

## Hippocampal Programmed Cell Death after Status Epilepticus: Evidence for NMDA-Receptor and Ceramide-Mediated Mechanisms

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**Summary:** *Purpose:* Status epilepticus (SE) can result in acute neuronal injury with subsequent long-term age-dependent behavioral and histologic sequelae. To investigate potential mechanisms that may underlie SE-related neuronal injury, we studied the occurrence of programmed cell death (PCD) in the hippocampus in the kainic acid (KA) model.

*Methods:* In adult rats, KA-induced SE resulted in DNA fragmentation documented at 30 h after KA injection. Ceramide, a known mediator of PCD in multiple neural and non-neural tissues, increased at 2–3 h after KA intraperitoneal injection, and then decreased to control levels before increasing again from 12 to 30 h after injection. MK801 pretreatment prevented KA-induced increases in ceramide levels and DNA fragmentation, whether there was reduction in seizure severity or not (achieved with 5 mg/kg and 1 mg/kg of MK801, respectively).

*Results:* Both ceramide increases and DNA fragmentation were observed after KA-induced SE in adult and in P35 rats. Ceramide did not increase after KA-induced SE in P7 pups, which also did not manifest any DNA fragmentation. Intrahippocampal injection of the active ceramide analogue C2-ceramide produced widespread DNA fragmentation, whereas the inactive ceramide analogue C2-dihydroceramide did not.

*Conclusions:* Our data support the hypotheses that (a) *N*-methyl-D-aspartate-receptor activation results in ceramide increases and in DNA fragmentation; (b) ceramide is a mediator of PCD after SE; and (c) there are age-related differences in PCD and in the ceramide response after SE. Differences in the ceramide response could, potentially, be responsible for observed age-related differences in the response to SE. **Key Words:** Kainic acid—Status epilepticus—Programmed cell death—Ceramide—NMDA receptor.

Apoptosis is a morphologically distinct type of cell death characterized by cellular shrinkage and condensation; large, round, or crescent-shaped chromatin clumps within the nucleus; and relative preservation of cytoplasmic organelles (1). Programmed cell death (PCD) refers to an ordered process that leads to cell death through predetermined and reproducible mechanisms that usually require de novo protein synthesis. PCD is characterized by certain biochemical features such as DNA fragmentation into oligonucleosomal fragments (2). Although generally used to refer to the same final outcome, apoptosis and PCD are not always identical, especially because PCD has recently been shown to occur in neurons with the morphologic features of necrosis (3,4). A number of studies have reported the occurrence of PCD after

status epilepticus (SE) in adult rats, in the kainic acid (KA), pilocarpine (PC), and perforant-path stimulation models (5–9). The mechanisms leading to PCD after SE remain largely unknown. Some studies suggested that *N*-methyl-D-aspartate (NMDA)-receptor activation is necessary for PCD after SE (4,6,10). Others failed to show such an effect (5). The reason for this discrepancy is probably that different studies used different doses of MK801, resulting in different degrees of blockade of NMDA receptors. In addition, none of these studies controlled for the severity of seizure activity after MK801 administration, and therefore did not exclude the possibility that the observed protective effects of MK801 may be due to decreases in seizure severity per se, and not to NMDA-receptor blockade.

The sphingolipid ceramide is a mediator of intracellular signaling pathways of PCD in several cell lines and tissues including neuronal tissues (11–19). The potential role of ceramide in SE-induced PCD has not been previously investigated.

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The immature rat brain is resistant to the long-term histologic and behavioral sequelae of SE in certain models such as the KA model (20). It has not been investigated whether this resistance is secondary to a resistance to PCD or to other mechanisms. Whether those mechanisms involve ceramide is not known.

In this study, our goals were to (a) determine whether the occurrence of SE-induced PCD is associated with increases in ceramide levels; (b) determine whether stereotaxic injection of the active ceramide analogue C2-ceramide induces PCD in the hippocampus; (c) establish the effects of NMDA-receptor blockade on ceramide levels and on PCD; and (d) determine the time course of ceramide increases during and after SE. In this article we use the terms PCD and apoptosis as referring to two distinct, although related, processes as defined earlier and not interchangeably, as did some previous authors.

## MATERIALS AND METHODS

### Experimental designs

#### *Experiment I: DNA fragmentation and ceramide levels in the kainic acid model*

The goals of this experiment were to investigate whether DNA fragmentation and changes in ceramide levels occur after KA-induced SE in three different age groups.

Six groups of Sprague–Dawley rats (seven to eight per group) were treated as follows:

Group 1 (Adult/KA): Adult rats (250–350 g) that received intraperitoneally (i.p.) a convulsant dose of KA (15 mg/kg), and underwent SE for  $\geq 4$  h.

Group 2 (Adult/Saline): Adult rats that received i.p. vehicle only.

Group 3 (P35/KA): P35 rats that received i.p. the same dose of KA as group 1, and underwent SE for  $\geq 4$  h.

Group 4 (P35/Saline): P35 rats that received i.p. vehicle only.

