Behavioral Performance of Rats Following Neonatal Hypoxia-Ischemia

RICHARD S.K. YOUNG, M.D.,* JOHN KOLONICH, B.S.,† CYNTHIA L. WOODS, M.S.,† AND SUSAN K. YAGEL, B.S.†

SUMMARY The behavioral performance of rats subjected in the neonatal period to hypoxia-ischemia at either 37°C or 21°C was compared to that of sham-ligated animals. Performance on complex motor tests was significantly delayed only in the hypoxic-ischemic 37°C rats. However, cognitive testing disclosed significant delay of spatial learning in animals subjected to hypoxia-ischemia at 21°C and those with gross infarction at 37°C. There was enhanced avoidance learning in the animals with gross infarction in the hypoxia-ischemia 37°C group. Hypoxic-ischemic damage in the neonatal rat at 37°C results in transient delay of complex motor skills, but longer lasting cognitive changes. Hypoxia-ischemia during hypothermia produces no motor deficits, although there may be similar alterations in learning.

Methods

Seven day old Sprague-Dawley rats of both sexes (Charles River Laboratories) were anesthetized with halothane (1.5–3.5%) in oxygen by mask inhalation as previously described.1,2 The right common carotid artery was exposed and ligated. The animals were returned to their dams for a 3 hour recovery period. Following the recovery period, the animals were placed in air-tight 500 ml jars with continuous flow of humidified gas (8% O₂, 92% N₂) for 4 hours. During the hypoxic exposure, hypothermia was induced (or normothermia maintained) by immersing the jars in a water bath thermostatically regulated to maintain a temperature of 37°C or 21°C. Following the hypoxic exposure, animals were returned to their dams.

Control animals were similarly anesthetized; their carotid arteries were visualized, but not ligated. The control animals were then subjected to the same experimental protocol as the experimental animals, except that they were placed in jars containing room air, rather than the hypoxic gas mixture.

Behavioral Testing

Animals were examined daily for appearance of six motor milestones according to a standardized neurologic developmental battery for rats.9 The specific skills of head lifting, walking, righting, cliff avoid-
ance, free falling (mid-air righting), and bar walking were judged as present or not present, according to published criteria. The day of mastery of the skill was noted.

Spatial learning was assessed with the Morris Water Maze. Animals were placed in a circular tub (diameter, 64 cm) filled to a depth of 15 cm with approximately 18°C water. Instant (powdered) skim milk was dissolved in water to decrease visual cues. A clear glass platform was present inside the pool; its top surface was 15 mm below the surface of the water. A trial consisted of placing a rat by hand into the water facing the wall of the pool. If the rat successfully localized the platform on a given trial, it was permitted to remain on the platform for 10 sec. If the animal was unsuccessful in locating the platform, the trial was terminated after 120 seconds. The number of trials necessary to correctly localize the platform in less than 10 seconds on 5 consecutive trials was noted.

Maze training was begun at 31 days of age. A commercially available shuttle box was used for avoidance training (Lafayette Instruments). An opening (7.8 cm diameter) separated two identical compartments (19 x 15 x 15 cm). During a trial, a light and a buzzer (conditioned stimuli) would activate in the compartment in which the rat was located. Continuous foot shock (unconditioned stimulus) would activate in the compartment. On the platform, the trial was terminated at 120 seconds. The number of trials necessary to correctly localize the platform in less than 10 seconds on 5 consecutive trials was noted.

Maze training was begun at 31 days of age. A commercially available shuttle box was used for avoidance training (Lafayette Instruments). An opening (7.8 cm diameter) separated two identical compartments (19 x 15 x 15 cm). During a trial, a light and a buzzer (conditioned stimuli) would activate in the compartment in which the rat was located. Continuous foot shock (unconditioned stimulus) would activate in the compartment. On the platform, the trial was terminated at 120 seconds. The number of trials necessary to correctly localize the platform in less than 10 seconds on 5 consecutive trials was noted.

Neuropathologic Examination

The animals' brains were removed for neuropathologic examination when behavioral testing was completed (37 days). Animals were anesthetized with pentobarbital and percutaneously perfused-fixed with formalin-acetic acid-methanol. The perfused brain was removed, further fixed for 24 hours and then cut coronally, embedded in paraffin, sectioned and stained (hematoxylin and eosin). Stained sections at the level of the anterior commissure, thalamus and cerebellum-brainstem were examined without knowledge of the animals' experimental status. The sections were judged as to whether gross infarction (extensive areas of necrosis visible at low magnification) was or was not present.

