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Changes of CA1 Excitability in Rats after Prenatal Methylazoxymethanol Treatment

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Experimentally induced cortical disorganization exhibits many anatomical features which are characteristic of cortical malformations in children with early-onset epilepsy. We used an immunocytochemical technique and extracellular field potential recordings from the dorsal hippocampus to determine whether the excitability of the CA1 pyramidal cells was enhanced in rats with experimentally induced hippocampal dysplasia. Compared with control rats, the MAM-treated rats displayed a decrease of paired pulse inhibition. When $GABA_A$ receptor antagonists were blocked with 10 μM bicuculline, the amplitude of the second population spike of the MAM-treated of rats was similar to that of the first population spike, as was in the control rats. The MAM-treated rats had fewer somatostatin and parvalbumin-immunoreactive neurons than the control rats. These results suggest that the enhanced neuronal responsiveness of the *in vivo* recording of the CA1 in this animal model may involve a reduction of CA1 inhibition.

Key Words: Somatostatin, Parvalbumin, Heterotopia, Epilepsy, Dysplasia, Hippocampus

INTRODUCTION

A wide variety of animal models are available for investigating mechanisms of epileptogenesis associated with cortical disorganization (Baraban et al, 1995). The inhibitory systems have been shown with various models of cortical malformations such as GABAergic innervation in heterotopia (Baraban et al, 2000), a change in function/distribution of GABA transporters in the heterotopic region (Calcagnotto et al, 2002), the reduction in the density of parvalbumin- and calbindin-positive interneuron in irradiated rats (Roper et al, 1999) and defects in inhibition in the normotopic dysplastic cortex of irradiated rats (Zhu et al, 2000) and in heterotopic gray matter (Chen et al, 2003). The administration of methylazoxymethanol acetate (MAM) during gestation disrupts cell migration in the central nervous system, resulting in a range of pathologies such as cortical dysplasia, heterotopias, microdysgenesis and lissencephaly (Chen et al, 1986; De Lanerolle et al, 1989). It is of interest to note that, although these models are well established to mimic the structural aspects of human early-onset epilepsy syndrome, is direct evidence for seizure generation in dysplastic tissue is lacking. Irradiated rats demonstrate spontaneous electrographic seizures in vivo (Kondo et al, 2001), however, the site of seizure onset in these animals is unknown. Possibilities include normotopic dysplastic cortex, heterotopic cortex, and hippocampus.

Heterotopic neurons in the MAM-model lack potassium channels and exhibit burster firing properties (Castro et al, 2001; Sancini et al, 1998), and heterotopia receives abundant GABAergic innervation in the MAM-exposed rats (Baraban et al, 2000). Recordings of the slices of MAM-treated rats suggest that the dysplastic neocortex and subcortical heterotopia can be induced to exhibit epileptiform bursting (Baraban et al, 2000), however, *in vivo* electrophysiological characteristics of the hippocampal neurons have not yet been examined in this model. Heterotopic CA1 pyramidal neurons appear to have atypical electrophysiological and morphological characteristics (Chevassus-Au-Louis et al, 1998a) and may also form abnormal connections with neocortical regions (Chevassus-Au-Louis et al, 1998b). Evidence, therefore, suggests that the dysgenic neurons in several models may form pathological connections with each other.

In the present study, we examined the neurochemical features and synaptic responses in the context of *in vivo* network excitability of CA1 neurons in intact animals.

METHODS

Animals

Female timed pregnant Sprague-Dawley rats were injected i.p. with 25 mg/kg MAM dissolved in 0.9% saline on the 15th day of gestation (E15). All animals were maintained in a 12 : 12 h light : dark cycle and provided with food and water *ad lib*.

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ABBREVIATIONS: MAM, methylazoxymethanol acetate.

In vivo recording

Rats (250~300 g) were anesthetized with urethane (1.3 g/kg) and fixed in a stereotaxic apparatus with a small-animal thermoregulatory device. 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. Stimulus intensity (×T) was standardized by determining stimulus threshold for a pyramidal cell population spike (T). 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. In some experiments, the recording electrodes also contained 10 μM bicuculline methiodide (Sigma), which leaked into the focal region of the CA1 near the electrode tip (Buckmaster et al, 1997a; Smith et al, 1999; Choi et al, 2004). Recording sites were verified by using a standard method of dye placement or by tracing the recording electrode tract penetrating into hippocampus with post hoc tissue processing and mapping. Pairs of stimuli were delivered at interstimulus intervals of 30~250 msec. Comparisons between the groups were made with two-tailed and unpaired Student t-test.