Group 5 (P7/KA): P7 rats that were injected i.p. with a convulsant dose of KA (3 mg/kg), and underwent SE for  $\geq 4$  h.

Group 6 (P7/Saline): P7 rats injected i.p. with vehicle only.

Previous studies showed that, in the KA model, the immature rat brain at P7 was resistant to long-term behavioral sequelae and memory impairments that often follow SE (20). In this experiment, we studied the P7 age group to determine whether it was also resistant to SE-induced PCD. We also studied P35 rats because this age group corresponds to early childhood in the human, an age during which temporal lobe epilepsy commonly begins (21).

Rats were killed at 30 h after the KA/saline injections ( $\sim 24$  h after the end of SE) with a lethal i.p. dose of sodium pentobarbital. The 30-h time point was chosen because it occurred  $\sim 24$  h after the onset of SE, a time point shown in previous studies to coincide with peaking or near peaking of the PCD process (22,23). After pentobarbital injection rats were perfused with saline and were then rapidly dissected. The left hemisphere was immediately homogenized, lipids were extracted, and then lipid phosphate and ceramide levels were determined. The right hemisphere was fixed in 10% formalin and embedded in paraffin. Slides 5- $\mu$ m thick were stained by using the hematoxylin-eosin (H&E), the terminal deoxynucleotidyl transferase (TdT) dUTP nick end-labeling (TUNEL) stain, and the Hoechst nuclear stain. The sections were taken at the level of separation of the dorsal and the ventral hippocampus.

#### *Experiment II: The effects of NMDA-receptor blockade on DNA fragmentation and on ceramide levels in the kainic acid model*

The goals of this experiment were to investigate whether, in the KA model, MK801 administration can prevent ceramide increases and DNA fragmentation.

Four groups of adult Sprague–Dawley rats (six to nine rats per group) were treated as follows:

Group 1 (Control) received an i.p. injection of vehicle only.

Group 2 (KA) received an i.p. injection of a convulsant dose of KA (15 mg/kg).

Group 3 (KA/MK801-1) received MK801 (1 mg/kg, i.p.) and then a convulsant dose of KA (15 mg/kg, i.p.).

Group 4 (KA/MK801-5) received MK801 (5 mg/kg, i.p.) and then a convulsant dose of KA (15 mg/kg, i.p.).

Rats were killed, and the brains were processed as in experiment I.

#### *Experiment III: The effects of C2-ceramide injection in the adult rat hippocampus*

The goals of this experiment were to determine whether hippocampal injection of the biologically active ceramide analogue C2-ceramide results in DNA fragmentation, at 24 h after injection, as compared with the inactive analogue C2-dihydroceramide (24).

Three groups of adult Sprague–Dawley rats (250–300 g), were treated as follows:

Group 1: Seven rats, injected with 1  $\mu$ l of the vehicle alone. The vehicle consisted of a 1:99 solution of dimethyl sulfoxide (DMSO) in phosphate-buffered saline (PBS).

Group 2: Seven rats, injected with 10 ng of C2-dihydroceramide in 1  $\mu$ l of vehicle.

Group 3: Seven rats injected with 10 ng of C2-ceramide in 1  $\mu$ l of vehicle.

In all the groups, the right dorsal hippocampus was stereotaxically injected. The coordinates, from the bregma, were lateral,  $-0.30$  cm; anteroposterior,  $-0.43$  cm; and vertical,  $-0.4$  cm. All rats were killed 24 h after the hippocampal injection, and the brains were rapidly dissected and fixed in formalin for later paraffin embedding and TUNEL staining.

#### *Experiment IV: Time sequence of ceramide increases after status epilepticus*

The goals of this experiment were to determine the time sequence of ceramide increases during and after SE.

Adult Sprague–Dawley rats (10 groups, four to seven rats/group) were studied. Animals were injected i.p. with 15 mg/kg KA, underwent SE, and were killed before KA injection (control group), and at 1, 2, 3, 4, 6, 12, 18, 24, and 30 h after KA injection. Rats were killed in the same way as in experiment I, the brains were removed, and the hippocampi were dissected. Ceramide levels were then determined in the hippocampus.

#### *Experiment V: Comparison of ceramide-to-phosphate ratios in the hippocampus and the rest of the hemisphere after kainic acid–induced status epilepticus*

The goals of this experiment were to (a) determine whether ceramide levels increase in both the hippocampus and in the rest of the hemisphere after SE; and (b) determine whether the results obtained in the whole hemisphere reflected those of the hippocampus.

Fifteen adult Sprague–Dawley rats (300–350 g) were divided into two groups.

Group 1: Control rats that did not receive KA and were killed at the same time as those in group 2. The hippocampi were dissected, and the ceramide-to-phosphate ratios were separately determined in each rat in the hippocampus and in the rest of the hemisphere.

Group 2: KA rats that were injected with a convulsant dose of KA (15 mg/kg), killed at 30 h after KA injection ( $\sim 24$  h after the end of SE), and the brains treated in the same way as in group 1.

