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Neuronal Migration Disorders Increase Susceptibility to Hyperthermia-Induced Seizures in Developing Rats

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Summary: *Purpose:* Retrospective studies suggest that adult patients with intractable epilepsy may have a history of febrile seizures in childhood. Risk factors for a febrile seizure may include the rate of increase in the core temperature (T-core), its peak (T_{max}), the duration of the temperature increase, or an underlying brain pathology. Recently, neuronal migration disorders (NMD) have been diagnosed with increasing frequency in patients with epilepsy, but the link between NMD, febrile seizures, and epilepsy is unclear. We studied rat pups rendered hyperthermic to ascertain the incidence of seizures, mortality, and extent of hippocampal cell loss in each group.

Methods: We exposed 14-day-old rat pups with experimentally induced NMD (n = 39) and age-matched controls (n = 30) to hyperthermia (core body temperature >42°C).

Results: The incidence of hyperthermia-induced behavioral seizures and mortality rate were significantly higher in rats with NMD than in controls ($p < 0.05$). The longer duration of hyperthermia resulted in a higher incidence of behavioral seizures and higher mortality rate ($p < 0.05$). In rats with NMD, hyperthermia resulted in hippocampal pyramidal cell loss independent of seizure activity; the extent of neuronal damage correlated positively with the duration of hyperthermia. In control rats, occasional neuronal loss and astrocytosis occurred only after prolonged hyperthermia.

Conclusions: In immature rats, NMD lower the threshold to hyperthermia-induced behavioral seizures and hyperthermia in the presence of NMD may cause irreversible hippocampal neuronal damage.

Key Words: Neuronal migration disorders—Febrile seizures—Hyperthermia—Hippocampal cell loss—Mortality.

Recent advances in magnetic resonance imaging allow the preoperative diagnosis of pathologies associated with epilepsy that were previously recognized only postoperatively or at autopsy. In particular, neuronal migration disorders (NMD) are being recognized with increasing frequency in the preoperative evaluation of patients with "idiopathic" epilepsy (1). Clinical studies suggest that there may be an association between NMD and epilepsy (2,3). Epidemiologic studies indicate that an underlying brain pathology, including NMD, may lower the threshold to seizures (4,5). Furthermore, NMD were the most common neuropathological finding in a recent study of children with medically refractory epilepsy treated surgically (6). NMD may lower the threshold to "reactive" seizures triggered by environmental stimuli such as fever (7).

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Febrile convulsions occur in ~2–5% of all infants and children aged <5 years (8,9). Although most children aged <5 years experience one or more episodes of "high fever," only a small percentage of them develop convulsions. Of the children who experience febrile seizures, another small percentage later develops epilepsy (10), at times intractable with medical treatment (11). There is still significant disagreement as to whether febrile seizures caused hippocampal damage in these children (10,12) and whether such seizure-induced damage led to the development of epilepsy (4,13). Indeed, previous febrile seizures were a significant predictor of seizure recurrence in children with symptomatic seizures (5). An explanation for the association between the increased incidence of febrile seizures with lesional epilepsy is the possibility that the small number of children who develop this condition have NMD. Presumably, abnormal migration of neurons predisposes these children to febrile seizures and epilepsy is a later consequence of NMD. In this case, the febrile seizures do not appear to be the cause of epilepsy. Alternatively, NMD may lower the

threshold to febrile seizures but may also render the brain more susceptible to seizure-induced hippocampal damage which then leads to mesial temporal sclerosis (MTS) and medically refractory epilepsy. Therefore, the effect of a first febrile seizure on the risk of future seizures has important clinical implications, since treatment with antiepileptic drugs (AEDs) early in life has undesirable side effects, particularly on cognitive development (14).

Histological features of NMD in humans may range from a few ectopic neurons to severe disruption of the cortical cytoarchitecture (15). Recently, we characterized an experimental rat model of NMD with histological features similar to human NMD (16). Experimental NMD was induced in all offspring of pregnant rats exposed transplacentally to an alkylating agent on pregnancy day 15 (17). Histological features of experimentally induced NMD included cortical laminar disorganization, ectopic neurons in the subcortical white matter and in cortical layer I, persistent granular layer, marginal glioneuronal heterotopia, and discrete areas of neuronal ectopia in the CA1 subfield of the hippocampus. Furthermore, using in situ hybridization (ISH), we examined expression of AMPA/kainate-type glutamate, and γ-aminobutyric acid$_A$ (GABA$_A$) mRNA in rats with experimentally induced NMD. In these rats, the expression of GluR2 and GABA$_A$ α_1 mRNAs in hippocampal neurons in the areas of ectopia was lower than their expression in ectopic CA1 pyramidal neurons. Baseline EEG recordings did not disclose any spontaneous seizures; however, rats with severe NMD had significantly higher slow wave activity as compared with controls. In the present study, we used this model to determine if NMD lower the threshold to hyperthermia-induced seizures and if hippocampal neuronal damage occurs under such circumstances.

