A Rat Model of Severe Neonatal Hypoxic-Ischemic Brain Injury

Philip H. Schwartz, PhD; Walid F. Massarweh, MD; Harry V. Vinters, MD; and Claude G. Wasterlain, MD

Background and Purpose: Perinatal hypoxic-ischemic brain injury is a common problem with severe neurological sequelae. In this report we describe in detail a simple model of hypoxia-ischemia in the neonatal rat that gives rise to severe neocortical infarction and to selective hippocampal neuronal necrosis.

Methods: Seven-day-old Simonsen Wistar rat pups underwent bilateral carotid artery ligation under methoxyflurane anesthesia and, after a 4 to 6-hour recovery, were exposed to 60 minutes of hypoxia (6.5% O₂); they were perfusion-fixed 3 days later for histological study. Brain temperature was monitored throughout this treatment.

Results: We found that 64±3% of neocortex above the rhinal sulcus was infarcted; this infarction was evenly distributed through the cerebral hemispheres. In the hippocampus, neuronal necrosis was selective for the internal (hilar) layers of granule cells of the dentate gyrus, with relative sparing of CA1 pyramidal cells. In addition, brain temperature was tightly controlled throughout the experimental manipulations.

Conclusions: The present model is easy and sensitive and provides an infarct of sufficient severity and homogeneity to make it well suited for pharmacological and biochemical studies directed toward therapeutic amelioration and mechanisms of hypoxic-ischemic brain damage, respectively. In addition, the pattern of damage in the hippocampus is quite different from that seen in the adult brain, which should be helpful in studying the ontogeny of selective vulnerability. (Stroke 1992;23:539–546)

KEY WORDS • animal models • anoxia • cerebral ischemia • rats

Perinatal hypoxic-ischemic brain injury is a common problem with severe neurological sequelae that include cerebral palsy, mental retardation, and seizures.¹⁻³ Rat models have been described for the study of neonatal hypoxia-ischemia⁴⁻⁶ but exhibit considerable variability or do not provide a cortical infarct of sufficient homogeneity for biochemical sampling.

Rice et al⁴ described a modified Levine⁷ preparation in immature rats that consisted of unilateral carotid ligation followed by exposure to a hypoxic (8% O₂) environment for several hours. We have altered this model by ligating both carotid arteries and increasing the severity (i.e., 6.5% versus 8.0% O₂) and reducing the length (i.e., 1 versus 3 hours) of hypoxic exposure.⁸⁻⁹ This accomplished our goal of obtaining severe, reproducible neocortical infarction, revealed a pattern of hippocampal lesions that strikingly differ from that seen in older animals, and may provide a unique tool for studying the ontogeny of selective vulnerability. In the present study we present the details of the methodology used as well as those of the quantitative histological findings. In addition, we discuss the rationale of the methodology and the application of statistical analyses for comparing control versus drug-treated animals.

Materials and Methods

Timed-pregnant female Wistar rats were purchased from Simonsen Labs (Gilroy, Calif.) and were kept on a 12:12 hour light:dark cycle with free access to food and water. Seven-day-old pups (n=134, taken from 33 different litters; day of delivery=day 1) were anesthetized by placement in an air-tight jar containing cotton soaked with methoxyflurane (Metofane, Pitman-Moore, Inc., Washington Crossing, N.J.). Anesthetized pups were placed on a circulating water pad (37°C) and continuing anesthesia was delivered as needed through a small plastic tube, adapted to the pups' nose and mouth, containing cotton soaked with methoxyflurane. Both carotid arteries were exposed through a midline anterior neck incision, isolated from vein and nerve, doubly ligated with 4-0 surgical silk, and severed between the two ligatures (right artery first). The total surgery time averaged 6 minutes, and the time between the ligation of each artery averaged 2 minutes. The pups were allowed to recover for 4–6 hours without the dams. The first and last 30 minutes of recovery took place in a container in a 37°C waterbath, the rest in a warmed (34°C) cage.
Ten to 12 ligated pups, representing animals from at least two different litters, were exposed to a warmed (37°C), humidified atmosphere of 6.5% O₂/93.5% N₂ (Ohio Anesthesia Machine, Airco, Inc., Madison, Wis.) at a flow rate of 1.0 l/min in an airtight 2.0-l plastic container placed in a waterbath at 37°C. Pups were exposed to this environment for periods of 15, 30, or 60 minutes, designated HI₁₅ (n=5), HI₃₀ (n=10), and HI₆₀ (n=116), respectively. The pups were allowed to recover for an additional 30 minutes in room air in a 37°C
FIGURE 1. Photomicrographs of hypoxic-ischemic neocortical infarction (light areas) in three rostrocaudal levels of the brain of a representative rat pup of the HI\textsubscript{M} group. There was an average of 77.7\% of the neocortex infarcted above the rhinal sulcus in this animal. Panel A: A4.4\,mm, 87.0\% neocortical infarction; panel B: A3.2\,mm, 86.4\%; and panel C: A2.0\,mm, 82.0\%. Percentages of damage in the three levels not shown were as follows: A7.0\,mm, 73.7\%; A5.6\,mm, 84.6\%; and A0.8\,mm, 52.6\%. Hematoxylin and eosin stain. Original magnification x3.3; bar, 788 \,

