Pilocarpine-Induced Seizure Susceptibility in Rats Following Prenatal Methylazoxymethanol Treatment

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Several rodent models of cortical malformation are available for the study of cellular mechanisms associated with early-onset epilepsy, but few are associated with spontaneous seizures. We examined the effect of pilocarpine on the spontaneous seizure development and excitability of the CA1 pyramidal cells of rats after prenatal treatment with methylazoxymethanol (MAM). Pilocarpine induced status epilepticus (SE) onset latency was greater for normal rats than for MAM-treated rats. After several days of normal behavior following pilocarpine treatment, the duration of spontaneous seizures were greater in MAM-pilocarpine rats than in normal-pilocarpine rats. When compared with the normal rats, electrical stimulation of afferent fibers resulted in more robust population responses in the CA1 region in all groups. At interstimulus intervals of 30 and 70 ms, the MAM-pilocarpine rats displayed a decrease in paired pulse inhibition versus the conventional MAM rats. A loss of somatostatin- and parvalbumin-immunoreactive neurons was apparent in the normal-pilocarpine rats, MAM-pilocarpine rats, and conventional MAM rats. These results indicate that pilocarpine induces spontaneous seizures and hyperexcitability in MAM-pilocarpine rats.

Key words epilepsy; model; pilocarpine; cortical malformation epilepsy

In the present study, we examined the facilitated effect of MAM-treated animals on seizure induced by pilocarpine.

MATERIALS AND METHODS

Animals Female rats (Sprague-Dawley) with known in-semination times were obtained. Pregnant rats were injected with 25 mg/kg MAM dissolved in 0.9% saline. Intraperitoneal injections were made on embryonic 15 d. Experimental procedures were performed in accordance with the animal care guidelines of NIH and the Korean Academy of Medical Sciences. All animals were maintained in a 12-h light–dark cycle and were provided with food and water ad libitum.

In Vivo Recording Sprague-Dawley rats were anesthetized with urethane (1.3 g/kg), according to procedures reported elsewhere. Briefly, the recording electrode was located in the hippocampus (AP: -3.8 mm from bregma; L: 2.5 mm). A concentric bipolar stimulating electrode was inserted into the contralateral fimbria-fornix (AP: -1.3, L: 1.0, V: 4.8 mm) to stimulate commissural inputs in the CA1 area. Ten field potentials were recorded with electrodes made from glass micropipettes blunted to an outer diameter of ~15 μm and filled with 1 M potassium acetate. The stimulus intensity was standardized by the stimulus threshold for a population spike T, which was determined by delivering stimuli (frequency 0.1 Hz, duration 100 μs) of increasing intensity until a small-amplitude population spike was evoked consistently. Commissural pathway stimulation was made using single or paired pulses. Paired pulse stimulation was used to assess inhibition in the CA1 network. Pairs of stimuli were delivered at interstimulus intervals of 30 ms, 50 ms, 70 ms, 100 ms, and 250 ms that generated inhibition of the second population spike of the pair. Population spike amplitude ratios were calculated by dividing the amplitude of the second response of the pair by that of the first response. Therefore, amplitude ratios <1 indicated paired pulse inhibition, and amplitude ratios >1 indicated facilitation. Statistical analysis was carried

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out with one-way analysis of variance (ANOVA) following by LSD *post-hoc* analysis. Comparisons between the groups were made with an unpaired Student *t*-test. The quantitative values are expressed as means±S.E.M.

**Pilocarpine Treatment** Sprague-Dawley rats (250—300 g, *n=61*) were injected intraperitoneally with atropine at a dose of 1 mg/kg prior to injection of pilocarpine in order to reduce the peripheral effects of pilocarpine. Normal rats and MAM rats were injected (i.p.) with a dose of 320 mg/kg of pilocarpine in 0.9% NaCl. On the day of injection, behavioral observation continued for 2 h after pilocarpine injection. Pilocarpine-induced seizures were graded according to the Racine scale using stage 1—5: Stage 0, in which the rats showed no convulsion; stage 1, in which rats showed head bobbing, tremor, backward walking, wet dog shake; stage 2, intermittent forepaw myoclonus, rearing and falling; stage 3, continuous chronic convulsion; stage 4, tonic flexion and stage 5, respiratory arrest. To improve the chances of survival, animals received 5-mg/kg of diazepam at 90 min after the onset of SE. After recovering from acute seizures, rats were observed at least 4 h/d, 2—3 d/week for chronic, spontaneous seizures.

