Long-Term Neuroprotective Effects of Hypothermia on Neonatal Hypoxic-Ischemic Brain Injury in Rats, Assessed by Auditory Brainstem Response

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ABSTRACT

A method to assess long-term neurofunctional outcome of hypothermia on immature brains has not yet been clearly established. To investigate the effects of hypothermia on long-term neurofunctional outcome, we studied brainstem function using auditory brainstem response in adult rats after neonatal hypoxic-ischemic brain injury. Seven-day-old rats underwent a combination of left common carotid artery ligation and subsequent exposure to 8% O_2 for 1 h ($n = 17$). The rats were divided into three groups: hypothermia group ($n = 6$), normothermia group ($n = 6$), and sham control group ($n = 5$). During recovery from the hypoxic-ischemic insult, body temperature was reduced to 30°C for 24 h in the hypothermia group, but was kept at 37°C in the normothermia and sham control group. Three months later the rats were assessed by auditory brainstem response, then killed.

The normothermia group showed increased III-V latencies and wave V abnormalities. Hypothermia significantly ameliorated wave V abnormalities. Injury to the ipsilateral inferior colliculus was also reduced in the hypothermia group compared with that in the normothermia group, and the degree of damage assessed histologically correlated well with auditory brainstem response findings. The current study demonstrates that postischemic hypothermia may provide effective and long-lasting neurofunctional as well as histopathologic protection to the immature brain. (*Pediatr Res* **53:** 57–61, 2003)

Abbreviation
ABR, auditory brainstem response

Perinatal hypoxic-ischemic brain injury is still one of the major causes of mortality and permanent neurologic disability. At present, there have not been any treatments that are proven to be effective, and the emphasis thus far has been on supportive therapy (1).

Although hypothermia has been reported to be the most effective method of neuroprotection in humans as well as in various animal models (2–4), long-term neuroprotective outcome of hypothermia has not yet been clearly established. Previous studies in immature animals have shown that postischemic hypothermia protects in the short-term (*i.e.* 1- to 7-d survival period) perspective (5–7), but there is no evidence with regard to long-term (*i.e.* 1- to 3-mo survival period) neurofunctional efficacy. At present, only one report has examined the effect of hypothermia on long-term neurofunctional outcome after neonatal cerebral hypoxia-ischemia, using some sensorimotor tests such as the postural reflex, but it failed to show long-term neurofunctional protection (8).

ABR is an early component of the auditory evoked potential, and provides objective information on the function of neural auditory pathways through the brainstem (9). Clinically, it has been widely used in evaluating brainstem function in perinatal asphyxiated infants (10–12). Using the ABR, we have recently described brainstem dysfunction in adult rats exposed to hypoxia-ischemia in the neonatal period (13). In the present study, we examined whether hypothermia can improve functional outcome in the adult rat after neonatal cerebral hypoxia-ischemia, using ABR.

METHODS

Subjects. This experiment was performed on 17 seven-day-old Sprague-Dawley rats obtained from S.L.C, Suita City,

Japan. All experimental protocols were approved by the Osaka University Animal Care and Use Committee. The procedure for the hypoxic-ischemic injury was derived from the model developed by Rice *et al.* (14). The pups were anesthetized with halothane (induction, 4.0%; maintenance, 2.0%) in room air, then the left common carotid artery was surgically ligated with 5-0 surgical silk. After the procedure, the pups were returned to their dams and allowed to recover and to be fed for 1 h. The pups were then placed in a chamber maintained in a water bath with the temperature kept at 37°C. The chamber was perfused with a mixture of humidified 8.0% oxygen balanced with nitrogen for 1 h. Then pups were divided into three groups, a hypothermia group ($n = 6$), a normothermia group ($n = 6$), and a sham control group ($n = 5$). Immediately after hypoxic exposure, the hypothermia group was placed in room temperature until the rectal temperature reached 30°C, and then placed in a chamber in a water bath at a stable temperature of 30°C for 24 h. The normothermia group remained in the chamber in the water bath at 37°C for 24 h. The chamber was circulated with room air. The core temperature of the rat pups was measured by rectal probe. The sham control animals underwent only the same surgical procedure without arterial ligation or hypoxic exposure. After this, the animals were returned to the mother and reared normally. Animals were kept in a room (12 h/12 h light/dark cycle) and weaned at 3 wk of age.