MATERIALS AND METHODS

Animals

The experimental procedures were conducted in time-pregnant Sprague-Dawley rats (Taconic Farms, Germantown, NY, U.S.A.) and their litters. Pregnancy day 1 in the rats was defined as the day of sperm cell detection by means of vaginal swab. Rat pups were kept with the dam before and after exposure to hyperthermia. All rats were maintained on a 12-h light/dark cycle with an average room temperature of 23°C and a relative humidity of 55%. Food and water were available ad libitum. The protocol was approved by our institutional animal care and use committee, and rats were monitored according to the guidelines of our center for laboratory animal sciences at Mount Sinai Medical Center.

Induction of NMD

Pregnant rats received a single intraperitoneal (i.p.) injection of methylaxozymethanol (MAM 25 mg/kg, Sigma Chemical, St. Louis, MO, U.S.A.) with a 25-gauge needle on day 15 of pregnancy (16). MAM was dissolved in isotonic saline to achieve a final concentration of 3 mg/ml. Age-matched control rats were injected with an equivalent volume of isotonic saline. Before the injection, careful aspiration was performed to rule out the presence of amniotic fluid and ensure that the injection was intraperitoneal. In cases of intrauterine penetration with the needle, as determined by the presence of yellow fluid in the syringe after aspiration or postinjection vaginal discharge, the rats were excluded from the study.

Experimental groups

The effects of hyperthermia on seizure susceptibility, mortality rate, and hippocampal neuronal damage were studied in 14-day-old rat pups. This age was selected because it corresponds to infancy in humans (18). Rat pups were randomly assigned to one of three groups based on the duration of exposure to core temperature (T-core) ≥42°C: 90, 120, or 150 s (described herein). These three exposures were chosen based on our pilot study, which showed a high mortality rate with >150-s exposures and minimal effects on the EEG with <90-s exposures.

Each hyperthermia group consisted of age- and weight-matched experimental rats with MAM-induced NMD and control rats. Average body weight of MAM-treated 14-day-old pups was 26 ± 0.5 g (range 22–32 g); that of controls was 30 ± 0.4 g. Our pilot study suggested that body weight may affect the mortality rate; MAM-treated pups with body weight <27 g had a higher mortality rate than age-matched rats with heavier body weight. Therefore, rats weighing <27 g were excluded from the study. Each group contained pups belonging to four different litters to avoid interlitter variability after their exposure to MAM.

EEG and physiological monitoring

T-Core and EEG activity were continuously monitored before, during, and after exposure to hyperthermia. Epidural EEG recording electrodes were implanted on the day before the hyperthermia experiments. Rats were anesthetized with intramuscular injections of xylazine and ketamine (15 and 30 mg/kg, respectively). The rat's head was placed in a stereotaxic holder, and the scalp was anesthetized with 1% lidocaine. Through a linear scalp incision,

a high-speed drill was used to make two holes 5 mm anterior and posterior to the coronal suture on each side of the sagittal suture. Epidural screw electrodes were implanted in the calvarium and stabilized with dental acrylic cement. The rats were placed on a heating pad to recover from anesthesia and were then returned to the dam. The epidural electrodes were connected with cables to an EEG (model 8-10, Grass Instruments, Quincy, MA, U.S.A.) for standard bipolar recordings.

T-Core was measured to the nearest 0.1°C with a lubricated thermistor probe attached to a digital thermometer (homeothermic unit, Harvard Apparatus, South Natick, MA, U.S.A.). The probe was inserted at least 1 cm beyond the anus and secured to the rat's tail with tape.

Induction of hyperthermia

Hyperthermia was induced by placing the rats in a covered translucent acrylic plastic hyperthermia chamber (24 × 10 × 12 cm) in a 99°C water bath. Room air circulated in the box through 2-cm inlet and outlet holes; separate holes were provided for the temperature probe and the EEG wires. The airflow was adjusted to maintain the temperature of the chamber at 56°–58°C. The bottom of the chamber was lined with a thick layer of tissue paper to prevent burn injuries. When the temperature reached 56°–58°C, the water bath was turned off and the rat was placed in the chamber. Rats were kept in the chamber for 90 s (group I), 120 s (group II), or 150 s (group III) after T-core exceeded 42°C. The mortality rate for control 14-day-old pups kept in the hyperthermia chamber with T-core ≥42°C for >150 seconds was >50%. Therefore, we chose 150 s as the longest exposure. The other two groups were exposed to T-core ≥42°C with 30-s decrements.

