Evidence of Enhanced Kindling and Hippocampal Neuronal Injury in Immature Rats with Neuronal Migration Disorders

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Summary: Purpose: Neuronal migration disorders (NMD) are often found in patients with epilepsy. However, the mechanisms linking these two pathologies are not yet fully understood. In this study, we evaluated whether NMD increased kindling seizure susceptibility and seizure-induced acute neuronal damage in the immature brain.

Methods: Experimental NMD were produced by exposing pregnant rats (gestation day 15) to methylazoxymethanol acetate (MAM, 25 mg/kg, ip). Seizures were induced in rat pups (postnatal day 15) transplacentally exposed to MAM and controls by hippocampal kindling. Afterdischarge (AD) threshold and duration, seizure stage, and number of stimulations required to reach each seizure stage were recorded. Acute seizure-induced damage was histologically assessed in Nissl-stained and silver-impregnated hippocampal tissue 24 h after kindling.

Results: Rat pups with NMD had a significantly lower AD threshold than controls (91 ± 18 vs. 163 ± 23 μA; p < 0.05). Furthermore, rats with NMD required fewer stimulations to reach seizure stage 3.5 and 4 than did controls. Additionally, rats with NMD had longer AD the second day of stimulation (2,094 ± 416 s vs. 1,755 ± 353 s; p < 0.05). Histologic examination revealed that in rats with NMD, acute seizure-induced neuronal hippocampal damage occurred bilaterally in CA3 hippocampal neurons.

Conclusions: The lowered AD threshold and more rapid kindling to stages 3.5 and 4 indicate that in the presence of severe NMD, hippocampal kindling is facilitated. Furthermore, this study suggests that in the immature brain, seizure-induced hippocampal neuronal damage occurs if there is an underlying pre-existing pathology. Key Words: Damage—Development—Epilepsy—Methylazoxymethanol acetate—Seizures.

Data from clinical and laboratory experience suggest that there is a link between neuronal migration disorders (NMD), increased seizure susceptibility, and epilepsy (1–7). NMD, characterized by abnormal neuronal migration resulting in cortical dysplasia, are often found in patients with a history of medically refractory epilepsy (3). Furthermore, we recently showed in the experimental setting that NMD cause increased seizure susceptibility to chemically induced seizures (5,7).

The mechanisms underlying the increased seizure susceptibility in the presence of NMD and subsequent epilepsy are still being investigated. Seizure-induced neuronal damage with subsequent formation of a glial “scar” is one of the processes thought to be the basis of epilepsy after seizures in adults (8). Clinical and experimental data, however, suggest that the immature brain is resistant to seizure-induced neuronal damage (9,10). Furthermore, there is clinical evidence that status epilepticus in children without antecedent brain injury does not result in recurrent seizures later in life, suggesting that a “dual pathology” may be one of the causes for further epilepsy (11,12). Thus, an underlying brain abnormality such as NMD may be prerequisite for seizure-induced neuronal damage.

Electrical kindling induced by repeated administration of local subconvulsive electrical stimulations, leading to the progressive development of seizures, is a well-established experimental model of epilepsy (13–16). In the normal immature brain, kindled seizures do not cause neuronal damage (17,18). In this study we determined whether the presence of NMD in the immature brain causes increased seizure susceptibility to kindling and whether, in this setting, kindling induces seizure-induced acute neuronal damage. Kindling was induced in 15-day-old rat pups, and acute seizure-induced neuronal damage was assessed 24 h after kindling.

MATERIALS AND METHODS

Animals

The experimental procedures were conducted in timed-pregnant Sprague–Dawley rats (Taconic Farms,
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Germantown, NY, U.S.A.) and their litters. Gestation day 1 corresponded to the day of sperm cell detection by means of vaginal swab. Rat pups remained with their dam. All animals were maintained on a 12 h light–dark cycle with an average room temperature of 23°C, humidity of 55% with food and water available ad libitum. Experiments were conducted according to our Institutional Animal Care and Use Committee (IACUC) and Center for Laboratory Animal Science (CLAS) guidelines and approved protocols.