ABR. At 3 mo of age, ABR was recorded from the left side in response to click stimulations (10 Hz) from a speaker located 1cm from one ear. Each animal was briefly anesthetized with ether, and s.c. needle electrodes were placed under the left external meatus (recording), vertex (reference), and under the right external meatus (ground). The stimulus intensity was a 136-dB sound pressure level. One thousand samples were averaged and filtered (50 to 6000 Hz). Interpeak latencies were determined for waves I-III, I-V, and III-V. Amplitude of each wave was determined from the positive peak to the following trough, and the amplitude ratio of wave V to wave I was calculated in each group.

Tissue Preparation. For histologic analysis, the animals were deeply anesthetized with an overdose of sodium pentobarbital (100 mg/kg, i.p.) and transcardially perfused with 0.9% saline solution at 4°C, followed by 4% paraformaldehyde in 0.1 M PBS, pH 7.4 at 4°C. Brains were removed and immersed in the same solution at 4°C for 24 h, dehydrated with a graded series of ethanol and xylene, embedded in paraffin, and cut into 5-μm-thick coronal sections at the level of the midbrain. Sections were deparaffinized in xylene and rehydrated in graded ethanol concentrations before staining. Slide-mounted sections were stained with hematoxylin and eosin for viewing. Sections were analyzed with a Macintosh computer using the NIH Image program, version 1.62, by a research assistant unaware of the treatment groups. With the aid of a microscope, the areas of the inferior colliculus in the ipsilateral and contralateral brainstem were determined. The area ratio of the inferior colliculus was calculated as follows:

Area ratio = area of ipsilateral inferior colliculus / area of contralateral inferior colliculus

Statistical Analysis. Means were computed for each group of animals and analyzed using one-way ANOVA with *post hoc* Fisher's protected least significant difference test. Significance was accepted at $p < 0.05$ level.

RESULTS

As we reported previously (15, 16), the rectal temperature remained rather constant and stayed at approximately 0.5°C above the water bath temperature during the experimental procedure (data not shown).

A typical ABR pattern from 3-mo-old sham control and normothermic hypoxia-ischemia rats consisted of five waves within the first 5 ms after click stimulation (Fig. 1). In the normothermic hypoxia-ischemia rat, there were abnormalities in both a prolongation of wave V latency and a decreased amplitude of wave V compared with the sham control rat. There were no significant differences for wave I latencies. Figure 2 shows the mean interpeak latency differences for group comparisons for waves I-III and III-V. Analysis of the interpeak latency differences did not detect significant group differences for wave I-III interpeak latencies (Fig. 2*A*). On the contrary, wave III-V interpeak latency was significantly longer in the normothermia group than in the sham control group ($p < 0.05$; Fig. 2*B*). Although there was a clear tendency for the hypothermia group to show a shorter interpeak latency, wave III-V interpeak latency between the normothermia group and the hypothermia group did not significantly differ. However, the amplitude ratio of wave V to wave I was significantly lower in the normothermia group than in either the sham control group or in the hypothermia group ($p < 0.05$; Fig. 3).

Giving special emphasis to the inferior colliculus, which usually receives widespread input from other structures of the auditory system (17), we histologically studied the midbrain after ABR recording. Figure 4 shows the effect of hypothermia after neonatal hypoxia-ischemia on midbrain damage in 3-mo-old rats. Figure 4*A* shows a representative coronal brain section at the inferior colliculus level assessed 3 mo after neonatal hypoxia-ischemia. A mild atrophic appearance in the ipsilateral inferior colliculus was observed in the normothermia group (Fig. 4*A*). The injury to the inferior colliculus was assessed in each experimental group. The area ratio of the inferior colliculus was 0.834 in the normothermia group. Hypothermia significantly reduced this damage to 0.939 (Fig. 4*B*) at 3 mo of age.

To evaluate whether there was a correlation between the degree of midbrain damage and functional deficits, correlation coefficients were calculated between the area ratio of inferior colliculus and ABR findings. Figure 5 presents the correlation of the two ABR measurements to the area ratio of inferior colliculus as assessed histologically. All ABR measurements exhibited highly significant correlation coefficients.