**Fixation and Timm Staining** Two months after treatment, an overdose of urethane was administered and animals were perfused with 11 of 0.37% sulfide solution and then 11 of 4% paraformaldehyde solution (pH 7.4). Brains were removed and 40 μm thick sections were mounted on glass slides and dried. The sections were placed in Timm developer solution (gum arabic, sodium citrate, citric acid, hydroquinone, silver nitrate) for 60—70 min in darkness at room temperature. Subsequently, sections were washed with distilled water for 15 min and were lightly counterstained with cresyl violet to stain Nissl substance, dehydrated, cleared, and coverslipped with Permount.

**Immunohistochemistry** Sections were cut to 40 μm on a sliding microtome. These free-floating sections were incubated with 4% normal goat serum (Vector) for 1 h at room temperature. Then, sections were incubated in mouse anti-parvalbumin (diluted 1:4000, Sigma) and in rabbit anti-somatostatin (diluted 1:1000, Peninsula Laboratories) in PBS containing 1% normal goat serum for 1 h at room temperature. After washing three times for 10 min with PBS, sections were incubated sequentially, in biotinylated goat anti-rabbit IgG (1:500) for somatostatin and in biotinylated goat anti-mouse IgG (1:500) for parvalbumin. They were diluted in the same solution as the primary antiserum and in HRP-streptavidin. Between the incubations, the tissues were washed three times with PBS for 10 min. The tissues were visualized with Dako 3,3′-diaminobenzidine (DAB) chromogen and mounted on gelatin-coated slides. In order to establish the specificity of the immunostaining, a negative control test was carried out with pre-immune serum instead of a primary antibody. The negative control resulted in the absence of immunoreactivity in all structures.

**Quantitative Analysis** An investigator who was blind to the experimental group treatment performed semi-quantitative analysis. Each sample consisted of four sections, which were used for Nissl-staining, Timm staining, somatostatin, and parvalbumin immunocytochemistry. Sections from normal rats, normal-pilocarpine rats, MAM-treated rats, and MAM-pilocarpine rats were processed together. The number of interneurons labeled for somatostatin and parvalbumin were obtained from the hippocampal region in the normal rats, normal-pilocarpine rats, MAM-treated rats, and MAM-pilocarpine rats. For immunocytochemically labeled tissue, caps were defined as immunoreactive somata that came into focus while focusing down through the light microscope (Nikon). Statistical analysis was carried out with ANOVA following by LSD *post-hoc* analysis. Comparisons between the groups were made with an unpaired Student *t*-test. The quantitative values are expressed as means±S.E.M. The extent of neuron loss in the MAM-treatment rats was calculated with the following formula: 100—(number of neurons in MAM-treated rat[s]÷mean number of neurons in the control group)%. The Timm-staining sections were estimated semi-quantitatively by an investigator who was blind to the experimental treatment using the rating scale of Tauck and Nadler, where zero indicated little or no Timm staining in the granule cell layer; 1 signified mild, patchy staining in the granule cell layer; 2 designated moderate continuous staining through the granule cell layer with discontinuous, punctate staining in the inner molecular layer; and 3 denoted a continuous band of intense staining throughout the inner molecular layer. Six sections were analyzed per rat; the regions analyzed were similar for all animals, with a wide range of the temporal half of the hippocampus analyzed. Mossy fiber sprouting scores are reported as the mean±standard error of the mean (S.E.M.). An unpaired Student’s *t* test was used to determine differences in mossy fiber sprouting scores.