Statistical Analysis

Statistical analysis was performed on a Tektronix 4051 computer (Wilsonville, OR) using the Plot 50 Statistics Package. The statistical routines perform an F-test (analysis of variance) to determine whether a significant difference exists between the different treatment groups. In addition, contrasts are provided to permit more specific questions regarding the difference between any 2 treatment groups. To define a contrast (comparison of means), let Mi represent the mean of all observations at level i. When the sum of all Ci's equals zero, the comparison of means (contrast) may be said to be: T = Cimi, where, T = difference in means represented by a particular choice of the coefficients Ci.

Results

Behavioral Effects

Gross motor skills (head lifting, walking, righting, cliff avoidance) appeared at approximately the same time in all 3 groups of animals (table 1). The development of more complex motor skills (free fall and bar walking) was significantly delayed in the hypoxia-ischemia-37°C group, but not in the hypoxia-ischemia-21°C group.

Cognitive testing showed that the hypoxia-ischemia-37°C group and the hypoxia-ischemia-37°C-infarction groups required approximately twice as many trials to master the Morris Water Task than did control animals (table 1). Animals who experienced initial difficulty in learning the Morris Water Task eventually performed as well as control animals once mastery was achieved.

In contradistinction to the Morris Water Task, the shock avoidance task was learned significantly faster by the hypoxia-ischemia-37°C-infarction group compared to the control, the hypoxia-ischemia-21°C and the hypoxia-ischemia-37°C no-infarction groups (table 1). Some of the animals in these 3 latter mentioned groups did not learn the shock avoidance task within the requisite 5 days.

Neuropathology

Light microscopic examination of the sham-operated animals revealed no abnormalities. None of the hypoxia-ischemia-21°C animals suffered infarction, although their right hemispheres appeared smaller than their left. Infarction developed in 3 of 8 hypoxia-ischemia-37°C animals. Injury was maximal in cerebral cortex, hippocampus, basal ganglia and thalamus (fig. 1). The remaining hypoxia-ischemia-37°C animals had no overt infarction, but had ipsilateral hemiatrophy.

Discussion

The morphologic consequences of the Levine procedure in the neonatal rat during both normothermia and hypothermia have been described and are consistent with the present findings. Ischemic cell change may occur in as many as 90% of animals subjected to the Levine procedure at 37°C. It is important to note such cell change may not be apparent 35 days after hypoxia-ischemia. Therefore, in analyzing our results we separated the animals in the hypoxia-ischemia-37°C group on the basis of whether overt infarction was present.

There have been only limited observations on behavioral effects of unilateral carotid artery ligation and hypoxia in the neonatal rat. Silverstein and Johnson
observed ipsilateral turning up to 10 minutes after emergence from the hypoxia chambers. Rice et al. observed no differences in reflex ontogeny and behavior up to 50 hours after hypoxic exposure. The present study extends the findings of Rice et al by demonstrating that the ontogeny of gross motor milestones in the neonatal rat is not delayed by unihemispheric injury.

In addition, the present study discloses that hypoxia-ischemia at 37°C significantly retards the development of motor behaviors requiring a greater degree of coordination regardless of degree of histologic damage. Similar impairment of motor skills in rats subjected to unihemispheric injury as neonates was described by Kolb et al.10

It is our contention that the 6 motor tasks performed by the animals are, in large part, independent of each other. A development sequence exists in which some of these motor behaviors are mastered earlier than others.10 For instance, primitive behaviors, such as righting, precede more complex behaviors such as walking on a narrow bar. While righting and bar walking both require locomotor ability, bar walking probably also involves visual orientation and more complex vestibular function. Head lifting requires no locomotor ability, but rather, focuses on orientation and axial musculature. During the free-fall test, no ambulation is required, but rather, the animal must orient its body during the fall. Free-fall, or mid-air righting, is a complex maneuver which is not usually mastered until the fifteenth day of life. That the free-fall test differs substantially from righting on a surface is clearly evident from the description of the free-fall test by Altaian and Sudarshan.10

A more conservative analysis of the possible independence of the motor and cognitive test results would necessitate the use of a multiple comparison procedure such as the Dunn Multiple Comparison test.12,13 Use of the former tests shows significant differences between saline and hypoxia-ischemia-21°C and the hypoxia-ischemia-37°C animals in 3 instances (table 1).