Induction of NMD

NMD was induced by transplacental exposure to methylazoxymethanol acetate (MAM; Sigma, St. Louis, MO, U.S.A.) at gestation day 15. This time was chosen to ensure that the MAM injections would induce severe NMD, as previously described (7). MAM was dissolved in physiologic saline with a final concentration of 3 mg/ml. Pregnant rats were intraperitoneally injected with 25 mg/kg MAM. Control rats were injected with an equivalent volume of drug vehicle at the same gestational age. By using a 25-gauge needle, aspiration was carefully performed before the injection to rule out the presence of amniotic fluid. Rats that had intrauterine penetration with the needle or postinjection vaginal discharge were excluded from the study.

Implantation of EEG electrodes

Rats were anesthetized with ketamine and xylazine (15 and 30 mg/kg, i.m., respectively) at postnatal day (PN) 14. Bipolar electrodes (MS 303/2; Plastic One Inc., Roanoke, VA, U.S.A.), were stereotactically implanted in the right hippocampus by using the following coordinates from bregma: AP, −3.2 mm; LAT, +2.5 mm; VERT, −3.0 mm. The mouth bar was set at −3.5 mm. In each rat, the electrode was fixed to the skull with dental acrylic and three anchoring screws. After the surgery, rats were allowed to recover from the anesthesia on a heating pad and then returned to their mothers.

Kindling paradigm and seizure stages

Kindling was initiated 24 h after surgery. EEG recordings were obtained before each stimulation (baseline) in each animal and immediately after each stimulation to record the complete seizure afterdischarge (AD). To detect differences in seizure threshold between MAM-exposed and control pups, rats were stimulated for 1 s with 50 μA current intensity at 60 Hz. Stimulation were delivered every 2 min with 50-μA increments until the first AD was seen on the EEG (NMD, n = 16; control, n = 16). The stimulation when the first AD was observed was called stimulation 1, and the current intensity was the AD threshold (ADT) for a given rat. The current intensity was then increased by 100 μA above the threshold level (stimulation 2), and kindling was continued every 15 min for a total of 30 stimulations: 20 stimulations the first day and 10 the following day. Seizures were graded based on motor behavior as follows: stage 1, mouth clonus; stage 2, head bobbing; stage 3, unilateral forelimb clonus; stage 3.5, alternating forelimb clonus; stage 4, bilateral forelimb clonus; stage 5, bilateral forelimb clonus with loss of balance; stage 6, wild running, jumping, rolling, and vocalizing; and stage 7, tonic posturing (18). Duration of AD was measured in seconds.

Histology

Rats were killed 24 h after completion of kindling together with nonkindled MAM-exposed and normal controls. Rats were perfused with 0.9% NaCl and then 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4). Brains were extracted, left in cold buffered paraformaldehyde overnight, and then cut at 50 μm with a vibratome and left in distilled water at 6°C until stained. Serial sections were stained with thionin to assess the placement of electrodes and perform neuronal counts. Alternate sections were stained with silver impregnation to assess acute neuronal damage. Silver staining was performed by using a modified technique (19).

In brief, slices were immersed in three changes of water (5 min each) followed by two changes of pretreating solution (5 min each) consisting of 9% NaOH and 1.2% NH₄NO₃, and then left in an impregnating solution of 9% NaOH, 16% NH₄NO₃, and 50% AgNO₃ (9 min). Subsequently sections were rapidly washed (three changes in 5 min) in a mixture of 1.2% NH₄NO₃ and anhydrous Na₂CO₃ in 95% ETOH and dH₂O, followed by ≥1 min in the developer, which consisted of 1.2% NH₄NO₃ and 0.5 g anhydrous citric acid in 37% formalin, 95% ETOH, dH₂O, and 9% NaOH (pH 5.8–6.1). Section were then mounted onto gelatin-coated slides, dried, dipped (three changes, 10 min each) in 5% glacial acetic acid, washed in water, dehydrated in a series of alcohol (70–100%), immersed in xylene, and coverslipped with permount mounting medium.

Tissue analysis and neuronal counts

Severity of NMD was graded as previously reported, based on the presence of cortical dysplasia and hippocampal neuronal ectopia (7). To decrease variability, only those rats with severe NMD were included in the study.

Seizure-induced hippocampal neuronal damage was assessed by neuronal counts of thionin-stained sections. Neurons were counted in the dentate and hippocampal subfields CA1, CA2, CA3 in dorsal hippocampus bilaterally. In MAM-treated rats, counts were not performed in the ectopic regions. 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. Four sections at 50-μm intervals were counted in each animal. Neuronal counts of MAM-treated (n = 16) and control rats (n = 16) after kindling were compared with those of age-matched MAM-treated (n = 4) and control rats (n = 4) not exposed to kindling.