Rats were killed and the brains were processed as in experiment I.

### **Experimental procedures**

#### *Determination of ceramide-to-phosphate ratios*

Ceramide levels were determined by using the diacylglycerol kinase assay (25). These were compared with lipid phosphate levels, which also were determined by using the method of the same authors. During each experiment, both assays were performed on all the samples simultaneously. In all the assays, the correlation coefficient of the standard curve was  $>0.99$ .

#### *Histologic and immunohistochemical methods*

The occurrence of DNA fragmentation was assessed by the occurrence of TUNEL-positive cells. In some experiments, we also performed the Hoechst nuclear stain. The TUNEL stain was performed as previously described (26). The Hoechst stain was performed as described by Lopez et al. (6). The severity of the observed lesions on H&E stain was established on a scale of 0 to 4 similar to that previously described by us: 0, no lesions; 1, a minimal lesion localized to part of the hippocampal area of interest (e.g., CA1) and affecting only 1–25% of the cells in the region being studied (25); 2, cellular lesions affecting 25–50% of the cells in the area of interest, with preservation of the general cellular architecture; 3, further cellular lesions affecting 50–75% of the cells in that region; and 4, complete disruption of the normal cellular architecture and complete, or almost complete, loss of normal cellular elements affecting 75–100% of the cells.

In the TUNEL stain, abnormal fluorescence was determined by comparison with negative controls (saline-injected control rats), and with positive controls (artificially fragmented DNA by the addition of DNase). Similarly, in the Hoechst stain, abnormal nuclei were determined by their condensed chromatin that stains intensively. In both of these stains, scoring also was performed according to a scale from 0 to 4: 0, no difference in fluorescence from controls; and 4, all or almost all (75–100%) of the cells in the area of interest had the same fluorescence as the positive controls. Scores of 1, 2, and 3 indicated 1–25, 25–50, and 50–75% affected cells in the area of interest. The scoring of all groups in all the experiments was blinded.

#### *Determination of seizure number and duration using videotape techniques*

All rats that received KA in all the experiments were directly observed to confirm the occurrence of SE. In addition, in experiment II, we used videotape techniques to determine the number and the duration of all forelimb clonus (FLC) episodes in each rat. The rats were monitored with a wide-angle lens, which allows monitoring of six rats simultaneously. We reviewed the videotape at 3 times regular speed. We then calculated the total duration of FLC for each rat and used that as a measure of seizure severity.

#### **Statistical analysis**

Analysis of variance (ANOVA) was used to compare differences among the groups in the ceramide-to-phosphate ratios. If one-way ANOVA demonstrated significance ( $p < 0.05$ ), then post hoc analysis with the Least Significant Differences (LSD) test was performed to compare paired groups. The Kruskal–Wallis test was used to compare serial ceramide-to-phosphate ratios, H&E, TUNEL, and Hoechst stain scores among the

groups. We compared the scores of each specific subfield (CA1, CA2, CA3/CA4, and granule cells) among the groups. When subfield comparisons did not achieve statistical significance, we added the scores of all the subfields to achieve with a total histologic severity score, and compared these total scores among the groups. If any of these comparisons showed significance ( $p < 0.05$ ), then paired Kruskal–Wallis analyses were performed to compare paired groups.