DISCUSSION

In the present study, postischemic hypothermia provided effective and long-lasting neuroprotection to the immature auditory system. To our knowledge, this is the first study that proved persistent neuroprotective effects of the hypothermia up to adulthood, by demonstrating neurofunctional as well as histologic improvements from hypoxic-ischemic insults given at neonatal period.
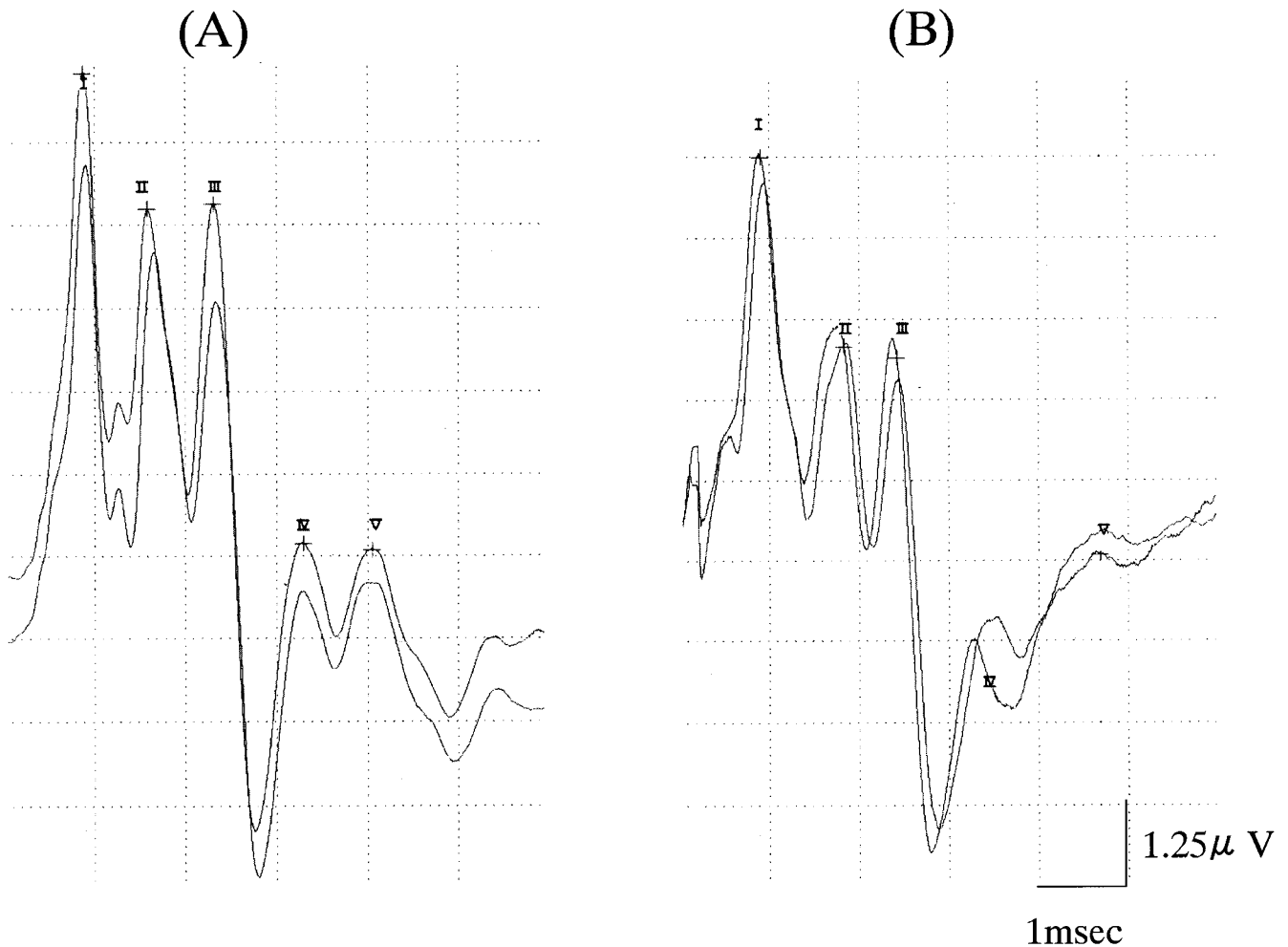
(A)

(B)

Figure 1. Typical ABR recordings in 3-mo-old sham control (*A*) and normothermic hypoxia-ischemia (*B*) rat. Note that a prolongation of wave V latency and decreased amplitude of wave V were seen in normothermic hypoxia-ischemia rat.