Waterbath, and then were kept in a warmed (34°C) cage until sacrifice. In preliminary experiments, it was observed that some pups were either ignored or cannibalized by the dams; thus, in order to maintain equality of nutrition among the pups, they were not returned to the dams but were injected subcutaneously, twice daily, with 5\% dextrose solution (5\% of body weight). As previously reported,\textsuperscript{8,9} pups who were kept with their dams and survived gained approximately 24\% of their body weight during the 3-day recovery period, whereas those administered glucose lost 18\%.

Three days after hypoxic exposure, pups were anesthetized (50 mg/kg pentobarbital) and perfusion-fixed with 4\% isotonic neutral buffered paraformaldehyde preceded by a 5-ml flush with heparinized normal saline. The heads were removed and placed for 24 hours in the same fixative at room temperature. Serial 8-\mu m paraffin-embedded coronal sections were stained with hematoxylin and eosin and examined, unblinded, under the light microscope.

As a previous study\textsuperscript{4} had shown that neither hypoxia nor carotid ligation alone produced neuropathology, we attempted to confirm these findings with controls that included untreated animals of the same age and litter as the experimental animals (n=3), as well as animals subjected to 60-minute hypoxic exposure (n=3) alone and animals with bilateral carotid ligations without hypoxia (n=3).

In neocortex the percentage of infarcted area, clearly visible due to a lack of staining, was calculated from coronal sections at six stereotactic levels of the neocortex using an image analyzer (IBAS, Kontron Bildanalyse, FRG). The areas examined were as follows: A7.0\,mm, at the genu of the corpus callosum and the anterior olfactory nuclei; A5.6\,mm, at the rostral striatum and nucleus accumbens; A4.4\,mm, at the caudal striatum and the preoptic area; A3.2\,mm, at the internal capsule and the anterior tip of the dorsal hippocampus; A2.0\,mm, at the middle of the dorsal hippocampus and the habenular nuclei; and A0.8\,mm, at the ventral hippocampus and mammillary body.\textsuperscript{10} The percentage of infarcted cortex (outlined on the screen of the image analyzer) was obtained by dividing the surface of infarcted cortex by the surface of the neocortex above the rhinal sulcus, in each section examined.

Counting of individual hippocampal neurons was carried out under the light microscope (\times400) at the level of the dorsal hippocampus in a single transverse section (A2.0\,mm) per animal. Necrotic neurons, defined as those with pyknotic nuclei and eosinophilic cytoplasm,\textsuperscript{11} were counted in the dentate granule cell layer, the dentate hilus (CA4), and the pyramidal cell fields of CA3, CA1, and the subiculum. In some of the HI\textsubscript{M} animals (n=10), the total number of neurons was counted in each area to calculate the percentage of necrotic neurons.
In areas of patchy damage (thalamus, evaluated at A2.0mm, and caudate/putamen, evaluated at A4.4mm), a subjective grading scale (0–5) was used to assess the degree of damage in the HI15 and HI30 groups and in those pups from the HI60 group in which total hippocampal cell counts were made. Grade 0 indicates no damage; grade 1, up to 10 ischemic neurons; grade 2, 11–20; grade 3, 21–50; and grade 4, more than 50 ischemic neurons per high power field in the most severely affected area. Grade 5 shows infarction in addition to selective neuronal necrosis. Both left and right hemispheres were evaluated individually, and the results are expressed as a percentage of individual hemispheres showing a particular grade.