**RESULTS**

**Pilocarpine-Induced Seizures and Spontaneous Seizures** Data for this study were obtained from a total of 61 rats treated with pilocarpine (31 MAM rats and 30 normal rats). The mortality rate for the pilocarpine treated normal rats (normal-pilocarpine rats) was 3.3% and 38.7% of the pilocarpine treated MAM rats (MAM-pilocarpine rats) expired within 2-h after being injected with pilocarpine. The surviving rats typically displayed behavioral changes 1—6 min after pilocarpine injection (e.g. wet-dog shakes, slight body tremors, salivation, head bobbing, and stiffening of the tail). In a subset of rats, we observed different types of seizures of different stages in both normal-pilocarpine rats and MAM-pilocarpine rats. The onset time of SE was 34.05±5.05 min in the normal-pilocarpine rats, 22.72±1.83 min in the MAM-pilocarpine rats. These results appeared significantly prematurely in the MAM-pilocarpine rats as compared to the normal-pilocarpine rats (*p*<0.05, Fig. 1A). Spontaneous seizures were observed in the SE survivors from each experimental group. Spontaneous tonic-clonic seizures were observed in 45.5% of the normal-pilocarpine rats (5/11 rats) and in 92.3% of the MAM-pilocarpine rats (12/13 rats) that survived the pilocarpine-induced SE, which began several days after the pilocarpine treatment. The seizure duration of MAM-pilocarpine rats was 140.84±47.85 s and normal-pilocarpine rats was 83.81±31.96 s (Fig. 1B).

**In Vivo Experiments** As the stimulation intensity increased, a population spike was evoked (Fig. 2A). Further increases in the stimulus intensity resulted in a larger-amplitude population spike in all groups (Fig. 2A). Although the response to the increased stimulus intensity was graded in all
groups, the population spike in the MAM-pilocarpine rats and conventional MAM rats tended to reach the maximum amplitude at a lower stimulation intensity than the population spike in the normal animals (Fig. 2A).

Paired-pulse responses were analyzed in normal rats, MAM-treated, rats and MAM-pilocarpine rats (Fig. 2B). The normal rats exhibited fast inhibition, facilitation, and slow inhibition of the second population spike of the pair at 30, 70, and 250 ms interstimulus intervals. At the 30 and 50 ms interstimulus intervals, there was inhibition of the amplitude of the second population spike. At 70 ms interstimulus intervals, there was facilitation of the amplitude of the second population spike. At 100 and 250 ms interstimulus intervals, there was a slow inhibition of the amplitude of the second population spike. Compared with the normal rats, MAM-pilocarpine rats and MAM-treated rats displayed less paired-pulse inhibition. At interstimulus intervals of 30 and 70 ms, the second population spike of the MAM-pilocarpine rats was inhibited instead of facilitated, as in the conventional MAM rats. At 50, 100, and 250 ms interstimulus intervals, the MAM-pilocarpine rats were more facilitated than the conventional MAM rats. Spontaneous seizure activity was observed in the MAM-pilocarpine rats (Fig. 2C). Spontaneous seizure activity was observed in 28.6% of the MAM-pilocarpine rats (2/7 rats). They are not exhibit in the normal rats and MAM-treated rats.

**Immunohistochemistry** Parvalbumin-immunopositive interneurons were found mainly in the CA1 and CA3 subfields, in the dentate granule cell layers, and in the dentate hilus (Fig. 3). The density of parvalbumin-immunopositive interneurons appeared reduced in the hippocampus of the normal-pilocarpine rats, MAM-treated rats, and MAM-pilocarpine rats (Fig. 3). The reduction was 1.6% ± 0.15 for the normal-pilocarpine rats, 74.1% ± 0.03 for the MAM-pilocarpine rats and 70.8% ± 0.08 for the conventional MAM rats in the dentate gyrus, and 52.8% ± 0.06 for the normal-pilocarpine rats, 90% ± 0.02 for the MAM-pilocarpine rats and 82.8% ± 0.06 for the conventional MAM rats in the CA1, and 44.2% ± 0.04 for the normal-pilocarpine rats, 69.8% ± 0.06 for the MAM-pilocarpine rats, and 59.5% ± 0.08 for the conventional MAM rats in the CA3 (Fig. 3M).