Immunhistochemistry

Sections with 50 μm thickness were cut on a sliding microtome. These free-floating sections were incubated with 4% normal goat serum (Vector) for 1 hour at room temperature. Then, sections were incubated with mouse anti-parvalbumin (diluted 1 : 4000, Sigma) and rabbit anti-somatostatin (diluted 1 : 1000, Peninsula Laboratories) in PBS containing 1% normal goat serum for 1 hour at room temperature. After washing three times for 10 min with PBS, sections were sequentially incubated, with biotinylated goat anti-rabbit IgG (1 : 500) for sommatostatin and biotinylated goat anti-mouse IgG (1 : 500) for parvalbumin diluted in the same solution as the primary antiserum and with HRPstreptavidin. 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 showed no immunoreactivity in all structures.

Quantitative analysis

Quantitative analysis was performed by an investigator who was blind to the experimental group treatment. Each sample consisted of three sections, which were used for Nissl-staining, and somatostatin and parvalbumin immunocytochemistry. Sections from the MAM-treated and control rats were processed together. The number of interneurons labeled for somatostatin, and parvalbumin were obtained from the CA1 region in the control and MAM-treated rats. For immunocytochemically labeled tissue, caps were defined as immunoreactive somata that came into focus while focusing down through the light microscope (Nikon). A statistical analysis was performed using a Student t-test. The extent of neuron loss in the MAM-treated rats was calculated with the following formula: 100 - [number of neurons in MAM-treated rat (s) ÷ mean number of neurons in the control group]% (Buckmaster et al, 1997b).

RESULTS

Examples of the CA1 field responses to the paired-pulse stimulation of the fimbria-fornix (30 ms interstimulus interval) in the control and MAM-treated rats are shown in Fig. 1A. The amplitude of the first population spike was typically maximum near 5T. Although the amplitudes of the population spikes to the first stimulus were similar (4.75±1.56 mV for control; 4.07±1.18 mV for MAM-treated), the MAM-treated rats had larger amplitude population spike responses to the second stimulus (5.37±1.29 mV) than the control rats (1.48±0.57 mV). Compared with the control rats, the MAM-treated rats showed a significant increase ($P<0.05$) at 30 ms interstimulus intervals.

The mean population spike amplitude ratios of the control over the MAM-treated rats are plotted in Fig. 1B. The control rats exhibited inhibition, facilitation, and inhibition of the second population spike of the pair at 30, 70 and

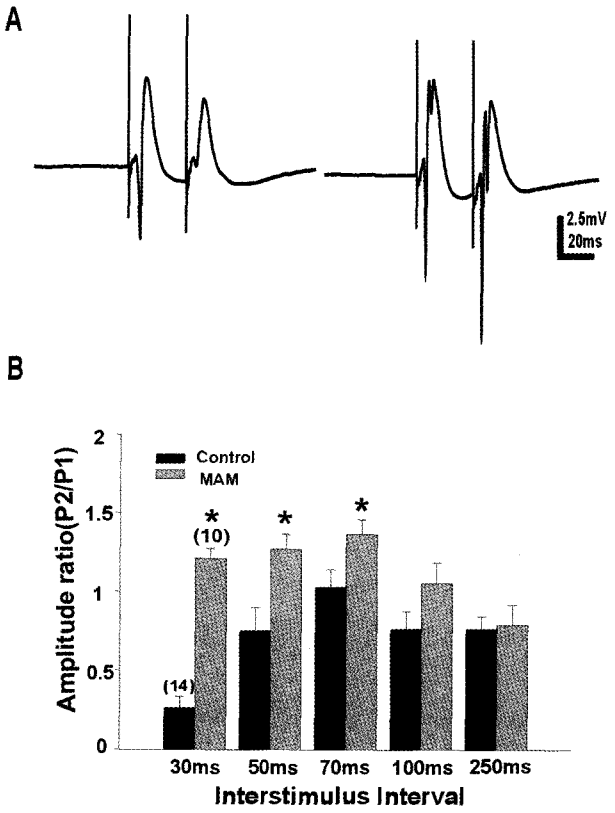

Fig. 1. Paired-pulse responses in hippocampal CA1. (A) Field-potential responses to paired-pulse stimulation of fimbria-fornix (30 ms interstimulus interval, 0.1 Hz stimulus frequency, 5×T stimulus intensity) in control (n=14) and MAM-treated rats (n=10). (B) Paired-pulse inhibition presented as the ratio of Response 2 to Response 1 evoked by stimuli presented at interstimulus intervals between 30~250 ms (stimulus intensity at 5×T). *represents $P<0.05$ compared to the control rats. Error bars indicate S.E.M.

250 msec interstimulus intervals. Compared with the control rats, the MAM-treated rats displayed less paired-pulse inhibition: At 30, 50 and 70 msec interstimulus intervals, the MAM-treated rats showed facilitation of the amplitude of the second population spike.