In addition to T-core and EEG, the mean, minimum, and maximum temperature of the hyperthermia chamber, the time to maximum core body temperature (T_{max}) after the rat was placed in the chamber, and the duration of T-core >42°C were monitored throughout the experiment. Brain temperature was not monitored.

Histology and immunohistochemistry

Four weeks after their exposure to hyperthermia, the surviving rats were anesthetized and perfused intracardially with 4% buffered paraformaldehyde (pH 7.4). The brains were removed, post-fixed in buffered paraformaldehyde for 24–48 h, embedded in paraffin, and cut with a microtome into 10-μm coronal sections. Alternate sections were stained with hematoxylin and eosin or cresyl violet or immunohistochemically stained for glial fibrillary acidic protein (GFAP) by the peroxidase-antiperoxidases technique (GFAP kit, Dako, Santa Barbara, CA) (19) to identify astrocytes.

The brain of rats that died during the exposure to hyperthermia were immediately removed, frozen at −32°C, sectioned with a cryostat in 10-μm sections, stained with thionin, and analyzed to confirm the presence of NMD.

We counted neurons in hippocampal subfields CA1, CA2, CA3, and CA4 (20) by examining cresyl violet-stained sections at 400× with a custom-made image-analysis system interfaced with a Macintosh computer and IPLab software (Signal Analytics, Vienna, VA, U.S.A.). Three high-power fields were quantified in each region and averaged. In each animal, both hippocampi were analyzed on one histological section obtained at the level of the amygdala.

We compared the neuronal counts of MAM-treated and control rats after exposure to hyperthermia with those of age-matched MAM-treated (n = 5) and control rats (n = 5) not exposed to hyperthermia. There was no significant difference in neuronal density between MAM-treated rats and control rats not exposed to hyperthermia in the hippocampal areas in which counts were performed. Areas of neuronal ectopia in the CA1 subfield were not used for neuronal counts. Data are reported as percentages of residual neurons in rats exposed to hyperthermia as compared with rats not exposed to hyperthermia within each treatment.

Statistical analysis

Factorial analysis of variance (ANOVA) with Bonferroni's correction was used to analyze the following variables within and between groups: baseline T-core, body weight, T_{max}, time to T_{max}, duration of T-core >42°C, and neuronal count. ANOVA post-hoc comparisons were made by the unpaired t test. Repeated-measures ANOVA with Bonferroni's correction was used to analyze changes in temperature over time during the hyperthermia experiments in each group. Seizure and mortality rates were analyzed by contingency table analysis with the chi-square statistic. For all analyses, differences were considered significant at $p < 0.05$.

RESULTS

Temperature data

The temperature data are summarized in Table 1. The range of T-core at baseline for rats with NMD was 37.1° ± 1°C; for controls it was 36.9° ± 2°C. After rats had been in the chamber for 1 min, T-core

TABLE 1. *T_{max} and time to T_{max}, in rats exposed to T-core ≥ 42°C for 90, 120, or 150 s*

Duration of T-core >42°C	No. of rats		T_{max} (°C)[a]		Time to T_{max}[a]	
	NMD	Control	NMD	Control	NMD	Control
90 s	5	4	43.3 ± 0.3	42.9 ± 0.8	11 min 48 s ± 48 s	12 min 42 s ± 36 s
120 s	14	7	43.1 ± 0.4	43.1 ± 0.1	13 min 24 s ± 42 s	13 min 54 s ± 24 s
150 s	20	19	43.1 ± 0.1	43.1 ± 0.1	13 min 48 s ± 1 min 6 s	14 min 48 s ± 36 s

T_{max}, maximum core temperature; T-core, core temperature; NMD, neuronal migration disorder.
[a] Values are mean ± SE. Times are given in minutes and seconds.

increased at a rate ≥0.3°C/min in all groups. In rats exposed to T-core ≥42°C for 120 or 150 s, T-core continued to increase after the rat was removed from the hyperthermia chamber, reached its peak ≤3 min after removal from the chamber, and returned to baseline after 7 min 12 s ± 12 s. Pups reached T_{max} 13 min 02 s ± 53 s after being placed in the hyperthermia chamber, and T_{max} was 43.2° ± 0.2°C. T_{max} did not differ significantly among groups.

Hyperthermia-induced seizures

Fourteen of 39 rats with NMD and 1 of 30 control rats developed behavioral seizures. In the NMD group, 8 of 14 rats had two seizures and 57% of rats had seizures while in the hyperthermia chamber. The only control rat that had a behavioral seizure experienced the seizure after exposure to T-core ≥42°C for 150 s and outside the hyperthermia chamber.