## RESULTS

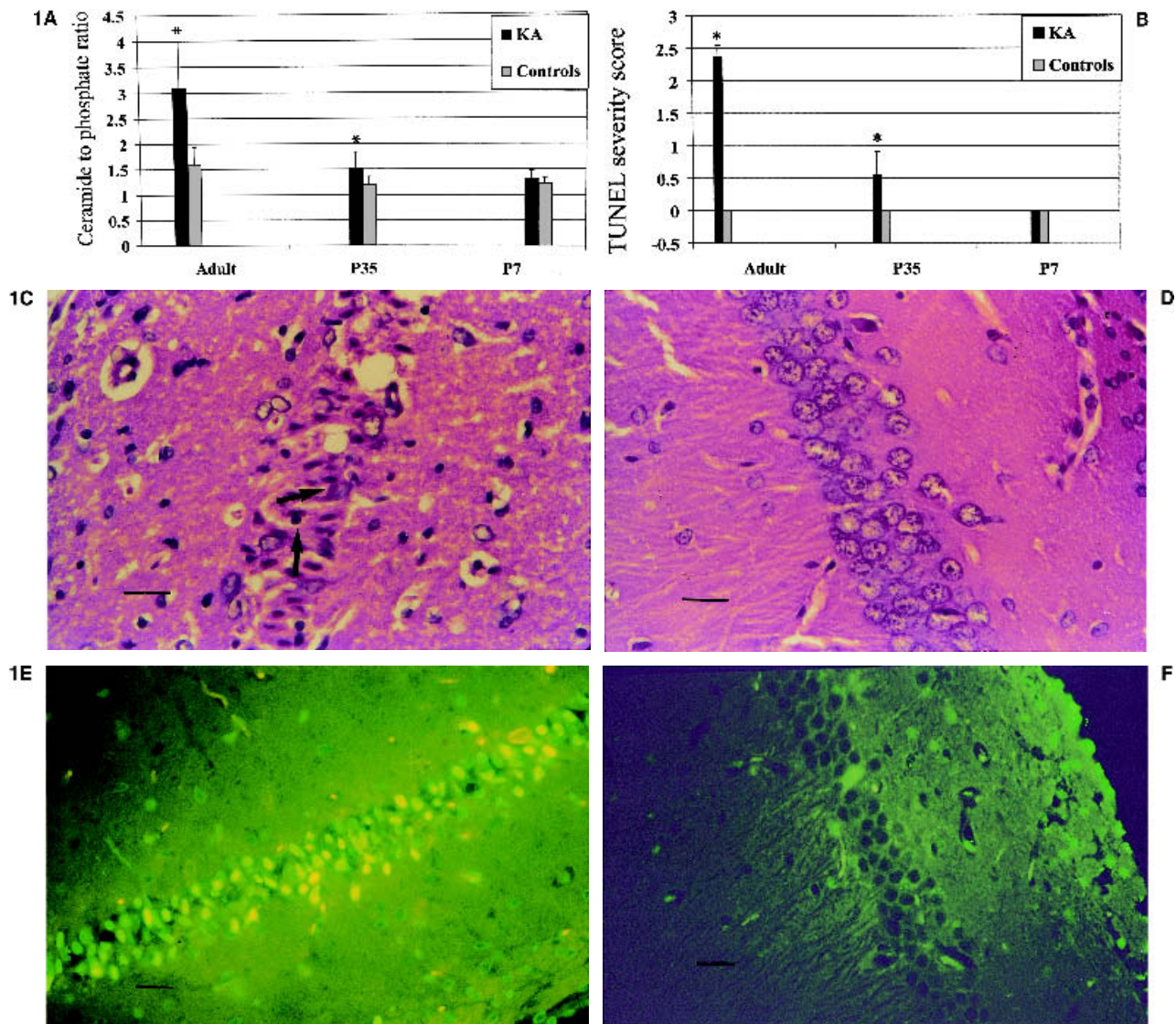
### Experiment I: DNA fragmentation and ceramide levels in the kainic acid model

Experiment I showed that both adult and P35 rats manifested increased ceramide-to-phosphate ratios and

DNA fragmentation 24 h after SE, whereas P7 rats showed neither (Fig. 1A and B). Findings on Hoechst and H&E were consistent with those of the TUNEL stain.

There were significant differences in the *ceramide-to-phosphate ratios* (mean  $\pm$  SEM) between the Adult-KA group and the Adult-Saline group ( $3.1 \pm 0.90$ ,  $1.59 \pm 0.35$ ;  $p = 0.0005$ ), and between the P35-KA and the P35-Saline groups ( $1.52 \pm 0.32$ ,  $1.2 \pm 0.16$ ;  $p = 0.01$ ), but not between the P7-KA and the P7-Saline groups ( $1.32 \pm 0.16$  and  $1.22 \pm 0.12$ ;  $p = 0.12$ ).

The *TUNEL stain* showed that the mean CA1 subfield scores of the Adult-KA and P35-KA groups, but not those of the P7-KA group, were higher than those of their respective controls. Specifically the CA1 subfield scores



**FIG. 1.** **A:** Ceramide (pmol) to phosphate (nmol) ratios in adult, prepubescent, and immature rats after kainic acid (KA)-induced status epilepticus (SE) compared with their respective controls. **B:** TUNEL scores of CA1 region of the same groups. Data are plotted as means  $\pm$  SEM. \* $p < 0.05$  compared with controls. **C:** Hematoxylin and eosin stain of the CA1 region of a P35 rat after KA-induced SE as compared with a control P35 rat (**D**). **C:** Neurons with necrotic morphology (arrows) in the CA1 region at 30 h after KA injection. Such neurons were intensely TUNEL positive (**E**) as compared with those of control rats (**F**). Scale bar, 20  $\mu$ m.

of the Adult-KA and the Adult-Saline groups were:  $2.37 \pm 0.18$  and  $0 \pm 0$  ( $p = 0.0002$ ). The P35-KA and the P35-Saline group scores were  $0.55 \pm 0.36$  and  $0 \pm 0$  ( $p = 0.021$ ). The P7-KA and the P7-Saline group scores were  $0 \pm 0$  and  $0 \pm 0$  ( $p = 1$ ). Scores in the CA3/CA4, CA2, and granule cell regions were not different from those of controls in any of the age groups ( $p \leq 0.13$ ).