In three HI15 animals, brain temperature was monitored with a microthermocouple radio transmitter temperature probe (model XM-FH, Mini-Mitter Co., Inc., Sunriver, Ore.) that had been implanted into the parietal cortex at the same time that the carotid arteries were ligated. The probe consisted of a transmitter portion 8 mm in diameter and 10 mm in height and a 25-gauge thermocouple portion 4 mm long. The total weight of the unit was 1.11 g. After a 4–6 hour recovery, the animals were exposed to hypoxia as described above. Serial cortical temperature readings were recorded before, during, and after hypoxia.

Statistical analysis of the data was performed using SAS (SAS Institute, Cary, N.C., version 6.04) and included two-tailed paired and unpaired Student’s $t$ tests and Fisher’s exact test. Results are expressed as mean±SE, and $p<0.05$ was considered significant.

### Results

No infarction or selective neuronal necrosis was detected in untreated animals or those having hypoxia or bilateral carotid ligation alone. The mortality rate for the duration of the experiment was zero for the 15-minute hypoxic exposure group, 10% for the 30-minute exposure group, and 32% for the 60-minute exposure group. For the HI30 group, the single mortality occurred during hypoxic exposure; for the HI60 group, 90% of the mortalities occurred during hypoxia and the other 10% during the 3-day recovery period.

Bilateral cortical infarcts were seen in nine of the 10 rats in the HI15 group and in 72 of 74 of those in the HI30 group; a single unilateral infarct was seen in one animal of the HI15 group. Infarcts usually were not distributed symmetrically, but there appeared to be no relation to the order of carotid artery ligation. In the HI15 group, one animal suffered a left-sided infarct; in the HI30 group, all animals had bilateral infarcts, with four animals showing larger infarcts on the left side and five animals showing larger infarcts on the right side. Of those in the HI60 group that showed infarction, 10% had bilaterally symmetrical infarcts, whereas the remainder were evenly divided between those with a larger left-sided and those with a larger right-sided infarct.

As preliminary experiments demonstrated that the HI15 group showed an infarction rate and severity that met our goals, this group was most extensively studied. Infarction in the HI15 group was sharply demarcated and was easily measured with the image analyzer (Figure 1). The infarcts predominantly involved the cortex in the territory of the middle cerebral arteries, although they sometimes extended into the territory of the anterior cerebral arteries. Rarely did infarction extend below the rhinal sulcus. The neocortical lesions ranged from a few islands of microinfarcts (mainly in layers 3–5) to transcortical infarcts in layers 2–6 (Figure 1) that, in some cases, extended into the underlying striatum and involved the intervening white matter. In areas of less than complete infarction, a columnar pattern was seen; however, occasional areas also showed a laminar pattern of necrosis predominating in the deeper cortical layers. Selective neuronal necrosis was also seen in some cortical areas with preservation of glial cells and vascular endothelium.

The extent of cortical infarction was clearly related to the duration of hypoxic exposure: in the HI15 group, the infarcted area covered 7±14% of the neocortical surface above the rhinal sulcus (n=5), whereas in the HI30 group it covered 33±10% (n=9) of neocortex ($p<0.05$ versus HI15 by Student’s $t$ test) and in the HI60 group 64±3% (n=74, $p<0.01$ versus HI15 by Student’s $t$ test). The percentage area of cortex infarcted in the HI60 group at six levels of the neuraxis was as follows: A0.8mm, 52±4; A2.0mm, 69±4; A3.2mm, 69±4; A4.4mm, 69±4; A5.6mm, 67±4; and A7.0mm, 63±5. These values show that the infarction was rather evenly distributed through the cerebral hemispheres (Figure 1). Of the HI60 pups, 11% showed 0–25% of neocortex above the rhinal sulcus infarcted, 89% showed more than 25% cortex infarcted, and 68% showed more than 50% infarcted.

