Behavioral/Environmental Intervention Improves Learning After Cerebral Hypoxia-Ischemia in Rats

I-Ching Chou, MD; Tatyana Trakht, BS; Carina Signori, BS; Julia Smith, EdD; Barbara T. Felt, MD; Delia M. Vazquez, MD; John D.E. Barks, MD

Background and Purpose—In premature infants, many of whom experience ischemic brain insults, the environment of rearing influences cognitive outcome. We developed a model to evaluate the effect of rearing conditions on learning after unilateral cerebral hypoxia-ischemia (HI) in 7-day-old (P7) rats. We hypothesized that neonatal handling would benefit rats recovering from an episode of HI.

Methods—Seventeen litters of P7 Long-Evans rats underwent either HI (right carotid ligation followed by 1.5 hours in 8% O₂) or control procedures. From P8 to P14, randomized litters were either handled (15 minutes of separation from dam per day) or nonhandled. After P55, learning was tested in the Morris water maze. To evaluate injury severity, hippocampal, cortical, and striatal volumes were measured.

Results—In water-maze performance, ANCOVA revealed an interaction between handling and severity of hippocampal damage. Among HI rats, handled rats learned faster when hippocampal damage was moderate (P<0.01, repeated-measures ANOVA), with no benefit when damage was mild or severe.

Conclusions—These observations suggest the beneficial cognitive effect of neonatal handling was limited to animals with moderate damage. Neonatal handling in post-HI rats may be a useful model in which to study mechanisms underlying the benefits of post-HI developmental intervention. (Stroke. 2001;32:2192-2197.)

Key Words: early intervention ■ hypoxia ■ ischemia, newborn ■ rats ■ social environment

Premature infants have an increased risk of neurodevelopmental disabilities. Epidemiological data suggest biological factors, eg, episodes of cerebral hypoxia-ischemia during early postnatal life, do not explain the full extent of neurodevelopmental deficits. For example, when reared in poverty, premature infants have poorer cognitive outcome than peers reared in nonimpoverished environments. Furthermore, early intervention programs are of greater benefit to infants “at risk,” whether defined in biological or socioeconomic terms, than to normal infants. Yet, the central nervous system (CNS) mechanisms underlying the beneficial effects of early intervention (or the adverse effects of poverty) after a neonatal brain insult are poorly understood. Human investigations of the CNS effects of the environment or behavioral interventions are hindered by multiple potential confounders. Mechanisms might be more readily studied in well-controlled animal experiments.

Rat models are used extensively to model early human brain development because the rat brain at postnatal day 7 (P7) approximates that of the premature human. Episodes of unilateral cerebral hypoxia-ischemia (HI) in P7 rats result in neuronal loss in the cortex, basal ganglia, and hippocampus. As in humans, rats that experience cerebral HI have motor and cognitive deficits. Compared with animals reared in isolation, daily handling with brief maternal separation, before weaning, results in adult rats with improved cognitive performance, attenuated emotionality, and a modest corticosterone response to stress. The benefit of handling appears to stem from the mother’s response, with increased quantity and quality of caregiving (eg, licking). The sensitive period for handling is from P1 to P14, with handling from P1 to P7 being most effective. However, because the neonatal cerebral HI paradigm has been most studied at 7 days’ postnatal age, and because we wished to model the effect of postinsult intervention, we limited handling to P8 to P14.

Few experiments have evaluated the impact of early life experiences on the cognitive function and stress response of animals that experience an early biological insult, and none have evaluated the impact of early intervention after neonatal cerebral HI. We hypothesized that the cognitive effect of early intervention on infant rats that experienced a biological CNS insult (HI) would be similar to that which is experienced by vulnerable human premature infants, specifically, early neonatal handling would be associated with improved learning.
Material and Methods

Animals
Adult Long-Evans rats were obtained from Charles River Laboratories (Wilmington, Mass). This strain is commonly used to investigate handling.13 Pups, bred in our animal facilities, were randomly and equally divided between litters of different paternity and culled to 10 pups per litter (5 males and 5 females). Litters were left undisturbed until P7. On P21, pups were weaned and separated by sex.

