjected to HI, there was an 18.0% reduction in cerebral hemisphere size ipsilateral to the ligation compared with the nonligated side, however, there was no change in brainstem area size ipsilateral to the ligation ($-0.003\% \pm 0.005\%$). We observed a 17.2% reduction in MBP-positive immunolabeling ipsilateral to the carotid ligation compared with the nonligated side. Compared with control animals, there was no significant difference in the cerebral hemisphere size, brainstem area, or MBP-immunolabeling on the nonligated side. These findings extend and confirm that the P3 HI insult produced outcomes comparable with previous studies (20,21).

Effects of HI on NTS catecholamine neurons. In animals subjected to P3 HI, there was a significant reduction in the total number of NTS TH-positive neurons compared with the total NTS TH counts in control animals. After P3 HI, there was a significant 35.7% reduction in numbers of NTS catecholamine neurons and a significant 35.3% NTS A2 noradrenergic neurons ipsilateral to the carotid ligation compared with counts in control animals on the corresponding right side (Figs. 1 and 2). The majority were lost from -2.25 to -0.5 mm relative to obex (Fig. 3). In contrast there was no effect of P3 HI on total NTS C2 adrenergic neuron counts ipsilateral to the ligation (Fig. 2) and there was no difference in C2 adrenergic counts from control and P3 HI along the entire rostro-caudal extent of the NTS (Fig. 3). For all NTS catecholamine counts, there were no significant differences between control and P3 HI counts on the nonligated side.

Effects of HI on VLM catecholamine neurons. In animals subjected to P3 HI, there was significant reduction in the total

Figure 1. Cross sections through the NTS immunolabeled for TH (A and B) in a rat subjected to P3 HI on the nonligated (A) and ligated side (B). Scale bar = 50 μ m.

Figure 2. Counts of NTS catecholamine neurons, A2 noradrenergic neurons and C2 adrenergic neurons on the nonligated (A) and ligated side (B) in control (■) and P3 HI animals (□). *** $p < 0.01$.

number of VLM TH-positive neurons compared with control animals. After P3 HI, there was a significant, 57.1%, reduction in numbers of VLM catecholamine neurons ipsilateral to the ligation compared with counts in control animals on the corresponding right side (Figs. 4 and 5). There was also a reduction in the number of TH-positive neurons on the nonligated side. Compared with control animals there was a significant reduction in the number of A1 noradrenergic neurons on both the ligated (58.6% reduction) as well as the nonligated side (35.0% reduction) in the VLM of P3 HI animals (Figs. 4 and 5). On the nonligated side, VLM A1 noradrenergic neurons were predominantly lost between -1.0 and -0.75 mm relative to obex (Fig. 6B). Ipsilateral to the ligation, VLM A1 noradrenergic neurons were predominantly lost from -1.5 to 0.5 mm relative to obex (Fig. 6E). In contrast, there was no effect of P3 HI on numbers of C1 adrenergic neurons compared with control counts on either side of the VLM (Figs. 5 and 6).

Effects of HI on locus coeruleus catecholamine neurons. After P3 HI, TH neurons were lost in the locus coeruleus (Fig. 7). Ipsilateral to the ligation in P3 HI animals, we observed a significant 24% reduction in the total number of TH-positive

neurons compared with the nonligated side. There was no significant difference between control and P3 HI TH-positive neuron counts on the nonligated side.

Effects of HI on D β H-positive immunolabeling in the forebrain. Because the major effect of P3 HI was on noradrenergic neurons in the brainstem, we investigated whether forebrain D β H-positive immunolabeling was also affected by P3 HI. After P3 HI, there was a significant 27.5% loss of D β H-immunolabeling ipsilateral to the ligation compared with control animals on the corresponding right side. There was no difference in D β H-immunolabeling between control and P3 HI groups on the nonligated side.

DISCUSSION

An outstanding finding of the present study was that damage occurs in the brainstem after P3 HI. This is commensurate with reports in human neonates describing neuronal losses in the brainstem after fetal asphyxia, HI, or acute perinatal asphyxia (4,10–13). We present novel evidence that brainstem neurons are lost and that numbers of catecholamine neurons in the locus coeruleus, NTS, and VLM are decreased 18 d after P3 HI. We found that only noradrenergic, but not adrenergic, neurons were affected by P3 HI. In addition, we report an associated reduction in forebrain D β H-immunolabeling. Together these findings suggest that P3 HI disrupts catecholaminergic networks in the immature rat brain and that specific neurochemically-defined populations in the brainstem, the noradrenergic neurons, are lost after P3 HI.