The hippocampus is less sensitive than the cortex in this model. In the HI15 group no hippocampal necrosis was found, and in the HI30 group very little damage was found; therefore, cell counts were not made in these two groups. In the hippocampus of the HI60 animals, selective neuronal necrosis, but no infarction, occurred in several regions. In the dentate gyrus extensive necrosis was observed in the inner layers of granule cells (Figure 2). The pattern of necrosis was strikingly laminar, with a complete sparing of the outer layers of granule cells, those closest to the molecular layer. At the level of the dorsal hippocampus (A2.0mm), the dentate gyrus granule cells had 127±35 necrotic neurons per section, whereas CA4 had 9±3, CA3 25±10, CA1 58±27 (Figure 3), and subiculum 87±31 necrotic neurons (n=26). In a set of 10 HI60 rats with total cell counts, the percentage of necrotic neurons was as follows: superior blade of the dentate gyrus, 13.5±6.2; inferior blade of the dentate gyrus, 4.3±2.3 ($p<0.01$ versus superior blade by paired Student’s $t$ test); area CA3, 4.9±3.0; area CA1, 1.1±3.0 ($p<0.01$ versus sum of dentate by paired Student’s $t$ test); and subiculum, 10.8±6.6% ($p<0.01$ versus CA3 or CA1 by paired Student’s $t$ test). The order of damage on a percentage basis, therefore, was dentate gyrus granule cells>subiculum>CA3>CA1.

Neuronal necrosis was also seen in the thalamus (Table 1) and involved the lateral thalamic nucleus, the nucleus reticularis thalami, and the ventral posterolateral nuclei. The extent of damage, i.e., the distribution of animals displaying the various grading scores, was related to the duration of hypoxic exposure ($p<0.05$; Fisher’s exact test was used to evaluate the distribution of the numbers of animals among the various grades versus the hypoxic interval). In the caudate/putamen
FIGURE 2. Selective neuronal necrosis (defined as pyknotic nuclei and eosinophilic cytoplasm) involving inner layers and predominately superior blade (upper) of dentate gyrus granule cells (some marked with arrows) in same rat pup as in Figure 1 (A2.0mm). In this animal there were 117 necrotic neurons in the superior blade; inferior blade, 38; CA4, 1; CA3, 2; CA1, 188; and subiculum, 183. Hematoxylin and eosin stain. Original magnification ×132; bar, 39.4 μm.

(Table 1), extensive neuronal necrosis involved the dorso-medial aspect of the caudate nucleus, spreading laterally. Again, the extent of damage was related to the duration of hypoxic exposure (p<0.05 by Fisher's exact test).

A few necrotic neurons were also observed in the superior colliculi, in the hypothalamus, in occasional granule cells of the cerebellum with sparing of the Purkinje cells, in the nucleus septi accumbens, and in the amygdala.

The average brain temperature for each HI M animal varied by no more than 0.4°C for the time period including the 30 minutes before hypoxic exposure, the hypoxic exposure, and the 30 minutes after hypoxic exposure; the group average was 35.0±0.3°C for this period.

Discussion

The present model provides a severe and reproducible hypoxic-ischemic insult that gives rise to a homogeneous cortical infarction without altering brain temperature. Brain damage in all areas was highly dependent on the length of the hypoxic exposure. The most severe damage was observed in the cortex in the distribution of the middle cerebral artery, a main tributary of the occluded carotid. In addition, a pattern of selective vulnerability seen in the hippocampus appears to be unique to the neonatal animal.

This model differs in several ways from the model described by Rice et al,4 and adapted by Silverstein et al and Ikonomidou et al,6 which uses unilateral carotid ligation followed by exposure to a hypoxic environment. The damage seen in these models permits comparisons
of a hypoxic-ischemic hemisphere to a hypoxic hemisphere in the same animal but is not as homogeneous as the present model in producing cortical infarction or is more severe in subcortical structures than in the cortex.

The severity and widespread nature of the lesions in the present model should be an asset for biochemical studies because of lesser dilution of sampled tissue by healthy surviving cells. In addition, the model is well suited for studies of the therapeutic efficacy of experimental drugs because the sharp boundaries of the infarct facilitate quantitative measurements.