Since Busto *et al.* (18) first reported using adult gerbils, numerous studies have reported the neuroprotective effects of hypothermia in various animal hypoxic-ischemic models, including immature animals (19–21). However, these studies are limited by the use of short survival times, the lack of functional assessment, or both. Hypoxia-ischemia–induced cell death has been shown to continue for weeks or months, and recent studies have demonstrated that histologic preservation does not necessarily translate into preserved neuronal function (22, 23). Accordingly, there is a need for long-term neurofunctional assessment after postischemic hypothermia. Fortunately, recent studies using adult animals show that prolonged postischemic hypothermia provides long-lasting functional protection (24, 25). When considering perinatal asphyxia, the result obtained in adult animals cannot be directly transposed onto neonatal animals. Both the mechanism of injury and response to the therapeutic interventions are likely to be different in neonatal animals. For example, immature brains are thought to be more resistant to hypoxia-ischemia and show a faster evolution of the injury (26). Resistance of immature brain is thought to be achieved by mechanisms such as lower energy demand (27), the ability use lactate and ketone bodies for energy (28), and early induction of heat shock protein (29). It is therefore
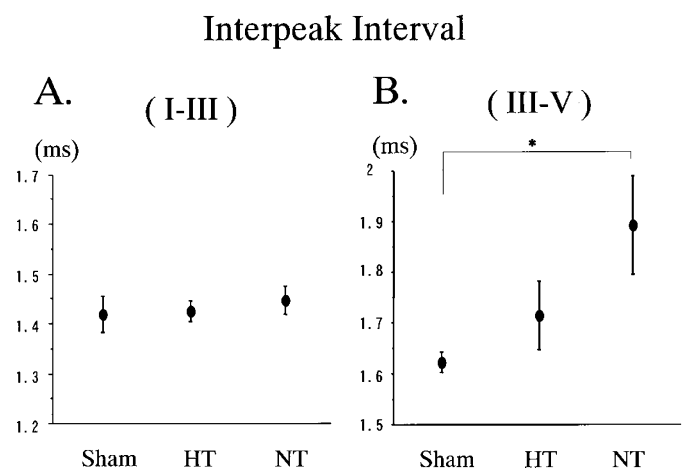
Interpeak Interval

Figure 2. Mean interpeak latency differences for waves I-III and III-V. The interpeak latency differences were not significantly different for wave I-III interpeak latencies (*A*). Latency of wave I was not significantly different. There were also no significant differences for wave I latencies. Wave III-V interpeak latency was significantly longer in normothermia group (*NT*) than in sham control group (*Sham*; *$p < 0.05$; *B*). Wave III-V interpeak latency between NT and hypothermia group (*HT*) did not significantly differ. Values are means; error bar indicates 1 SEM.
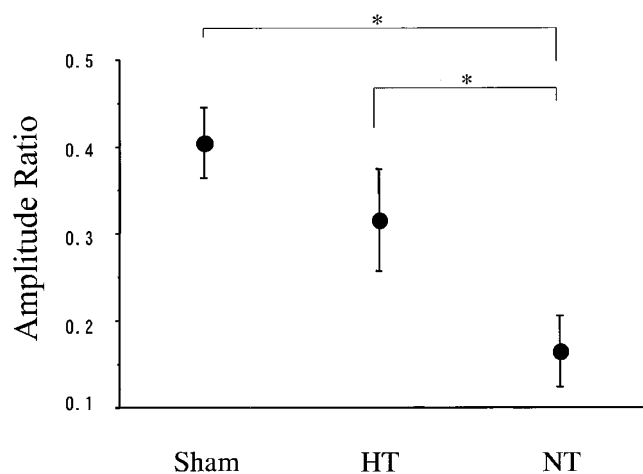
Amplitude Ratio of Wave V / Wave I

Figure 3. Amplitude ratio of wave V to wave I. Normothermia group (*NT*) was significantly lower than both sham control (*Sham*) and hypothermia (*HT*) groups (*$p < 0.05$). Values are means; error bar indicates 1 SEM.

essential that any therapeutic strategy intended for human infants should be thoroughly tested in newborn animal models.