Somatostatin-containing neurons are one of the major subpopulations of GABA neurons in the stratum oriens of CA1, in the CA3 and in the dentate hilus (Fig. 4). Relative to normal rats, the decrease of somatostatin-immunoreactive neu-
rons was 60.5% ± 0.16 for the normal-pilocarpine rats, 84.6% ± 0.04 for the MAM-pilocarpine rats and 76.8% ± 0.06 for the conventional MAM rats in the CA1, and 52.9% ± 0.11 for the normal-pilocarpine rats, 70.6% ± 0.05 for the MAM-pilocarpine rats and 85.9% ± 0.02 for the conventional MAM rats in the CA3 (Fig. 4M). In contrast with the parvalbumin-immunoreactive neuron staining, the MAM-pilocarpine rats had larger somatostatin-immunoreactive neurons than the conventional MAM rats.

**Mossy Fiber Sprouting**  Mossy fiber sprouting of dentate gyrus granule cell axons was assessed using a modified method for Timm histochemistry. Not all rats examined from both strains that reached SE on the day of the pilocarpine injection had mossy fiber sprouting. The average mossy fiber sprouting score of the MAM-pilocarpine rats that reached SE was 1.33 ± 0.21 (n = 6) and the average score for the normal-pilocarpine rats that reached SE was 1.40 ± 0.51 (n = 5; Fig. 5). These scores were not significantly different (p > 0.05).

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Fig. 3. Parvalbumin Containing Neurons in the CA1, CA3 and DG Hippocampus Subfields

Parvalbumin immunoreactivity in normal rats (n = 5; A, E, I), normal-pilocarpine rats (n = 5; B, F, J), conventional MAM rats (n = 5; C, G, K), and MAM-pilocarpine rats (n = 6; D, H, L). Cell loss of conventional MAM rats, normal-pilocarpine rats and MAM-pilocarpine rats. (M) * represents p < 0.05 compared to the normal rats. Error bar indicates S.E.M. Scale bar represents 500 μm.

Fig. 4. Somatostatin Immunopositive Interneurons in the CA1, CA3 and DG Hippocampus Subfields

Somatostatin immunoreactivity in normal rats (n = 5; A, E, I), normal-pilocarpine rats (n = 5; B, F, J), conventional MAM rats (n = 5; C, G, K), and MAM-pilocarpine rats (n = 6; D, H, L). Cell loss of conventional MAM rats, normal-pilocarpine rats and MAM-pilocarpine rats. (M) * represents p < 0.05 compared to the normal rats. Error bar indicates S.E.M. Scale bar represents 500 μm.

Fig. 5. Timm Staining in Normal Rats (n = 5), Conventional MAM Rats (n = 5), Normal-Pilocarpine Rats (n = 5) and MAM-Pilocarpine Rats (n = 6)

(A) Timm staining in the dentate gyrus of normal rats. (B) Staining in normal-pilocarpine rats that reached pilocarpine-induced SE and had spontaneous seizures. (C) Timm staining in the dentate gyrus of conventional MAM rats. (D) Staining in MAM-pilocarpine rats that reached pilocarpine-induced SE and had spontaneous seizures. The arrows represent the dark band of aberrant mossy fibers in the supragranular layer. Scale bar represents 500 μm.
DISCUSSION