Hippocampal tissue also was assessed for damage with the silver-impregnation technique. This technique selectively stains presynaptic neuronal elements, including dendrites, axons, and terminals of degenerating neurons, with a gray–black appearance. The degenerating neurons and their processes can be easily distinguished from the intact cell bodies and myelinated axons, which stain a light yellow–gold color. The presence or absence of silver-stained neurons was assessed in all hippocampal subfields in dorsal hippocampus bilaterally. In addition, we looked for degenerating neurons in adjacent cortical regions.

Statistical analysis
Student’s unpaired t test was used to analyze differences in seizure threshold (in μA), number of stimulations to reach each seizure stage, and duration of AD (in seconds). One-way analysis of variance (ANOVA) was used for cell counts in the various hippocampal regions. The data is reported as mean ± standard error (SEM); p < 0.05 was considered significant.

RESULTS
Kindling is facilitated by NMD
The ADTs were compared between rats with NMD (n = 16) and age-matched control rats (n = 16). Results in Fig. 1 indicate that the ADT for rat pups with NMD (91 ± 18 μA) was significantly lower than that of control rats (163 ± 23 μA; t(30) = 2.796; p = 0.0089). There were no differences in the AD durations between the groups at ADT. All the control and NMD rats kindled, and there were no differences in the behavioral manifestations of each kindled seizures stage. Figure 2 demonstrates differences in the pattern of kindling between the two groups. Rat pups with NMD required fewer stimulations to reach kindling stage 3.5 (t(30) = 3.515; p = 0.0014) and stage 4 (t(30) = 4.083; p = 0.0003). The number of stimulations to reach the other kindling stages did not reach statistical significance. Figure 3 reveals that, although the overall duration of AD was similar in both groups, rats with NMD had longer AD durations (2,094

![FIG. 1. Hippocampal ADT in immature rats with NMD. Rat pups with NMD has lower ADT than did age-matched controls. The bar graph shows the current in μA necessary to elicit the first AD. Data are expressed as mean ± SEM; NMD, n = 16; controls, n = 16; *p = 0.0089.](image)
Seizure Stages

FIG. 2. Development of hippocampal kindling young rats with NMD. Rat pups with NMD developed stage 3.5 and 4 seizures significantly faster than did controls. The bar graph shows the number of stimulations necessary to elicit each seizure stage; NMD, n = 16; controls, n = 16; *p < 0.0003.

Histologic findings

All rats transplacentally exposed to MAM had severe NMD, as previously described (7). These 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 (Fig. 4). In this study, we included rats with electrodes placed in the dorsal hippocampus (Fig. 4). In rats with NMD, the electrode was not located in hippocampal areas of ectopic neurons.

Kindling in the presence of NMD causes acute neuronal damage

All kindled rats were assessed for hippocampal neuronal injury or cell loss 24 h after kindling and compared with nonkindled rats. The following groups were examined: NMD rats with and without kindling and normal rats with and without kindling. At this time, we did not find any significant difference in neuronal cell counts between the two groups of NMD rats or the two groups of normal rats. However, the silver-impregnation method revealed differences between the kindled NMD rats and the three other groups. Throughout the CA3 hippocampal subfield, several darkened neurons were present in each of the kindled NMD rats. These cells were observed among intact cells, which were yellow–gold–brown in appearance. Positive silver-stained cells had the silver precipitate in the somata and the processes (Fig. 5). The silver-stained pyramidal cells were seen in both hippocampi without any relation to the electrode placement. In control rats, occasional silver-stained neurons were seen at the site of electrode insertion. Several adjacent cortical areas also were examined for damage. No positively stained silver cells were observed in any of the groups.