Hypoxia-Ischemia
Seventeen litters underwent interventions on P7. One or 2 male/female pairs per litter were assigned at random to 1 of 4 groups: (1) right carotid ligation under methoxyflurane anesthesia, followed 1 hour later by 1.5 hours in 8% O2 in glass jars, in a 37°C warm-air incubator (HI); (2) sham surgery and hypoxia (sham); (3) 3-hour separation (separated), to control for time apart from dams; or (4) nonseparation (nonseparated), in which pups remained with the dam. Each litter included all interventions. The University of Michigan Committee on Use and Care of Animals approved all procedures.

Handling
Eight litters were assigned at random to daily handling and 9 to nonhandling. Handling occurred 2 hours after lights on from P8 to P14 and consisted of removal of each pup to a cup for 15 minutes. Nonhandled litters were undisturbed. Time-lapse videotape recordings of maternal-pup interactions were conducted at P9 to P10 (24 hours) and coded by a single observer who was unaware of the treatment. The duration of rest with pups, rest away from pups, nursing with passive posture, nursing with arched-back posture, nesting, licking pups, self-grooming, eating and drinking, rearing, running, and moving were scored with a computerized logging program (The Observer, Noldus Information Technologies).

Water Maze
Beginning on P55, learning was tested using the Morris water-maze place-navigation task.17 A Plexiglas escape platform was submerged 1.5 cm below the surface in a fixed location within a circular water-filled pool (180-cm diameter, 45 cm high) rendered opaque with floating polystyrene beads, in a room containing multiple fixed concentric rings and lines radiating from the center. Rats, placed in the center of the arena, were observed for 2 minutes; the numbers of rearings and of segments entered with 4 paws were recorded. In addition, we noted the frequency of entries into the outer (adjacent to the wall) and inner (at least 1 segment removed from the wall) segments and the time spent in outer and inner segments.

Corticosterone Response to Stress
To document that our handling induced the reported alteration in glucocorticoid feedback sensitivity,19 in 50% of litters, serum corticosterone levels were measured during and after restraint, after the completion of behavioral testing (approximately P65). Testing was conducted within the trough of corticosterone circadian rhythm (8 AM to 1 PM). Animals were wrapped in a Velcro-fastened flexible plastic tube for 30 minutes. Blood samples (0.1 mL) were obtained by tail venipuncture before and 7, 30, 60, and 120 minutes after restraint. Samples were centrifuged (5 minutes, 5000 rpm), and plasma was stored at −80°C. Corticosterone was measured by radioimmunoassay (Coat-A-Count, Diagnostic Products Corp).

Neuropathology
After completion of testing, rats were anesthetized with pentobarbital, and brains were removed and frozen. Histopathological analysis was performed in the HI and sham groups. Cross-sectional areas of bilateral neocortex, striatum, and hippocampus were measured in regularly spaced coronal 20-µm cresyl violet–stained sections beginning at the anterior genu of the corpus callosum and continuing caudally to the posterior genu of the corpus callosum, with NIH Image software. Tissue volumes were calculated by summation of areas and multiplication by between-section distance.20 With this HI protocol, atrophy of the ipsilateral damaged structures evolves.21 Percent damage of each ipsilateral structure compared with the left side was calculated with volumes by the formula % damage = 100×(L – R)/L, where L is left and R is right.

Data Analysis
The effect of handling on each maternal behavior was evaluated for the light and dark phases separately by t test. The effect of handling on serial corticosterone levels after restraint was compared between groups by repeated-measures ANOVA (RM ANOVA). To analyze water-maze place-navigation performance, the average escape latency of 4 trials per day per animal was calculated. The effect of HI on place-navigation performance (ie, serial mean escape latency) was evaluated by RM ANOVA. The relationship between lesion severity (regional volumes) and water-maze performance (sum of all latencies) was evaluated by linear regression. The effect of handling on place-navigation performance was evaluated across all P7 intervention groups by RM ANOVA, with handling and sex factored independently of pathology. Next, the effect of handling on place-navigation performance was evaluated in the non-HI control groups (separately or combined) and in the HI group by RM ANOVA. For place-navigation performance, the possibility of an interaction between severity of brain damage (hippocampal, cortical, or striatal) and handling was evaluated by ANCOVA. The effect of handling on place-navigation performance in the HI group, stratified by empirical grading of hippocampal lesion severity, was also evaluated by RM ANOVA. The combined effects of HI and handling on retention in the free swim test and on time spent in the center of the open field were evaluated by 2-way ANOVA and by a post hoc examination with ANOVA within the HI group, factoring hippocampal lesion severity. For all ANOVAs, P7 intervention or handling, the sex were initially considered as independent variables. Whenever differences were determined to be nonsignificant, the data were collapsed across the respective variable. Post hoc comparisons were made with the Fisher protected least significant differences test.