The seizure rate did not correlate with T_{max} because T_{max} did not differ significantly among groups (Table 1). The seizure rate, was affected by the duration of T-core ≥42°C, however. Rats exposed to T-core ≥42°C for 150 s had the highest hyperthermia-induced behavioral seizure rate (Fig. 1). Rats with NMD exposed to 90 s of T-core ≥42°C had no seizures. T-Core at the time of behavioral seizures was not significantly different from T_{max}.

Behavioral manifestations of seizures included chewing, rapid synchronous movements of the extremities, and loss of upright posture. During behavioral seizures, the EEG activity was characterized by rhythmic spike discharges (Fig. 2). Behavioral seizures occurred in rats at 14 min 24 s ± 30 s (mean ± SE) after they were placed in the chamber and lasted 3–60 s (mean ± SE 23 s ± 3 s).

Hyperthermia-induced epileptiform electrographic changes occurred in 78% in rats with NMD and in 78% of controls. These changes were characterized by spikes, spike and wave, and polyspike and wave discharges. In rats with both electrographic and behavioral seizures, epileptiform discharges always preceded behavioral seizures. Electrographic paroxysms occurred at a mean of 13 min 24 s ± 36 s after exposure to hyperthermia (i.e., after the rat was placed in the chamber), when the T-core was 42.1° ± 0.2°C (mean ± SE), which was significantly lower than T_{max} ($p < 0.05$). There were no differences in baseline T-core, T_{max}, duration of T-core >42°C, or temperature of the hyperthermia chamber between rats that had EEG paroxysms and rats that did not. Latency and duration of epileptiform electrographic discharges did not vary between rats with NMD and controls.

Mortality rate

Rats with NMD had a significantly higher mortality rate than controls ($p < 0.05$) (Fig. 3). Comparison of morality rate of rats with NMD that had behavioral seizures with that of rats with NMD that did not have behavioral seizures showed behavioral seizures to have no impact on mortality rate. The presence of electrographic seizures did not increase the mortality rate because only 10 of the 28 rats with electrographic seizures died. On the other hand, comparison of rats with NMD exposed to T-core

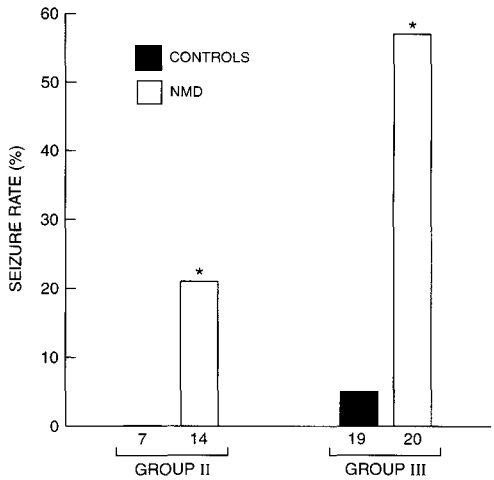

FIG. 1. Effects of hyperthermia [core temperature (T-core) ≥42°C] on behavioral seizures. In rats with neuronal migration disorders (NMD) exposed to T-core ≥42°C for 120 s (group II) or 150 s (group III) of hyperthermia, the rate of hyperthermia-induced behavioral seizures was significantly higher than in controls (*$p < 0.05$). Exposure to T-core ≥42°C for 90 s did not induce any behavioral seizures. Data are percentages of total animals in each group. Numbers on the abscissa refer to sample size in each group.