The *Hoechst stain* scores of the Adult-KA and P35-KA groups, but not those of the P7-KA group, were significantly higher than those of their respective controls in both the CA1 and CA3/CA4 subfields. The Adult-KA group and the Adult-Saline group scores were CA1,  $3.75 \pm 0.16$  and  $0 \pm 0$  ( $p = 0.0002$ ); CA3/CA4,  $1.75 \pm 0.24$  and  $1 \pm 0$  ( $p = 0.009$ ). The P35-KA group scores and the P35-Saline group scores were CA1,  $1.44 \pm 0.6$  and  $0 \pm 0$  ( $p = 0.028$ ); CA3/CA4,  $0.44 \pm 0.17$  and  $0 \pm 0$  ( $p = 0.027$ ). The scores of the P7-KA and the P7-Saline groups were CA1,  $0 \pm 0$  in both groups ( $p = 1$ ); CA3/CA4,  $0.9 \pm 0.17$  and  $1.09 \pm 0.09$  ( $p = 0.32$ ). Scores of the CA2 subfield and of the granule cell regions were not different from those of controls in any of the age groups.

On the *H&E stain*, there were significant differences between the Adult-KA and the Adult-Saline groups as well as between the P35-KA and the P35-Saline groups, but not between the P7-KA and the P7-Saline groups in both the CA1 and the CA3/CA4 subfields. The mean scores were the following: CA1: Adult-KA,  $3.87 \pm 0.12$ ; Adult-Saline,  $0.12 \pm 0.11$  ( $p = 0.0002$ ); P35-KA,  $1.88 \pm 0.63$ ; P35-Saline,  $0.0 \pm 0.0$  ( $p = 0.011$ ); CA3/CA4: Adult-KA,  $1.62 \pm 0.26$ ; Adult-Saline,  $0.37 \pm 0.26$  ( $p = 0.006$ ); P35-KA,  $0.88 \pm 0.3$ ; P35-Saline,  $0.0 \pm 0.0$  ( $p = 0.011$ ). The scores of the P7-KA and the P7-Saline groups were the following: CA1: both groups scored  $0.0 \pm 0.0$  ( $p = 1$ ); CA3/CA4:  $0.9 \pm 0.17$  and  $0.9 \pm 0.21$ , respectively ( $p = 1$ ).

The majority of damaged neurons in adult and in P35 rats in this experiment had eosinophilic cytoplasm and pyknotic, condensed nuclei, which is consistent with morphologic features of necrosis (26) (Fig. 1C). However, a few damaged neurons appeared to have a relatively intact cytoplasm with large basophilic chromatin clumps changes that are consistent with morphologic features of apoptosis. DNA fragmentation, as evidenced by TUNEL positivity, was seen in neurons with either necrotic or with apoptotic morphology. Similarly, most damaged neurons in the other experiments showed a necrotic morphology. Only a minority of the TUNEL-positive neurons showed an apoptotic morphology (Fig. 3).

### Experiment II: The effects of NMDA-receptor blockade on DNA fragmentation and on ceramide levels in the kainic acid model

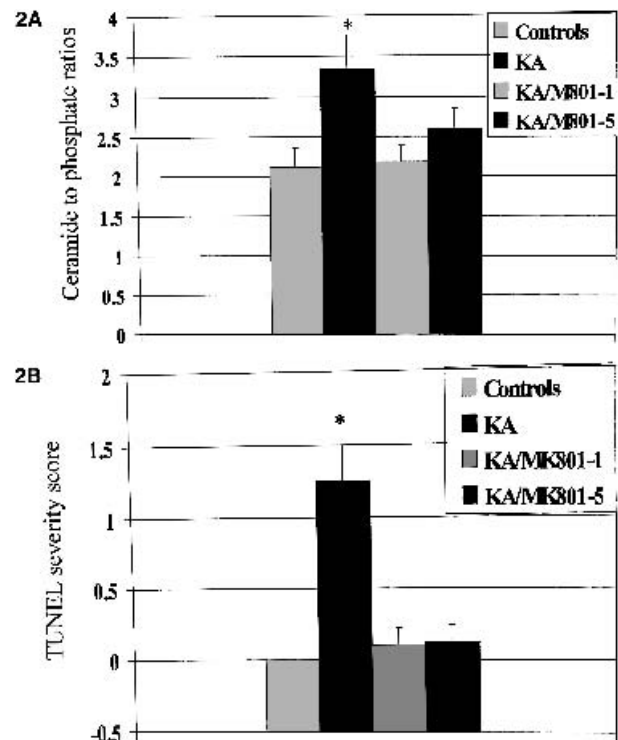
Pretreatment with MK801 prevented ceramide increases, as well as DNA fragmentation, even in the ab-

sence of a decrease in the duration of seizure activity (Figs. 2 and 3).