Results
Improved performance (ie, decreasing mean escape latencies) over time in the place-navigation task was a consistent finding across all groups (P<0.001, RM ANOVA). There
Figure 1. Cortical and hippocampal pathology. These coronal sections illustrate a normal (non-HI) adult rat brain and examples of mild, moderate (Mod), and severe (Sev) right hippocampal damage in adult rats that underwent right carotid ligation followed by 1.5 hours in 8% O₂ on P7. Note patchy cortical neuronal loss (arrowheads) associated with mild right hippocampal atrophy. Note cortical cavitation (*) associated with moderate and severe hippocampal atrophy. Cort indicates cortex; Hip, hippocampus; and Thal, thalamus.

were no differences by sex and no differences among the sham, separated, and nonseparated controls. Therefore, data for both sexes and for the 3 control groups were combined (HI, n=38; non-HI, n=119). There were no differences in the foot fault and swimming speed tests. The contralateral percentage of forepaw faults [(left/total)× 100; mean±SEM] was similar in both groups (HI 53±8%, non-HI 61±4%; P=0.3). All rats completed the swim speed test in ≤2 seconds.

Effect of HI on Water-Maze Performance
The severity of unilateral HI damage was heterogeneous (Figure 1) in both handled and nonhandled rats (Figure 2A). When the handled and nonhandled groups were combined, place-navigation performance was impaired in HI rats versus controls (Figure 3; P<0.001, RM ANOVA), and long-term retention in the free swim was reduced in HI rats (time in target quadrant [seconds, mean±SEM]: 120-second swim, HI 6.1±2.1%; non-HI 34.2±1.1%, P<0.001, 1-tailed t test; initial 20-second swim, HI 4.9±0.6, non-HI 6.0±0.4, P=NS). Among HI rats, improvement in performance across trials was slower and was dependent on the severity of regional damage. There was a negative linear relationship between the sum of all escape latencies and right-sided cortical, striatal, and hippocampal volumes (adjusted R²=0.215, 0.217, and 0.241, respectively; P<0.002 for each).

Effect of Handling on Water-Maze Performance
Analysis of all subjects (HI plus non-HI) revealed no differences between handled and nonhandled rats in the place-navigation task. Among HI subjects, with no consideration of damage severity, no improvement in performance was detected in the handled group. However, ANCOVA revealed a significant interaction between handling and both hippocampal and striatal percent damage (F(1,136)=7.32 and 5.18; P=0.008 and 0.024, respectively), which suggests that the efficacy of handling was dependent on the severity of hippocampal and striatal damage. The interaction between handling and cortical damage did not reach significance (F(1,136)=3.73, P=0.056). There were strong intercorrelations among all 3 regional damage percentages (striatum versus hippocampus 0.782; cortex versus hippocampus 0.805; cortex versus striatum 0.951), which suggests that all were indices of the same phenomenon. To elucidate the interaction between brain damage and handling, all brain lesions were empirically categorized, based on hippocampal percent damage, as either mild (≤10%), moderate (11% to 39%), or severe (≥40%; Figure 1). Percent hippocampal damage was different among the categories (mean±SE: mild, -1.7±1.3%; moderate, 26.9±2.1%; severe, 76.0±6.1%; P<0.0001). When HI subjects were stratified by hippocampal severity, the beneficial effect of handling was limited to rats with moderate damage.