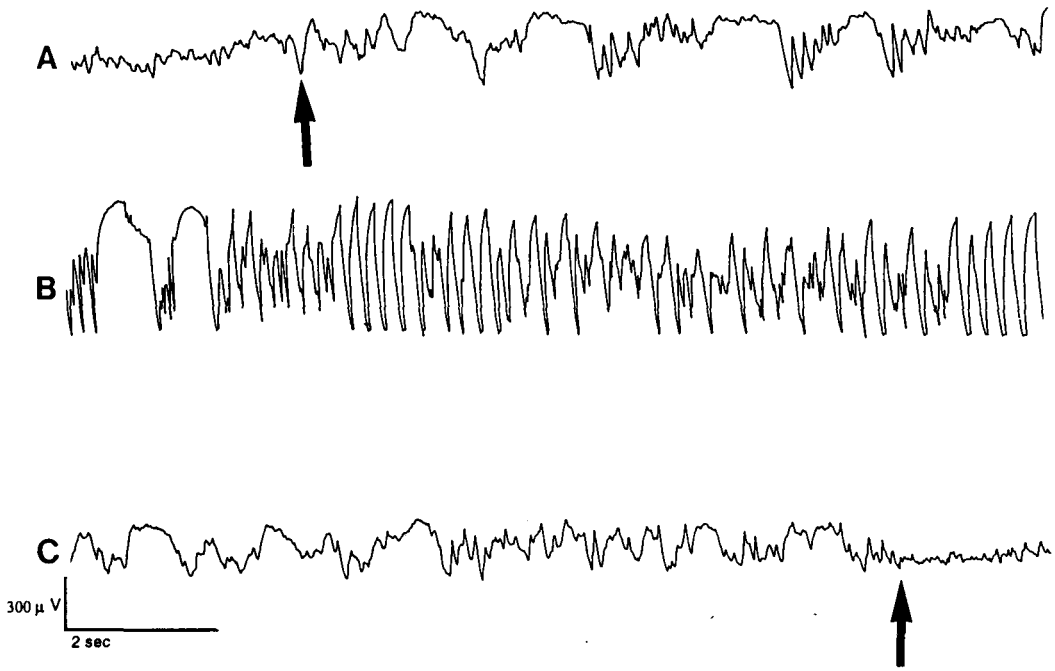

FIG. 2. EEG recording from a representative rat with neuronal migration disorder (NMD) during hyperthermia. **A:** The electrographic seizure occurred when core temperature (T-core) reached 42°C (arrow), and lasted 7 min 36 s. **B:** Two minutes 36 s after the electrographic seizures began, high-voltage rhythmic EEG discharges were apparent. These discharges corresponded to a behavioral seizure characterized by clonic movements of the mouth and all four limbs; the seizure lasted 40 s and occurred at a core temperature of 43.1°C. **C:** Twenty minutes after discontinuation of hyperthermia, the EEG tracing returned toward baseline (arrow); T-core was 37°C.

≥42°C for 120 s with those exposed to T-core ≥42°C for 150 s showed animals with longer exposure (150 s) to have a higher mortality rate.

Histological findings

All MAM-exposed rats had severe NMD characterized by cortical dysplasia and discrete areas of neuronal ectopia in the hippocampus. Hyperthermia induced neuronal loss in the CA1 and CA 3/4 pyramidal cell layer of the hippocampus in all surviving rats with NMD (n = 11) regardless of whether they had behavioral seizures. Furthermore, longer exposures to hyperthermia (T-core ≥42°C for 120 or 150 s) caused more significant neuronal damage than did exposure to T-core ≥42°C for 90 s. Neuronal damage was characterized by loss of pyramidal cells and damage to their cytoplasms, which was apparent as "dark neurons" on histological analysis (Figs. 4 and 5). Increased numbers of astrocytes were observed on GFAP-stained sections in the areas of neuronal damage (data not shown). Neuronal loss and astrocytosis were noted in 1 of 16 surviving control rats exposed to hyperthermia; this rat was exposed to prolonged hyperthermia and had no behavioral seizures.

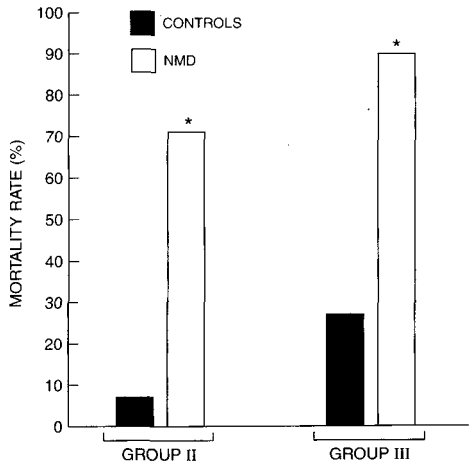

FIG. 3. Effects of hyperthermia [core temperature (T-core) ≥42°C] on mortality. In rats exposed to T-core ≥42°C for 120 s (group II) and 150 s (group III), the mortality rate was significantly higher in rats with neuronal migration disorders (NMD) than in controls (*$p < 0.05$). Data are percentages of total animals in each group.

Neuronal counts showed significant cell loss in the CA1 and CA 3/4 hippocampal subfield in rats with NMD exposed to hyperthermia as compared with control rats exposed to hyperthermia ($p < 0.05$) or rats with NMD not exposed to hyperthermia ($p < 0.05$). The neuronal count in control rats exposed to hyperthermia was not significantly different from that in control rats not exposed to hyperthermia (Fig. 5).