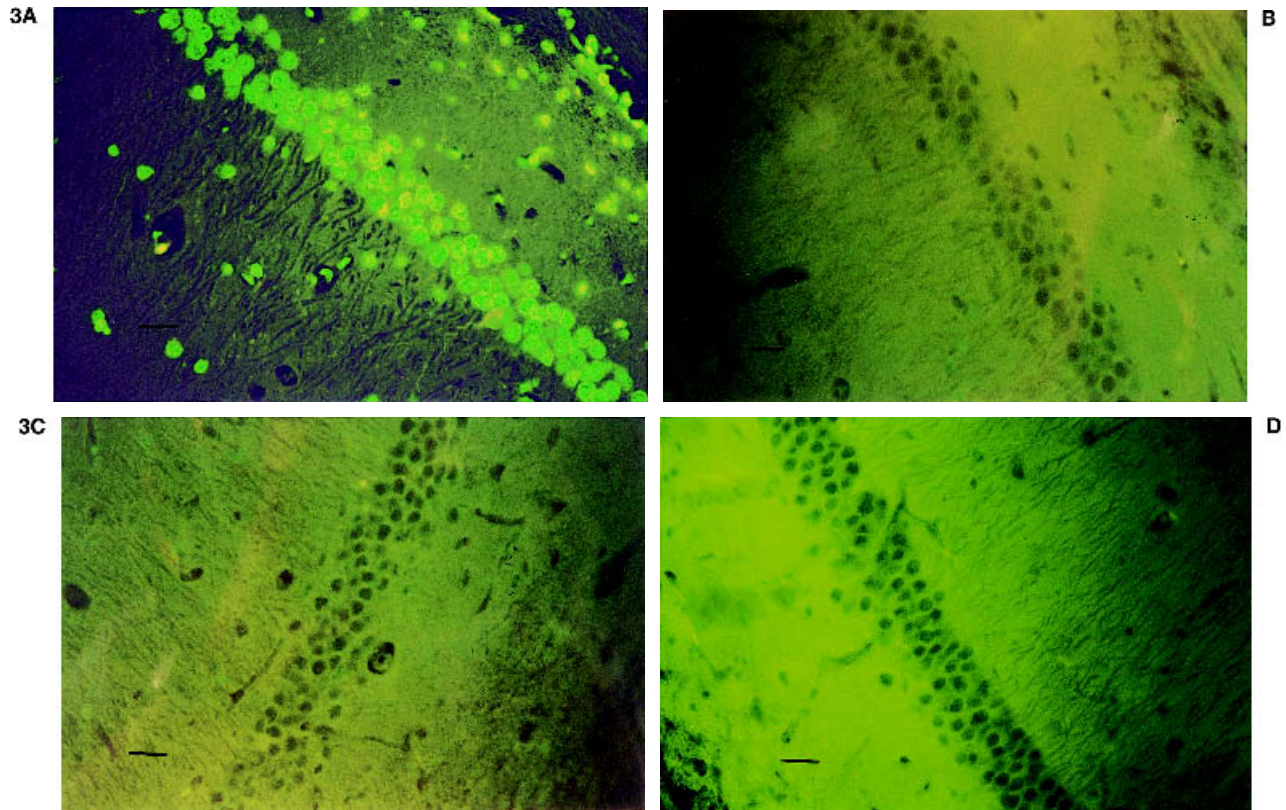
Only the KA group had an elevated *ceramide-to-phosphate ratio* as compared with controls ( $p = 0.008$ ). The ceramide-to-phosphate ratios were the following: control group,  $2.11 \pm 0.25$ ; KA group,  $3.34 \pm 0.42$ ; KA/MK801-1 group,  $2.18 \pm 0.2$ ; KA/MK801-5 group,  $2.6 \pm 0.25$ . Both of the KA/MK801 groups were not different from controls ( $p = 0.84$  and  $0.18$ , respectively) or from each other ( $p = 0.31$ ). Paired comparisons of the KA group with each of the KA/MK801 groups also showed the following levels of significance:  $p = 0.007$  and  $0.078$ , respectively.

On the *TUNEL stain*, we found that the KA group had higher total scores than the control group ( $p = 0.003$ ) and than each of the KA/MK801-1 and the KA/MK801-5 groups ( $p = 0.005$  and  $0.0031$ ), respectively. We also found that the last two groups were similar to controls ( $p > 0.3$  in both comparisons). The mean scores were as follows: control group,  $0.0 \pm 0.0$ ; KA group,  $1.25 \pm 0.25$ ; KA/MK801-1 group,  $0.12 \pm 0.11$ ; and KA/MK801-5 group,  $0.11 \pm 0.11$ .

The *H&E stain* findings were consistent with those of the TUNEL stain. Specifically the KA group had higher scores than the control group and than either of the KA/MK801 groups in the CA1 subfield. The mean scores of



**FIG. 2.** **A:** Ceramide-to-phosphate ratios in adult rats after kainic acid status epilepticus with or without MK801 pretreatment as compared with controls. **B:** TUNEL scores of the CA1 region of the same groups. Data are plotted as means  $\pm$  SEM. \* $p < 0.05$  compared with the control group.



**FIG. 3.** TUNEL stain of the CA1 region of adult rats after kainic acid (KA)-induced status epilepticus (**A**) as compared with a control rat (**B**) and with rats from 1-mg/kg and the 5-mg/kg MK801-pretreated groups (**C, D**). **A:** TUNEL-positive nuclei at 30 h after KA injection. Scale bar, 20  $\mu$ m.

the control, KA, KA/MK801-1, and KA/MK801-5 groups were  $1.2 \pm 0.37$ ,  $2.4 \pm 0.23$ ,  $1.37 \pm 0.32$ , and  $1.72 \pm 0.23$  ( $p = 0.033$ ,  $0.032$ , and  $0.041$ , respectively).