DISCUSSION

Electrical kindling, first introduced in 1967 (13), is a widely used model to investigate basic mechanisms of
neuronal migration disorders

FIG. 3. Cumulative AD duration rats during hippocampal kindling. Rat pups with NMD had longer duration of AD the second stimulation day compared with controls. Bar graph showing the duration of ADs (s) during the first and the second stimulation day; NMD, n = 16; controls, n = 16; *p = 0.0189.

epileptogenesis (14–16). Electrical kindling of the limbic system results in the expression of generalized seizures at the final stage. In this paradigm, it is possible to study factors involved in local epileptogenesis and in the propagation of seizures (20). Rats with NMD have altered characteristics in both local epileptogenicity and propagation of seizures. Our results indicate that the ADT, an index of epileptogenicity at the stimulated site, is lower in rats with NMD than in controls. A lower ADT usually denotes an increased local epileptogenicity propensity. It is interesting that this increase in local epileptogenicity is present in an area devoid of dysplastic neurons, as the kindling electrodes were not within the area of neuronal ectopia. It is not clear whether the ectopic neurons contribute to the decreased threshold. However, human studies of resected epileptic tissue that includes ectopias suggest that the altered epileptogenicity is a propensity of neurons near the ectopic lesion but not of the ectopic lesion per se (G. Mathern, unpublished observations). These findings are in agreement with a recent study (4) showing changes in the electrophysiologic features of hippocampal CA1 neurons in rats with MAM-induced NMD. In this study, the electrophysiologic features of ectopic neurons in the CA1 could not be assessed.

Rats with NMD require fewer stimulations compared with controls to reach kindled seizure stages 3.5 and 4. However, subsequent seizure stages are reached with equal number of stimulations in both groups. Several studies indicated that the progression of kindling from limbic sites involves the recruitment of a paleocortical network involving piriform, insular, and perirhinal cortices (20–23) with the eventual propagation to basal ganglia and other subcortical structures (24,25) and eventually the brainstem (18,26). The development of kindling is different in immature rats compared with adults. Immature rats develop stages 3.5 and 4 faster than do adults, but not stage 5 seizures. The increased seizure susceptibility of the immature brain to kindling is partly reflected by the early emergence of bilateral seizures, stages 3.5 and 4 (16,27). These observations suggest that the mechanisms involved in the control of seizure propagation are different for stages 3.5 and 4 than for stage 5. Burchfiel and Applegate (28) proposed that the develop-
ment of kindling represents the opening of "gates" that normally suppress seizures. Norepinephrine may be the neurotransmitter responsible for the emergence of stages 3.5 and 4 (15), whereas frontal lobe influences may be responsible for the emergence of stage 5 seizures (16,29). In our experiments, we found differences in the development of kindling between MAM rats and controls, indicating that the structures involved in stages 3.5 and 4 seizures are preferentially affected. These findings suggest that the neuronal migration abnormalities in rats with NMD may affect the occurrence of specific seizure stages. Finally, the longer AD durations we observed in kindled MAM rats during the second day of stimulations may indicate that, over time, rats with NMD are more susceptible to seizures than are controls. Germano and Sperber (30) reported similar findings in adult rats with NMD. These MAM rats have more prolonged kainic acid seizures than do controls.

We previously showed that in rat pups with severe NMD, kainic acid–induced status epilepticus causes chronic neuronal damage to CA3 hippocampal neurons (7). On the other hand, a single seizure induced by hyperthermia did not result in neuronal damage related to the seizure per se (5). The study shows that repeated seizures after kindling in the presence of severe NMD cause acute hippocampal neuronal damage bilaterally in areas remote from the electrode (stimulation) site in the absence of any overt cell loss. This is not the case with controls, in which kindling did not produce any injury other than that associated with the insertion of the electrode. The data in control rats confirm that the normal immature hippocampus is more resistant to seizure-induced neuronal damage than is the adult hippocampus.
(17; Haas et al. unpublished observations). However, if there is an underlying pathology, such as NMD, repeated seizures may cause neuronal injury.

This is the first documentation of hippocampal injury after kindling in developing rats. The intensity of the damage is much less than that we observed previously with kainic acid–induced seizures in rats with NMD. The difference may be related to the severity of the seizures (status vs. short repetitive seizures). It is possible that if additional kindling stimuli were delivered, the degree and extent of injury might have been greater, as Cavazos et al. (31) demonstrated in kindling of adult animals. In addition, the injury we observed occurred in rats with severe NMD. It is not known whether kindling induces hippocampal injury in developing rats with mild NMD. Further studies are necessary to assess whether the damage observed here will result in a hippocampal “scar” and cause future epilepsy.

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