Figure 2. Regional volumes in handled vs nonhandled rats. P7 rats underwent right carotid ligation followed by 1.5 hours in 8% O₂ (HI, n=38). Regional volumes (mm³, mean±SE) were calculated from bilateral area measurements after the behavioral studies (see Methods). Among all HI rats (A), there were no differences in ipsilateral cortical, striatal, or hippocampal (Hippo) volumes between handled and nonhandled subjects. Among rats with moderate (mod; 11% to 39% reduction in ipsilateral volume) hippocampal damage (B), there were also no differences in regional volumes between groups.

Figure 3. Effect of neonatal cerebral hypoxia-ischemia on adult water-maze performance. P7 rats underwent right carotid ligation followed by 1.5 hours in 8% O₂ (HI, n=38). Performance in the water-maze place-navigation task was evaluated beginning after P55 (see Methods). Results are presented as mean±SE escape latencies for each day. HI offspring showed higher escape latencies (ie, slower acquisition) than non-HI offspring (n=119) over 4 days (P<0.001, RM ANOVA). *P<0.05, †P<0.001, ‡P<0.0005, t test, comparing HI and non-HI groups on the same day.
Among rats with moderate hippocampal damage, handled rats found the platform faster than nonhandled rats over successive days (Table 1; \( P<0.01 \), RM ANOVA). The mean daily escape latencies of the handled rats with moderate hippocampal damage were indistinguishable from those of non-HI controls. There was no benefit from handling in rats with either mild or severe hippocampal damage (Table 1). Among the HI rats with moderate hippocampal damage, the severity of cortical, striatal, and hippocampal damage did not differ between handled and nonhandled rats (Figure 2B). Among HI rats, there were no differences between handled and nonhandled when damage severity was not taken into account. However, among HI rats with moderate and severe hippocampal damage, handled rats spent more time in the center of the open field than did nonhandled rats (mean\( \pm \)SEM: 28\( \pm \)3 versus 15\( \pm \)3 seconds; \( P<0.01 \), \( t \) test). Among rats with minimal HI damage and non-HI controls, there was no effect of handling on Open Field behavior.

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**Response to Novelty**

In the Open Field test, there were no differences between HI and non-HI rats. Among HI rats, there was no relationship between Open Field behavior and lesion severity. Overall, there were no differences between handled and nonhandled rats. Among HI rats, there were no differences between handled and nonhandled rats when damage severity was not taken into account. However, among HI rats with moderate and severe hippocampal damage, handled rats spent more time in the center of the open field than did nonhandled rats (mean\( \pm \)SEM: 28\( \pm \)3 versus 15\( \pm \)3 seconds; \( P<0.01 \), \( t \) test). Among rats with minimal HI damage and non-HI controls, there was no effect of handling on Open Field behavior.

**Maternal Behavior**

Mothers of handled pups spent more time during the dark phase licking and grooming their pups than did mothers of nonhandled pups (mean\( \pm \)SEM: handled 614\( \pm \)42 minutes, nonhandled 422\( \pm \)39 minutes; \( P<0.005 \), 2-tailed \( t \) test). There were no other differences in behavior that distinguished between mothers of handled and nonhandled pups.

**Glucocorticoid Response to Stress**

As adults, handled rats had lower basal plasma corticosterone concentrations, and after restraint, their corticosterone concentrations returned more rapidly toward basal values at 60 and 120 minutes than did those of nonhandled rats (\( P<0.05 \), RM ANOVA; Table 2). There was no difference in peak corticosterone values between groups. When HI rats were compared with non-HI controls, there was no difference in serial serum corticosterone levels.

**Discussion**

Consistent with previous reports,\( ^{1,2} \) hippocampal damage was associated with impaired performance in the water-maze place-navigation task. We found a direct relationship between place-navigation performance impairment and the severity of hippocampal, cortical, and striatal damage.