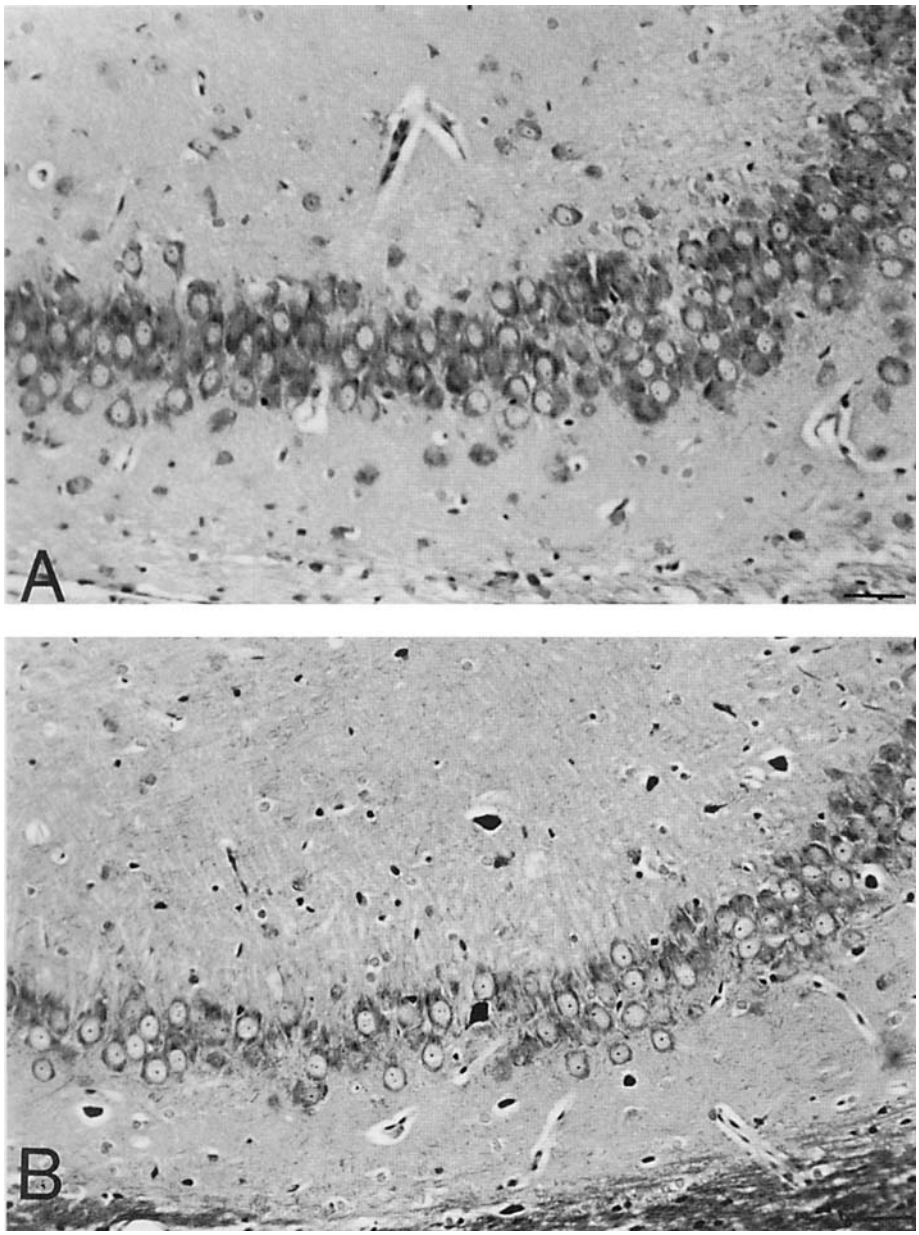

FIG. 4. Photomicrographs of 10-μm coronal rat brain sections at the level of the hippocampus showing CA 3/4 pyramidal cell neurons. Control rat **(A)** and rat with neuronal migration disorder (NMD) not exposed to hyperthermia **(B). C** and **D** shown on p. 908.

DISCUSSION

Epileptiform activity after temperature increase was previously observed in in vitro studies (20a). Our study is the first to document differences in seizure threshold to hyperthermia-induced seizures in the presence of NMD. In our study, we indicate that hyperthermia-induced seizures occur more frequently in rats with NMD. Furthermore, rats with NMD have a higher mortality rate after exposure to hyperthermia. Neuronal loss is also significantly higher in rats with NMD exposed to hyperthermia.