The mean duration of FLC *seizure activity* in the KA group, the KA/MK801-1 group, and the KA/MK801-5 group were  $1,341 \pm 187$ ,  $1,099 \pm 694$ , and  $288 \pm 151$  s, respectively. The KA/MK801-5 group had significantly fewer seizures than the KA group ( $p = 0.03$ ). The KA/MK801-1 group and the KA group seizure duration did not differ ( $p = 0.1$ ).

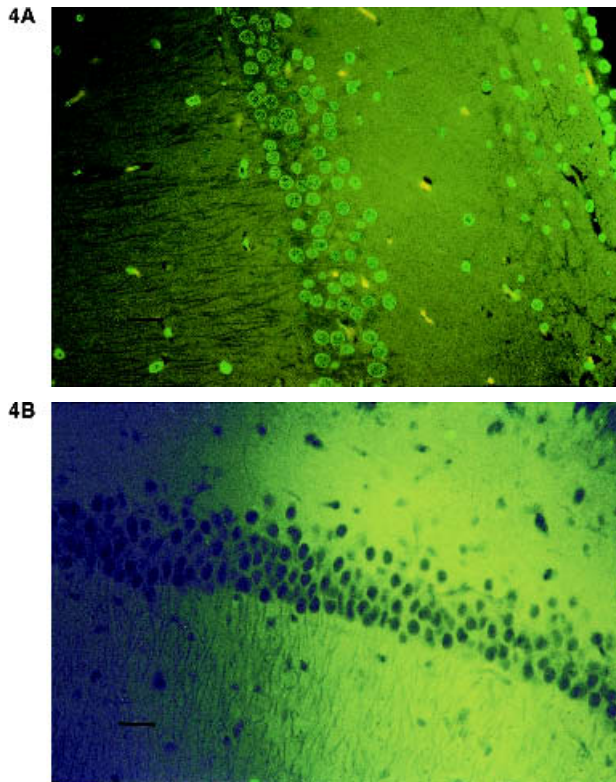
### Experiment III: The effects of C2-ceramide injection in the adult rat hippocampus

This experiment demonstrated that the hippocampal injection of C2-ceramide induced widespread DNA fragmentation in all hippocampal subfields (CA1, CA2, CA3/CA4, granule cells), whereas the injection of either the inactive ceramide analogue C2-dihydroceramide or the vehicle alone did not induce DNA fragmentation in any of the hippocampal subfields. Specifically, the mean total score (for the four areas CA1, CA2, CA3, and granule cell layer) of the C2-ceramide-injected group was  $13.42 \pm 0.33$ , of the C2-dihydroceramide group,  $1.27 \pm 0.12$ , and of the vehicle-injected group,  $0.0 \pm 0.0$  ( $p = 0.002$  and  $0.0001$  respectively; Fig. 4).

The amounts of C2-ceramide we used (10 ng in 1  $\mu$ l of vehicle) approximately correspond to increases in the ceramide-to-phosphate ratio (1.28) observed during the first peak of ceramide (1.11 and 1.42 at 2 and 3 h after KA injection, respectively). Of note is that the injected C2-ceramide induced TUNEL positivity in areas beyond the hippocampus, which indicates that ceramide can potentially activate the PCD cascade in areas of the brain other than the hippocampus.

### Experiment IV: Time sequence of ceramide increases after status epilepticus

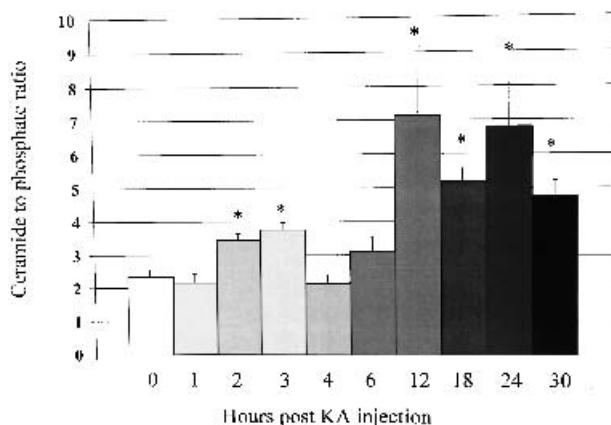
Experiment IV showed that ceramide levels increased at 2 and 3 h after KA injection, and then dropped back to control levels at 4 and 6 h after KA. Ceramide levels increased again at 12 h after KA and remained significantly elevated at 18, 24, and 30 h after KA injection. Ceramide levels at 2 and 3 h were significantly higher than those at 0 h ( $p = 0.0101$  and  $0.0105$ , respectively), 1 h ( $p = 0.014$  and  $0.02$ , respectively), and 4 h ( $p = 0.011$  and  $0.014$ , respectively; Fig. 5). Ceramide levels at 12, 18, 24, and 30 h were also significantly higher than those of the controls ( $p = 0.045$ ,  $0.0062$ ,  $0.01$ , and  $0.02$ , respectively).