The enriched maternal-pup interaction associated with handling resulted in improved place-navigation performance in adulthood, but this benefit was evident only in rats that experienced moderate HI damage. Early handling restored performance in rats with moderate damage to a level indistinguishable from non-HI controls. Handling had no effect on performance in rats with severe damage. This suggests that there is a ceiling effect, ie, a severity of damage above which handling has no benefit. However, it is possible that the small

### Table 1. Effect of Handling on Learning After Neonatal Cerebral Hypoxia-Ischemia

<table>
<thead>
<tr>
<th>Hippocampal Pathology†</th>
<th>Handled vs Nonhandled‡</th>
<th>n</th>
<th>Mean±SE Daily Escape Latency, s*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-HI§</td>
<td>Handled</td>
<td>65</td>
<td>Day 1: 83.7±3.4, Day 2: 57.4±4.4, Day 3: 34.4±4.0, Day 4: 22.0±3.2</td>
</tr>
<tr>
<td>Non-HI§</td>
<td>Nonhandled</td>
<td>65</td>
<td></td>
</tr>
<tr>
<td>Mild</td>
<td>Handled</td>
<td>54</td>
<td>Day 1: 87.4±3.3, Day 2: 55.8±4.6, Day 3: 32.8±3.5, Day 4: 22.7±2.8</td>
</tr>
<tr>
<td>Mild</td>
<td>Nonhandled</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Moderate</td>
<td>Handled</td>
<td>9</td>
<td>Day 1: 90.2±8.4, Day 2: 69.1±11.1, Day 3: 40.0±10.8, Day 4: 32.2±10.6</td>
</tr>
<tr>
<td>Moderate</td>
<td>Nonhandled</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Severe</td>
<td>Handled</td>
<td>5</td>
<td>Day 1: 89.2±9.3, Day 2: 56.9±12.4, Day 3: 41.5±10.6, Day 4: 24.4±4.2</td>
</tr>
<tr>
<td>Severe</td>
<td>Nonhandled</td>
<td>5</td>
<td></td>
</tr>
</tbody>
</table>

*Seven-day-old (P7) rats underwent right carotid ligation followed by 1.5 hours in 8% O\(_2\) (HI, \( n=38 \)). Performance in the water-maze place-navigation task was evaluated over 4 successive days beginning after P55 (see Methods). Results are presented as mean±SE escape latency.

†Tissue volume was calculated by summation of hippocampal cross-sectional areas and multiplication by between-section distance (see Methods). As an index of damage severity, the percent right-sided atrophy was calculated \( [100 \times \# \text{ of HI rats} / \text{R}/L] \). Hippocampal damage was classified according to percent atrophy as mild (11–39%), moderate (39–63%), or severe (≥64%).

‡All 3 non-HI control groups were combined (see Results).

§All 3 non-HI control groups were combined (see Results).

| Among rats with moderate hippocampal damage, mean daily escape latencies were shorter in the handled than nonhandled group (\( P<0.01 \), RM ANOVA). Handling had no effect among rats with mild or severe hippocampal damage. |
sample size with severe damage precluded detection of a handling effect.

Neonatal handling resulted in the expected increased maternal attentiveness to the pups, as measured by licking. In adulthood, the expected increased feedback sensitivity to circulating glucocorticoids was demonstrated in the handled animals; basal plasma cortisol concentrations were lower, and the stress-induced rise returned more rapidly toward basal values. This is consistent with the suggestion that the effects of early handling afford the animal better feedback regulation of the hypothalamic-pituitary-adrenal response to stress.22

There are several possible mechanisms by which our behavioral intervention might have improved place-navigation performance. We considered the possibility that early behavioral intervention after acute brain injury in neonatal rats might result in a change in lesion size. However, ipsilateral cortical, striatal, and hippocampal volumes after HI were similar in handled and nonhandled rats. The absence of differences in regional volumes does not preclude a neural basis for the difference in performance between groups, because tactile and other stimulation in neonatal rats can result in long-term changes in dendritic arborization.23 Our experiments were not designed to detect these changes. Behavioral interventions can alter brain levels of neurotrophic factors, which might be responsible for the long-term changes in function.24,25 The interaction between severity of hippocampal pathology and the beneficial effect of handling is consistent with the notion that place-navigation in the water-maze is hippocampus dependent.17 Because the severity of hippocampal damage was somewhat less than cortical and striatal damage, it is possible that the hippocampus had greater potential to respond to handling. Yet, given the strong intercorrelation between hippocampal, striatal, and cortical damage, we cannot exclude the possibility that cortex and striatum also contributed to the effect of handling.