To examine the population responses when synaptic inhibition was suppressed with the vicinity of the recording, the recording pipettes were filled with a solution containing 10 μM bicuculline. Bicuculline that leaked from the pipette tip presumably blocked the $GABA_A$ receptors near the recording electrode. The amplitude of the first population spike was typically maximum near 3T. The stimulation of the afferent fibers resulted in 2~3 population spikes in the CA1 region of the control animals, when bicuculline leaked from the recording pipette (Fig. 2A). When inhibition was suppressed, the amplitude of the second population spike of the MAM-treated rats was similar to that of the first population spike, as was in the control group (Fig. 2B).

In the control rats, most parvalbumin-immunoreactive somata were located in or adjacent to the stratum oriens, the pyramidal cell layer and the stratum radiatum (Fig. 3A). The MAM-treated rats displayed a pattern of parvalbumin immunoreactivity which was generally similar to that seen in the control rats (Fig. 3B), however, the MAM-treated rats displayed a loss of parvalbumin-immunoreactive neuron staining. The MAM-treated rats had significantly fewer parvalbumin-positive cell bodies than the control rats (Fig. 3E), and the mean numbers of labeled neurons were 21.80±2.67 in the MAM-treated rats, whereas 81.89±6.10 in the control rats ($p<0.05$, Fig. 3F). A comparison of the group means revealed that the MAM-treated rats lost an average of 73.4% of their parvalbumin-immunoreactive neurons.

In the control rats, somatostatin-immunoreactive neurons are located primarily in the stratum oriens (Fig. 3C). The MAM-treated rats displayed a pattern of somatostatin-immunoreactivity which was generally similar to that seen in the control rats (Fig. 3D). The MAM-treated rats, however, displayed a loss of somatostatin-immunoreactive neuron staining: A marked decrease of labeled neurons was observed in the stratum oriens as compared to the control rats. The mean numbers of labeled neurons were 8.06±0.98 in the MAM-treated rats, whereas 25.82±2.36 in the control rats ($p<0.05$, Fig. 3F). A comparison of the group means revealed that the MAM-treated rats lost an average of 68.8% of their somatostatin-immunoreactive neurons.

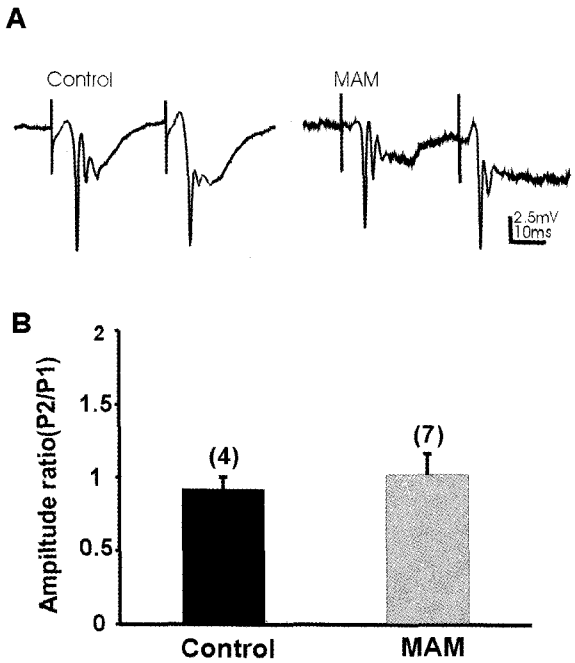

Fig. 2. Paired-pulse responses recorded with a 10 μM bicuculline-filled electrode in the hippocampal CA1. (A) Field-potential responses to paired-pulse stimulation of fimbria-fornix (30 ms interstimulus interval, 0.1 Hz stimulus frequency, 3×T stimulus intensity) in the control (n=4) and MAM-treated rats (n=7). (B) Population spike amplitude ratios. When inhibition was suppressed, the amplitude ratios of the MAM-treated rats were similar to that of control rats ($P>0.05$). Error bars indicate S.E.M.