Effects of P3 HI on NTS catecholamine neurons. We observed a significant loss of noradrenergic neurons in the NTS ipsilateral to the ligation 18 d after P3 HI but no change in NTS C2 adrenergic neuron counts, suggesting that the C2 adrenergic population is less vulnerable to HI-induced injury than NTS A2 noradrenergic neurons. Consistent with our findings, it has been reported that neurons localized in cranial

Figure 3. Rostrocaudal distributions of NTS catecholamine neurons (A, D), A2 noradrenergic neurons (B, E) and C2 adrenergic neurons (C, F) on the nonligated (A–C) and ligated side (D–F) in control (●) and P3 HI animals (○). * $p < 0.05$; *** $p < 0.01$.

Figure 4. Cross sections through the VLM immunolabeled for TH (A, B) in a rat subjected to P3 HI on the nonligated (A) and ligated side (B). Scale bar = 50 μ m.

Figure 5. Counts of VLM catecholamine neurons, A1 noradrenergic neurons and C1 adrenergic neurons on the nonligated (A) and ligated side (B) in control (■) and P3 HI animals (□). * $p < 0.05$; ** $p < 0.01$.

nerve nuclei, including the NTS, are severely damaged in premature infants with HI encephalopathy (10,13). The selective loss of A2 noradrenergic neurons could influence a number of functions including cardiovascular, respiratory, and autonomic regulatory mechanisms and thus may provide a neuroanatomical basis for certain deficits observed in preterm infants who have previously experienced a HI episode.

VLM catecholamine neurons. The VLM exhibited the greatest reduction in numbers of A1 noradrenergic neurons after P3 HI. In the VLM, effects were predominantly ob-

served ipsilateral to the ligation; however, the nonligated side also showed a significant reduction in numbers of VLM A1 noradrenergic neurons. There was no effect of P3 HI on VLM C1 adrenergic neuron counts suggesting that adrenergic populations are less vulnerable to HI-induced injury than noradrenergic neurons, as seen in the NTS.

The VLM has major roles in the regulation of the cardiovascular system, vasomotor tone, neuroendocrine control, and sympathetic outflow. The VLM A1 noradrenergic neurons in particular are critical in the control of neuroendocrine systems involving the hypothalamus (26–28) and disruption of neural inputs from the A1 neurons could have considerable effects on oxytocin, vasopressin, and ACTH secretion. Disruption of hypothalamic-pituitary-adrenal (HPA) axis function occurs after HI injury (29–31) and A1 and A2 neurons contribute to HPA axis responses (26,32). Thus, the observed losses of noradrenergic neurons might contribute to such neuroendocrine imbalances after a perinatal insult.

Locus coeruleus noradrenergic neurons. In the locus, coeruleus neuronal losses were observed ipsilateral to the ligation. Noradrenaline concentration is reduced in the adult locus coeruleus after middle cerebral artery occlusion (14) and thus a loss of locus coeruleus neurons, as seen in the present study, may contribute to such an effect.

The locus coeruleus provides the major source of noradrenergic inputs to the brain innervating widespread brain areas including the cortex, hippocampus, thalamus, and hypothalamus and has a significant role in the central processing of alertness, learning, adaptive behaviors, arousal, and sleep (8,9). Many of these functions are disrupted after perinatal HI injury and thus loss of locus coeruleus neurons may contribute to such deficits.

Forebrain DBH immunolabeling. After P3 HI, we observed a significant reduction in DBH-immunolabeling in the

Figure 6. Rostrocaudal distributions of VLM catecholamine neurons (A, D), A1 noradrenergic neurons (B, E) and C1 adrenergic neurons (C, F) on the nonligated (A–C) and ligated side (D–F) in control (●) and P3 HI animals (○). * $p < 0.05$; ** $p < 0.01$; † $p < 0.001$.