Despite the large number of patients affected by hyperthermia-induced seizures (21), why fever leads to seizures remains to be determined. Many children aged <5 years experience one or more episodes of high temperature, yet only 2–5% of all children develop febrile seizures (8,9). Experimental studies suggest that T_{max} may play a significant role in hyperthermia-induced seizures in the immature brain (7,22,23). Animal studies also suggest that seizures are most likely to occur when T-core increases faster than 0.3°C/min (24). In our study there was no significant difference in T_{max} between groups and T-core increased ≥0.3°C/min in all rats exposed to hyperthermia. Nevertheless, behavioral seizures occurred more frequently after 150-s exposure to hyperthermia than after 90-s exposure. Therefore, factors other than T_{max} and the rate of

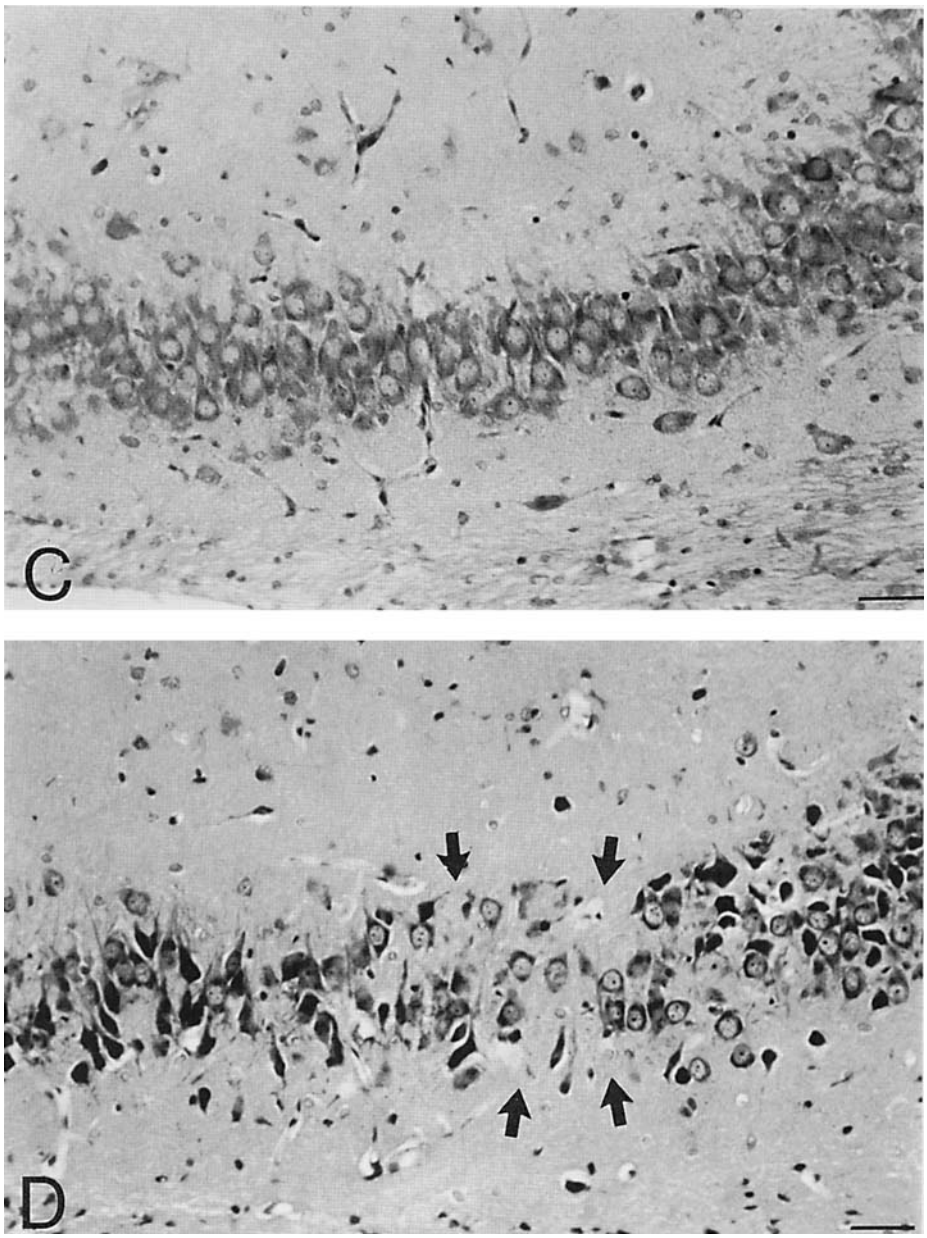

FIG. 4. *Continued.* Control rat **(C)** and rat with NMD 4 weeks after exposure to [core temperature (T-core) ≥42°C] for 120 s (group II). Hyperthermia-induced neuronal loss was significant **(D)** (arrows); this rat did not have behavioral seizures. Cresyl violet stain; bar = 200 μm.

temperature increase must account for hyperthermia-induced seizures.