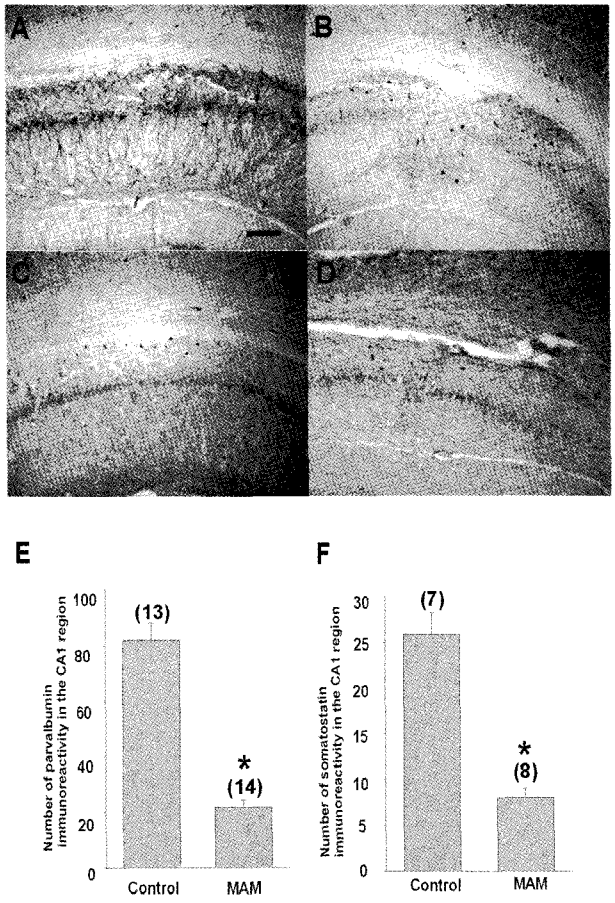

Fig. 3. Parvalbumin- and somatostatin containing neurons in the CA1 region. Parvalbumin immunoreactivity in control (n=13) (A) and MAM-treated rats (n=14) (B). Somatostatin immunoreactivity in control rats (n=7) (C) and MAM-treated rats (n=8) (D). MAM-treated rats had fewer parvalbumin- and somatostatin- immunoreactive neurons than control rats. (E, F) Number of parvalbumin- and somatostatin-immunoreactive neurons in the MAM-treated rats is significantly lower than the control rats. *represents $P<0.05$ compared to the control rats. Error bar indicates S.E.M. Scale bar represents 500 μm.

DISCUSSION

One of the main findings of this study is that the MAM-treated rats had paired-pulse facilitation instead of inhibition, similar to the control rats, demonstrating direct electrophysiological evidence for the hyperexcitability of the CA1 of the MAM-treated rats. In the CA1 field of the hippocampus, paired-pulse facilitation has been attributed to reduced feedback inhibition (Nathan et al, 1991). Similarly, the greater paired-pulse facilitation of the MAM-treated rats suggests less feedback inhibition in the CA1 of the MAM-treated rats than in the control rats (Buckmaster et al, 1994). In the present study, paired-pulse facilitation was studied at only one interstimulus interval (30 msec), however, a full range of interstimulus intervals (10~1,000 msec) and a plot of these data would provide more convincing evidence of such an abnormality, although it is possible that facilitation was altered.

An alternative mechanism underlying paired-pulse facilitation in the MAM-treated rats is augmented excitation, i.e. recurrent excitation. To test a possibility that enhanced excitatory circuitry contributed to the hyperexcitablity in the CA1 of the MAM-treated rats, paired pulse responses in the presence of GABA receptor antagonists were compared between the two groups. Our results showed that the amplitude of the second population spike of the MAM-treated rats was similar to that of the first population spike, as was in the control. This suggests that the augmented excitation could not fully explain the increased excitability in the MAM-treated rats. The degree of facilitation following bicuculline administration in the MAM animals did not appear to change much, however, it changed significantly in the control animals. Although this might suggest an alteration of GABAergic sensitivity, it may also mean that the MAM animals, are much less sensitive to the effects of GABA, because of no change compared to the untreated state. Multiple population spikes observed in our present study had not previously been shown under during the baseline recording conditions in the MAM-treated rats (Baraban et al, 2000). These discrepancies are most likely due to difference in the systems used for electrophysiological recording; We used in vivo system which preserves on excitatory and inhibitory circuit intact.

The loss of local GABAergic neurons has been suggested as a means by which excitatory circuits are disinhibited, especially in areas that normally have local recurrent inhibitory connections, which probably exist in the CA1 (Morin et al, 1998). The alteration of inhibition appears to be more complicated than a simple overall reduction in the total content of GABA neurons, since the specific types of interneurons appear to be selectively affected in the MAM cortex, changing the proportions of selective subtypes (Jacobs et al, 1999). In the present study, there were fewer parvalbumin- and somatostatin-immunoreactive neurons in the CA1 of MAM-treated rats than with the control rats. In the CA1 pyramidal cells, inhibitory inputs are mainly concentrated in the perisomatic region and on the dendrites in stratum lacunbosum-molecurale (Megias et al, 2001). Therefore, a substantial loss of somatostain-containing cells, which participate in dendritic inhibition and parvalbumin-containing cells that control action potential generation of principal cells (Miles et al, 1996), would 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 of GAD-containing neurons lost in the stratum oriens of CA1, a semiquantitative assessment of somatostatin- and parvalbumin-immunoreactive neurons suggests that the pattern of cell loss in the MAM-treated rats was similar to that occurred in animals that were vulnerable to pilocarpine-induced cell death (Buckmaster et al, 1997). A more detailed analysis of these differences would be necessary to determine the amount of damage caused by the MAM-treatment.

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