An important issue in the development of animal models for cortical dysplasia epilepsy is whether they exhibit behavioral and cellular changes that are similar to those found in cortical dysplasia epilepsy patients. Perhaps the most easily identified behavioral criterion for cortical dysplasia epilepsy development in animal models is the presence of recurrent, spontaneous seizures.29) Our data demonstrate that the MAM-pilocarpine and normal-pilocarpine rats develop spontaneous seizures and undergo mossy fiber sprouting after this treatment. In the MAM-pilocarpine rats that survived pilocarpine-induced SE, a silent period of several days followed in which no spontaneous seizures were observed. Following this silent period, the majority of the MAM-pilocarpine rats that reached SE began to have spontaneous seizures. This is also consistent with data showing that chronic epilepsy typically—but not always—develops over time after the pilocarpine treatment in rats.27,28) The rats in the present study were observed for 4 h/d, 2—3 d/week, implying that seizures probably occurred more frequently than observed during that time period. Based on the current results where most of the MAM-pilocarpine rats that underwent SE were observed to have spontaneous seizures, although seizures were observed in only 45.5% of the SE survivors of the normal-pilocarpine rats, MAM-pilocarpine rats might be predicted to be more susceptible to excitotoxic damage from pilocarpine, and by inference to be more likely to develop chronic epilepsy. Since a smaller percentage of normal-pilocarpine rats was observed to have spontaneous seizures and most of the MAM-pilocarpine rats of both strains that survived SE exhibited cell loss and developed mossy fiber sprouting, the lack of seizures observed in some rats may simply be an artifact of the limited observation time.

Recordings from the heterotopic neurons in the MAM-treated rats suggest that the heterotopic CA1 neurons were in communication with each other as well as with neurons in the overlying neocortex.9,10) The present results for the intact animals indicate that the synaptic excitability of the pyramidal neurons in the CA1 of the MAM-pilocarpine and MAM-treated rats with hippocampal dysplasia and subcortical nodular heterotopia is greater than that of the CA1 pyramidal neurons in the normal rats. Further studies will be necessary to ascertain the connectivity patterns of normotopic CA1 neurons and to determine the extent to which those connections render the neurons susceptible to epileptiform activity. The MAM-pilocarpine and conventional MAM rats had paired-pulse facilitation instead of inhibition, as in the normal rats. In addition, electrophysiological spontaneous responses were observed in the MAM-pilocarpine rats. This demonstrates that there is direct electrophysiological evidence regarding the hyperexcitability in the CA1 region.

In the present study, there were fewer parvalbumin-and somatostatin-immunoreactive neurons in the CA1 of MAM-pilocarpine and conventional MAM rats compared with the normal rats. In the CA1 pyramidal cells, inhibitory inputs are mainly concentrated in the perisomatic region and on the dendrites in the stratum lacunosum-moleculare.29) Therefore, a substantial loss of somatostain-containing cells, which participated in dendritic inhibition and parvalbumin-containing cells that control action potential generation of principal cells,30) can be expected to result in strong deficits of inhibition and imbalance in favor of excitation. Although we did not quantify the total number of neurons lost in the MAM-treated rats and the loss of GAD-containing neurons in the stratum oriens of CA1, semiquantitative assessment of somatostatin—and parvalbumin—immunoreactive neurons suggests that the pattern of cell loss in the normal-pilocarpine and MAM-pilocarpine rats after pilocarpine treatment was similar to that which occurs in animals that are vulnerable to kainate- and pilocarpine-induced cell death.31) In the pilocarpine model of chronic libic seizures, vulnerability of GABAergic interneurons to excitotoxic damage has been reported in the hippocampus CA1 region. The loss of GABA containing neurons corresponds preferentially to the degeneration of somatostatin.31) A more detailed analysis of these differences will be necessary to determine the amount of damage caused by the pilocarpine- and MAM-treatment.

The issue of whether an animal model undergoes cellular changes in the brain to render it more susceptible to spontaneous seizure generation is paramount.35) Several animal models of chronic epilepsy, including the pilocarpine and kainate models, have shown that adult rats develop aberrant mossy fiber sprouting with associated hippocampal cell loss following status epilepticus.29,36) These studies suggested that axon sprouting is correlated with an elevated propensity for increased network excitability. Recurrent spontaneous seizures and mossy fiber sprouting in MAM-pilocarpine rats is replicating similar experiments conducted on rats29) and albino mice.33) Difference between our MAM-treated model and other models29,34) was the seizure silence period. Latency to which seizure began to appear after SE induction was larger for other models (15 d) than for MAM-treated rats (7.9 d). The present study indicates that pilocarpine treatment facilitates seizure in the presence of preexisting brain malformations in MAM rats by exacerbating mossy fiber sprouting and some GABAergic hippocampal cell loss.

REFERENCES