Although the improved place-navigation performance of the handled offspring may represent improved spatial learning, we cannot exclude that handled rats more readily learned to use a more efficient search strategy, possibly owing to diminished anxiety. The possibility of improved nonspatial learning is supported by the unexpected effect of handling on performance in the free swim. Among the HI rats, handled rats spent fewer of the first 20 seconds in the quadrant formerly containing the platform. This could be interpreted as less retention in the handled rats or alternatively that handled rats adaptively switched strategies sooner than nonhandled rats.

Handling had no effect on place-navigation performance in non-HI controls and in HI rats with mild or nondetectable damage. This finding may have several possible explanations. In previous reports, glucocorticoid hypersecretion and feedback insensitivity in senescent rats (≥12 months) with loss of hippocampal neurons were offset by neonatal handling.26 Among our subjects, glucocorticoid hypersecretion was also demonstrated in nonhandled rats; thus, the beneficial effect of handling on learning in nonlesioned rats might have become apparent with aging. Previous reports and the present results are consistent with the possibility that the cognitive benefits of handling only become apparent in a brain that has experienced some neuronal loss. The duration of handling used was shorter than in most studies; Meaney et al22 found that handling over the second week of life was somewhat less effective than handling over the entire first 3 weeks. To model behavioral intervention after a biological insult to the premature infant brain, we induced injury on P7 and restricted handling to the post-HI period (P8 to P14), a design that may have reduced the power of handling.

To the best of our knowledge, other investigators have not examined the effect of early neonatal handling on cognitive outcome after cerebral HI. However, environmental enrichment can improve motor and cognitive function in adult rats after cerebral ischemia.27 Environmental manipulation has beneficial effects on learning after a variety of nonischemic insults to the developing rat brain, including bilateral posterior cortical ablations,28 prenatal ethanol exposure,29 and neonatal monosodium glutamate treatment.30

Our results are consistent with the notion that vulnerable populations may benefit the most from early intervention or conversely may be most at risk in an impoverished environment.31 Yet our data also suggest that there may be a threshold of disability above which interventions become less effective. Similarly, the results of human studies evaluating the effectiveness of early intervention for premature infants suggest that certain children benefit much more than do others.6,7

Neonatal handling in post-HI rats may thus be a useful model in which to study the mechanisms underlying the benefits of post-HI developmental intervention or, conversely, a model in which to study the adverse effects of an impoverished environment of rearing. We anticipate that in

### TABLE 2. Stress Axis Responsiveness: Effect of Handling

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>0 min</th>
<th>7 min</th>
<th>30 min</th>
<th>60 min</th>
<th>120 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonhandled</td>
<td>33</td>
<td>19.0±1.9</td>
<td>30.5±2.4</td>
<td>43.1±2.2</td>
<td>40.1±2.8</td>
<td>30.6±3.2</td>
</tr>
<tr>
<td>Handled†</td>
<td>30</td>
<td>12.5±1.6t</td>
<td>26.7±2.5</td>
<td>42.3±2.3</td>
<td>32.4±2.8§</td>
<td>19.6±2.4§</td>
</tr>
</tbody>
</table>

*Plasma cortisol concentrations before (t=0), during, and after termination of a 30-minute restraint stress (see Methods) in offspring from litters that were handled (n=30) or nonhandled (n=33).
†Corticosterone levels in handled animals were lower at baseline and returned more rapidly toward baseline (P<0.05, RM ANOVA).
‡P<0.05, §P<0.05, t test, comparing handled vs nonhandled rats at the same time point.
the future, this paradigm could be used to study the way in which the brain translates an enriched environment into improved function later in life, even in the face of a preexisting brain insult.

Acknowledgments
This study was supported in part by grants from the University of Michigan Center for Human Growth and Development Children in Poverty Small Grant Program and the C.S. Mott Children’s Hospital Research Fund. The authors thank Drs B. Lozoff, T. Schallert, and F. Silverstein for helpful advice and A. Crossland, Y.-Q. Liu, and G. Wheatcroft for valuable technical assistance.

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Stroke. 2001;32:2192-2197
doi: 10.1161/hs0901.095656

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