Evoked ABR is an objective measure of CNS integrity (9). Clinically, the ABR has been widely used for evaluating peripheral auditory function and neural brainstem integrity in perinatal asphyxiated infants (10–12). However, there have been surprisingly few studies in which ABR was applied to the assessment of long-term outcome in various animal hypoxic-ischemic models. One study reported an increased I-V interval and decreased wave V to wave I ratio in young rabbits subjected to hypoxia (30). However, the study was limited by the use of ABR immediately after hypoxic insult. In the present study, the ABR provides a stable and objective behavioral measure after neonatal cerebral hypoxia-ischemia. Moreover, the ABR measurements correlate to the extent of the injury, suggesting that this test is useful in evaluating treatment effects for a longer period of time. It is generally considered that the auditory nerves generate peak I, the cochlear nucleus, peak II, the superior olivary nucleus, peak III, the lateral lemniscus, peak IV, and the inferior colliculus, peak V in rat (31). Thus, increased III-V latencies and wave V abnormalities observed in the present study are probably attributed to generators lying primarily within the midbrain. The inferior colliculus receives widespread input from other structures of the auditory system (17). In the current study, hypothermia significantly reduced damages to the ipsilateral inferior colliculus in 3-mo-old neonatal hypoxia-ischemia rats. Although the basis for the change in electrophysiologic response in this model will remain uncertain until there is a more complete understanding of the brainstem auditory pathway, it is possible that the neuroprotective effects of hypothermia on the inferior colliculus may partly contribute to the preservation of ABR findings.

Indeed, it is generally thought the main lesions in this model involve the cortex, hippocampus, and striatum, but one report described reduced blood flow to the ipsilateral inferior colliculus in this neonatal hypoxic-ischemic model (32). We have

(A) Representative coronal section at the midbrain level
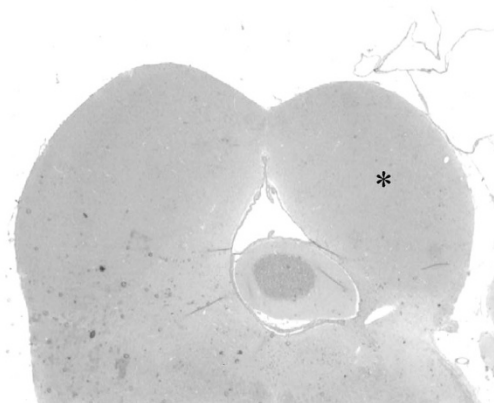
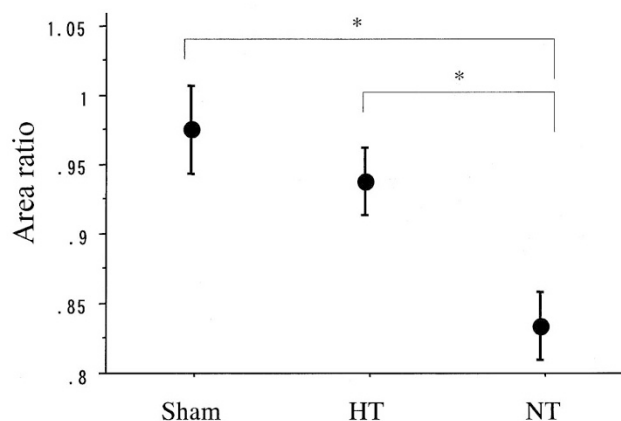Normothermic H-I rat