This model's reproducibility is an asset for pharmacological studies: 89% of the animals sustain moderate to severe neocortical infarction (>25% total neocortex infarcted), while 68% sustain severe damage (>50% total neocortex infarcted). Given this degree of variability, we can calculate the minimum amount of protection that can be detected in testing the therapeutic efficacy of neuroprotective agents. The variance of the data is ±32% in the present model; we accept the probabilities of making Type I (α) and Type II (β) errors of 0.05 and 0.1, respectively; and groups of 20 animals are reasonable. It is easy to calculate that, using β=0.1 and n=20, a 39% decrease in damage would be detected; by increasing the probability of making a Type II error to β=0.2 and increasing the n to 30, an 18% decrease would be detected.

As has been reported in other models of neonatal hypoxia-ischemia, the mortality rate and brain damage vary from litter to litter. To minimize these variations, the fasting time and interval between surgery and hypoxia are rigorously standardized because blood glucose concentrations could influence the amount of damage suffered from the insult and cerebral blood

FIGURE 3. Relative preservation of CA1 pyramidal cell field (some necrotic neurons shown with arrows) compared with dentate gyrus granule cell layer (some necrotic neurons shown with arrows) in same section and same animal as in Figure 2. Hematoxylin and eosin stain. Original magnification x66; bar, 19.7 μm.
flow after carotid ligation varies markedly with time after occlusion. The higher mortality rate in the present model compared with the unilateral model of hypoxia-ischemia, may reflect the greater severity of the insult or its bilateral nature. We have also found that the susceptibility to hypoxia-ischemia of neonatal rats within the same strain varies considerably between vendors, showing cortical infarction from bilateral ligation alone (Charles River Wistar). Pups of a different strain (Harlan Sprague-Dawley) also showed much greater brain damage than the Simonsen pups (P.H. Schwartz and C.G. Wasterlain, unpublished observations).

It is important to note that selective vulnerability within the hippocampal formation of these 7-day-old pups was strikingly different from that seen in adult ischemia models. A high proportion of dentate granule cells were necrotic. Although damage to the subiculum was similar to that seen in the dentate gyrus and may reflect the fact that this cell group already has adult levels of NMDA-receptor recognition sites at this age, CA1 pyramidal cells, which are the most vulnerable to ischemia in adults, were less severely affected. Interestingly, it has been reported in unilateral models that hippocampal damage is similar to that seen in adult ischemia models. While we have no explanation for this discrepancy, it is possible that slight age differences or the strain differences mentioned above may be a factor. Also, it is conceivable that it is the nature of the insult itself that contributes: a shorter ischemia time and more severe hypoxia in the present model compared with a longer ischemia time and less severe hypoxia in the unilateral model.

Because granule cells continuously multiply postnatally and migrate to the hilar side of the dentate gyrus granule cell layer, the layers of necrotic cells in this model represent the most recently generated granule cells. It is of interest to note that studies of the distribution of calbindin 28 kd at early postnatal ages show that this protein and, thus, calcium buffering capacity, is not present in the inner layers of the dentate gyrus. The sparing of CA1 and, to a lesser extent, of CA3 pyramidal cells is less surprising and could be due to the immaturity of Schaffer collateral or mossy fiber presynaptic terminals, which may protect the pyramidal cells in the same fashion as a lesion of the dentate gyrus granule cells or the Schaffer collaterals protects CA1 in adult animals. The 3-day delay between exposure and sacrifice was sufficient for cell necrosis to mature; therefore, it is unlikely that delayed cell death would account for the apparent preservation of some regions.

In conclusion, the present model is simple, sensitive, and well suited for pharmacological and biochemical studies. In addition, the pattern of damage in the hippocampus is quite different from that seen in the adult brain, a difference that should be helpful in studying the ontogeny of selective vulnerability.

Acknowledgments

The authors gratefully acknowledge the technical assistance of Lisa Marie Adams and the critical comments on the manuscript provided by Barney E. Dwyer, PhD, Denson G. Fujikawa, MD, and Anne M. Morin, PhD.

References

15. Kirino T: Delayed neuronal death in the gerbil hippocampus following ischemia. Brain Res 1982;239:57–69
A rat model of severe neonatal hypoxic-ischemic brain injury.
P H Schwartz, W F Massarweh, H V Vinters and C G Wasterlain

*Stroke.* 1992;23:539-546
doi: 10.1161/01.STR.23.4.539
*Stroke* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1992 American Heart Association, Inc. All rights reserved.
Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/23/4/539

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Stroke* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to *Stroke* is online at:
http://stroke.ahajournals.org//subscriptions/