Experimental and clinical studies suggest that an underlying brain pathology could increase seizure susceptibility (22,25,26). In particular, in vivo animal studies using an experimental model of NMD showed a decreased seizure threshold to kainic acid seizures in the immature brain (25,27). In vitro studies using the same experimental model suggested increased spontaneous epileptiform discharges in brain tissue with experimentally induced NMD (28). In the present study, we showed that behavioral seizures after prolonged hyperthermia occurred significantly more often in rat pups with NMD than in normal control rats. Therefore, two factors contribute to hyperthermia-induced behavioral seizures: the duration T_{max} and the presence of an underlying brain pathology that in itself does not cause seizures.

Experimental studies suggest that a lower seizure threshold after exposure to hyperthermia is an inherited predisposition (29,30). There is significant evidence that many cases of NMD may be genetically inherited (31,32). A previous study showed that probands with febrile seizures often had family members with epilepsy (33). Clinical studies have shown that a family history of febrile seizures and neurodevelopmental abnormalities are risk factors for a first febrile seizure in children (34). Similarly, environmental factors could also alter the genome

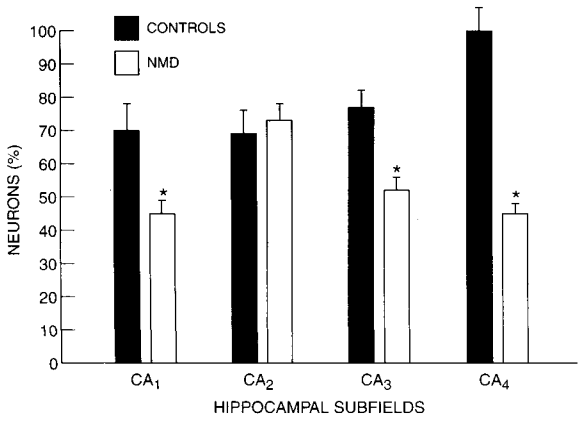

FIG. 5. Effects of hyperthermia on hippocampal neurons. Rats with neuronal migration disorder (NMD) exposed to hyperthermia had a significant neuronal loss in the CA1 and CA3/4 hippocampal subfields as compared with rats with NMD not exposed to hyperthermia and to control rats exposed to hyperthermia (*$p < 0.05$). Data are percentages of neurons in each hippocampal subfield as compared with rats not exposed to hyperthermia.

and increase the likelihood of seizures. These findings support the notion that an altered genome by either inherited or environmental factors may increase susceptibility to hyperthermia-induced behavioral seizures. In the present study, we altered the genome by methylation of nucleic acids during gestation in rats, which resulted in environmentally induced NMD (16).

In the present study, electrographic seizures after exposure to hyperthermia occurred as frequently in rats with NMD as in normal rats (78%). These findings suggest that prospective clinical studies might be used to examine electrographic changes in children with increased T-core (>42°C), but such studies may be difficult or impractical to perform. However, an increased T-core may cause electrographic seizures in humans but result in clinical convulsions only when an underlying brain pathology is present. The mortality rate after exposure to hyperthermia was significantly higher in rats with NMD. These data corroborate results of clinical studies suggesting that the mortality rate for patients with epilepsy is higher in the presence of an underlying brain pathology (35,36).

The connection between MTS, intractable epilepsy, and childhood febrile seizures is a controversial issue. MTS is characterized by neuronal loss and gliosis of mesiotemporal structures and is often evident in adult patients undergoing surgery for refractory seizures (37,38). The severity of hippocampal sclerosis (39) and hippocampal synaptic reorganization (40) is greater in patients who have a history of febrile seizures. Moreover, MTS has been associated with a long history of epilepsy and with early childhood convulsions (41). Although these clinical studies show an association between MTS and febrile seizures in patients with epilepsy, they do not indicate whether the relation is causal.

Seizures in children may not cause hippocampal damage as they do in adults. The immature brain is more susceptible to seizures (7,42–44) and yet more resistant to seizure-induced hippocampal damage (45,46). In addition, MTS is rare in children (47). However, the histological effects of hyperthermia in the absence of seizures in the immature brain have not been clearly documented. Several experimental studies have shown that the immature brain is more susceptible to febrile seizures (43,48), but did not describe the histologic findings after hyperthermia. However, one study in rats showed that hyperthermia during the neonatal period did not cause cell loss (49). That study differed from ours in that T_{max} was 42.7°C and the duration of T-core >42°C was not mentioned.

To our knowledge, this is the first report of hyperthermia-induced hippocampal damage in the immature brain. This damage occurred in our study in 11% of normal rat pups and in 100% of the rat pups with an underlying brain pathology (i.e., NMD) that by itself did not cause behavioral seizures. Our findings suggest that the hippocampal damage observed in young patients with epilepsy may be caused by hyperthermia and not necessarily by seizures as previously believed. Furthermore, the presence of underlying abnormalities such as NMD greatly enhances the development of hippocampal damage.

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