Although both control and experimental animals eventually appeared similar in feeding, walking and other motor behaviors by the third week of life, differences in cognitive performance remained. On one cognitive test, the Morris Water Task, performance of some of the hypoxic-ischemic animals was initially poorer than the control animals. However, the eventual performance of control and experimental groups was comparable suggesting that some delays in learning are

### Table 1

**Behavioral Performance of Control Rats vs. Rats Made Hypoxic-ischemic**

<table>
<thead>
<tr>
<th>Task</th>
<th>Controls</th>
<th>Hypoxia-ischemia 21°C</th>
<th>Hypoxia-ischemia, 37°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Gross motor</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. head lifting</td>
<td>10.5 ± 0.3</td>
<td>10.3 ± 0.3</td>
<td>10.3 ± 0.4</td>
</tr>
<tr>
<td>2. walking ability</td>
<td>10.8 ± 0.3</td>
<td>10.4 ± 0.2</td>
<td>10.7 ± 0.4</td>
</tr>
<tr>
<td>3. righting</td>
<td>9.0 ± 0.4</td>
<td>8.9 ± 0.3</td>
<td>10.0 ± 0.5</td>
</tr>
<tr>
<td>4. cliff avoidance</td>
<td>9.2 ± 0.6</td>
<td>10.1 ± 0.5</td>
<td>9.7 ± 0.8</td>
</tr>
<tr>
<td>5. free fall</td>
<td>14.2 ± 0.4</td>
<td>14.8 ± 0.3</td>
<td>16.0 ± 0.5</td>
</tr>
<tr>
<td>6. bar walk</td>
<td>14.2 ± 0.3</td>
<td>13.8 ± 0.3</td>
<td>16.0 ± 0.5</td>
</tr>
<tr>
<td>B. Cognitive</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Morris water task</td>
<td>9.3 ± 2.6</td>
<td>16.8 ± 2.3</td>
<td>22.0 ± 3.7</td>
</tr>
<tr>
<td>(trials needed to learn)</td>
<td>5.3 ± 0.5</td>
<td>5.3 ± 0.5</td>
<td>1.7 ± 0.7</td>
</tr>
</tbody>
</table>

Significance by Analysis of Variance.

* = significant difference (p < 0.05) vs Control.

- = significant difference (p < 0.01) vs Control.

* = significant difference (p < 0.05) vs Hypoxia-Ischemia-21°C.

** = significant difference (p < 0.01) vs Hypoxia-Ischemia-21°C.

e = significant difference (p < 0.05) vs Hypoxia-Ischemia-37°C-No Infarction.

*Significant by Dunn Multiple Comparison Test (p < 0.05).
transient. In contrast, the results of the shock avoidance task suggest that some of the cognitive differences between control and hypoxic-ischemic animals may be more long-lasting. Some of the control, hypoxic-ischemic-21°C and hypoxia-ischemia-37°C-no infarction animals failed to master the shock avoidance task even after 150 trials. It is also noteworthy that the hypoxia-ischemia-37°C-no infarction animals were slower to learn the Morris Water Task but faster at learning the shock avoidance task. The paradox of enhanced avoidance learning in brain-injured animals has been addressed by Krieckhaus et al. These investigators suggest that the cerebral injury reduces the species-specific “freeze-reflex” which is characterized by tense, crouching immobility in moments of stress or surprise.

The present experiments utilized only right hemispheric injury. Although anatomic asymmetries may exist in the rat, Kolb and colleagues maintain that there is no lateralization of function such as exists in man. The degree of neuropathologic damage may relate to the nature and severity of behavioral deficit. Animals in the hypoxia-ischemia-37°C group had delays in acquisition of more complex motor tasks. However, only those with overt infarction had abnormalities on cognitive testing.

The effect of hypothermia on subsequent cognitive development has also been of great interest. While studies of children who undergo cardiac arrest under hypothermia for repair of cardiac lesions show no deterioration of intellectual skills, anecdotal reports suggest that mild cognitive impairments might occur when conditions of hypothermia are not ideal. This report suggests that hypoxia-ischemia during hypothermia may completely spare motor delays, but nonetheless result in some degree of cognitive impairment.

Acknowledgments
We thank Drs. Edward Bixler and B. Kolb for advice and Betty Riccio for secretarial assistance. These experiments were supported by N.I.H. grant 1 R01 24605. Dr. Young is a recipient of a Clinician-Scientist Award from the American Heart Association.

References
Behavioral performance of rats following neonatal hypoxia-ischemia.
R S Young, J Kolonich, C L Woods and S K Yagel

*Stroke*. 1986;17:1313-1316
doi: 10.1161/01.STR.17.6.1313

*Stroke* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1986 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/17/6/1313

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/