(B) Area ratio of inferior colliculus

Figure 4. Effects of hypothermia after neonatal hypoxia-ischemia on brainstem damage in a 3-mo-old rat. *A*, representative coronal brain section of normothermic hypoxic-ischemic rat assessed 3 mo after left carotid ligation. A coronal section at the midbrain level showed a relatively mild atrophic appearance in the ipsilateral inferior colliculus (indicated by [squlo]). *B*, area ratio (area of ipsilateral inferior colliculus to area of contralateral inferior colliculus) in 3-mo-old rats ($n = 5$–6). Area ratio was significantly lower in normothermia group (*NT*) than in either sham control (*Sham*) or hypothermia (*HT*) group (*$p < 0.05$). Values are means; error bar indicates 1 SEM.

recently described an increased I-V interval in adult rats exposed to hypoxia-ischemia in the neonatal period, and the presence of activated caspase-3–positive cells in the ipsilateral inferior colliculus 24 h after neonatal hypoxia-ischemia, suggesting that the apoptosis signal pathway may be activated in this region (13). In addition to several neuroprotective mechanisms of hypothermia suggested in some studies (5, 33–35), we recently described that postischemic hypothermia blocks caspase-3 activation after neonatal cerebral hypoxia-ischemia (15). It is possible that hypothermia might suppress apoptotic cell death in this region, leading to the preservation of auditory function in the adult rat after neonatal cerebral hypoxia-ischemia.
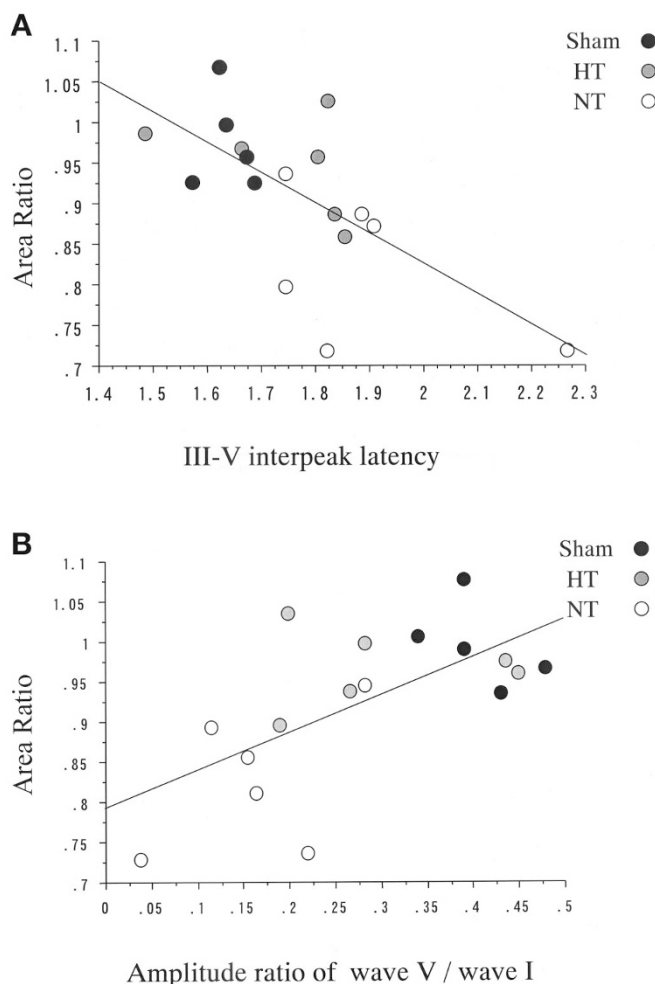
Figure 5. III-V interpeak latency ($r = -0.626$; $p = 0.0275$; A) and amplitude of wave V to wave I ($r = 0.620$; $p = 0.0295$; B) were plotted against area ratio (area of ipsilateral inferior colliculus to area of contralateral inferior colliculus) in sham control (*Sham*), hypothermia group (*HT*), and normothermia group (*NT*).

CONCLUSIONS

In summary, the current study demonstrated using ABR that the improvement of functional outcome by hypothermia can continue to adulthood in rats after neonatal cerebral hypoxia-ischemia. Postischemic hypothermia may provide effective and long-lasting neuroprotection for the immature brain. Although a recent clinical study failed to show long-term efficacy of hypothermia, it supported the safety of hypothermia with no evidence of late adverse effects in any infants (36). Our data strongly warrant further clinical investigation of hypothermia in hypoxic-ischemic encephalopathy.

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