Figure 7. Cross sections through the locus coeruleus immunolabeled for TH on the nonligated (A) and ligated side (B) in a rat subjected to P3 HI. Counts of TH-positive neurons (C) on the nonligated (■) and ligated side (□). ** $p < 0.05$. Scale bar = 50 μm .

forebrain ipsilateral to the ligation suggesting that losses of noradrenergic and adrenergic labeling occurred. The reduction in D β H-immunolabeling was greater than the cerebral hemisphere area change and thus it is unlikely that this effect was solely a reflection of hemisphere size. However, given the selective loss of brainstem noradrenergic neurons it is conceivable that the reduction in D β H-immunolabeling reflects the loss of noradrenergic and not adrenergic fiber networks in the brain. This is consistent with findings in the adult rat that cerebral ischemia produces a 30–40% reduction in noradrenaline concentration in the ipsilateral cortex (15) and that prenatal hypoxia significantly alters D β H-immunolabeling in the guinea pig forebrain (33). Interestingly, after prenatal exposure to hypoxia, brain regions that exhibit changes in *in vivo* TH activity at P21 are noradrenergic areas of the brainstem (17) and regions innervated by noradrenergic fibers such as the motor cortex area and hippocampus (18); further emphasizing the vulnerability of the noradrenergic system to perinatal insults.

Putative mechanisms affecting noradrenergic neuron survival. The mechanisms by which locus coeruleus, NTS, and VLM noradrenergic neurons are affected by P3 HI are not known. The brainstem itself is not believed to endure ischemia in models of HI that involve occlusion of the middle cerebral artery or the common carotid artery, particularly because the brainstem lies outside the vascular fields of these vessels. Instead blood flow to the brainstem increases during HI (34). In addition, unlike the forebrain, there was no change in brainstem area after P3 HI. Thus, the brainstem can be considered remote from primary HI injury sites in the forebrain and brainstem injury may evolve *via* secondary injury mechanisms. In this context, several postulated mechanisms may be responsible for the observed noradrenergic neuronal losses in the brainstem after P3 HI. Although our results indicate that there was not a global loss of neurons in the brainstem, it is also conceivable that other noncatecholamine populations of neurons could also be affected by P3 HI.

Factors that may have contributed to the degeneration of neural connections include excitotoxic mechanisms affecting axonal and cell body survival, neuroinflammation, loss of synaptic inputs, and target deprivation (35,36). The reduction in D β H-immunolabeling may be directly attributed to the degeneration of noradrenergic neurons in the brainstem. Alternatively, retrograde degeneration of noradrenergic projections may also have led to the loss of noradrenergic cell bodies in the locus coeruleus, NTS, and VLM. A further possibility is

that higher brain centers that sustain significant neuronal injury after HI, and with descending inputs to the brainstem noradrenergic neurons, might be responsible for the observed neuronal losses through loss of synaptic input and stimulation of brainstem neurons. For example, the cerebral cortex has direct projections to the NTS and the VLM (37) and in the case of the VLM there are bilateral neural pathways (38). The bilateral loss of VLM neurons may also result from NTS neuron losses because bilateral connections exist between the NTS and VLM (39,40) including direct, excitatory NTS inputs to VLM A1 noradrenergic cells (40). The precise sequence of events and potential brain regions involved in the loss of brainstem neurons are not known and future studies are required to at least clarify the temporal relationships of the observed changes.

The caudal distribution of NTS and VLM noradrenergic neurons may dictate their vulnerability to HI-induced injury. Based on functional divisioning in the NTS, caudally distributed A2 cells are influenced primarily by peripheral chemosensory inputs (41). After transient focal ischemia in the adult rat Nnos expression in the caudal VLM increases but decreases in the rostral VLM (42). Using nuclear Fos as a marker of neuronal activation, hypoxia primarily activates A2 noradrenergic neurons (43) and middle cerebral artery occlusion in adult rats induces Fos in TH-positive neurons of the NTS and VLM; the majority of Fos-positive TH neurons apparent in the VLM (44,45). These distribution patterns are similar to the selective injury patterns in the present study. The significance of these putative associations may be relevant to the long-term synaptic and metabolic status of the A1 and A2 neurons caused by secondary injury mechanisms after the P3 HI insult. Future studies may elucidate whether there are regional and/or inherent differences in cellular or molecular mechanisms pertaining to noradrenergic neurons compared with adrenergic neurons.

Concluding remarks. We provide evidence that brainstem catecholamine neurons in the locus coeruleus, NTS, and VLM are lost because of perinatal HI and that there are associated changes in forebrain D β H-immunolabeling. This study pinpoints changes in brainstem noradrenergic neuron populations suggesting that noradrenergic networks in the brain are particularly susceptible to injury after P3 HI. Autonomic, cardiorespiratory, movement, and attention disorders may occur in children previously exposed to perinatal HI and these functions involve brainstem catecholamine neurons. The locus coeruleus, NTS, and VLM noradrenergic neurons provide extensive neural inputs to many forebrain structures fulfilling

their important integratory roles in the CNS and thus disruptions to noradrenergic neurocircuitry could potentially contribute to HI-induced deficits.

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