**FIG. 4.** TUNEL stain of the CA1 region of an adult rat injected with C2-ceramide (**A**) as compared with an adult rat injected with C2-dihydroceramide. **B:** TUNEL-positive neurons. Scale bar, 20  $\mu$ m.

#### Experiment V: Comparison of ceramide-to-phosphate ratios in the hippocampus and the rest of the hemisphere after kainic acid-induced status epilepticus

Experiment V showed that the increases in *ceramide-to-phosphate ratio* after SE are attributable to the increases observed in the hippocampus. The hippocampal ceramide-to-phosphate ratio of the KA group was higher



**FIG. 5.** Ceramide-to-phosphate ratios at 0, 1, 2, 3, 4, 6, 12, 18, 24, and 30 h after kainic acid injection. Data are plotted as means  $\pm$  SEM. \* $p < 0.05$  compared with controls (0-h time point).

than that of normal (control) hippocampi ( $5.66 \pm 0.74$  and  $2.57 \pm 0.49$ ;  $p = 0.0002$ ). The ceramide-to-phosphate ratio of the rest of the hemisphere was not higher than that of the control group (KA group,  $2.30 \pm 0.12$ ; controls,  $1.72 \pm 0.11$ ;  $p = 0.43$ ). In the control group, there was no difference in the ceramide-to-phosphate ratio between the hippocampus and the rest of the brain ( $2.57 \pm 0.49$  and  $1.72 \pm 0.11$ ;  $p = 0.64$ ). In the KA group, there was a significant difference between the hippocampus and the rest of the hemisphere ( $5.66 \pm 0.74$  and  $2.30 \pm 0.12$ ;  $p = 0.00002$ ).

## DISCUSSION

A number of studies have demonstrated, in the adult brain, the occurrence of PCD in several models of SE including the KA and the LiPC models (8,27,28). However, to our knowledge, the occurrence of PCD in the immature brain has been investigated in only four previous studies. Thompson et al. (29) demonstrated the occurrence of PCD after perforant-path stimulation in immature, P14 pups. Sankar et al. (30) reported the occurrence of PCD after LiPC-induced SE in P14 and P21 rats. Santos et al. (31) described the occurrence of DNA fragmentation in the same model in P7 rats. Ribak and Baram (32) demonstrated the occurrence of hippocampal neuronal death in rats younger than P15 after corticotropin-releasing hormone (CRH)-induced SE. However, the histologic changes noted in that study were not consistent with apoptosis (4,6). None of those studies investigated the immature (P7) or prepubescent (P35) rats in the KA model. In our study, we showed that PCD occurs in prepubescent (P35) and in adult, but not in P7 rats after KA-induced SE. This is consistent with the previous documentation of long-term neurobehavioral sequelae (memory impairment, increased aggressivity, hyperactivity, and spontaneous recurrent seizures) and of histologic lesions after KA-induced SE in those age groups, but not in rats younger than 28 days (20,21). This suggests that PCD may be the factor responsible, at least in part, for those long-term sequelae of SE. If this is true, then factors that may be found, in the future, to interfere with PCD could potentially prevent at least some of the long-term sequelae of SE.

Our finding that ceramide increased in age groups that manifested PCD whereas it did not increase in P7 rats that did not manifest PCD suggests that ceramide is a mediator of PCD in the central nervous system after SE. It also provides a potential explanation for the observed differences in the response of the immature brain to the effects of KA-induced SE (33). Thus differences between the responses of the mature and the immature brain to SE may be, at least in part, due to the inability of KA-induced SE to produce increases in ceramide in immature rats. One potential criticism of this hypothesis

is that the KA doses needed to induce SE in the P7 rats are much smaller than those used in P35 and adult rats, and that the observed differences may be secondary to this dose discrepancy. However, the P7 rats underwent SE that was of severity (>4 h) similar to that of the older age groups, yet they did not manifest either PCD or increases in ceramide levels. The P7 age group was chosen because Holmes et al. (20) in 1988 demonstrated the absence of long-term memory and behavioral impairments in rat pups younger than P20 after KA-induced SE. Our observations thus suggest that increases in ceramide correlate with the occurrence of PCD after SE and are not dependent on the occurrence of SE per se.

We used several methods to confirm the presence of neuronal injury and/or PCD (H&E, TUNEL, Hoechst). In general, the results from those methods were highly consistent. However, the TUNEL stain in the CA3/CA4 region in experiment I did not achieve statistical significance, whereas the other two stains did. This may be related to the number of animals we used. Alternatively, it may be that the time point we chose (30 h after KA injection) was a time point when TUNEL positivity had subsided. PCD is a dynamic process in which some neurons, after starting the process of PCD, undergo necrosis secondary to energy failure (34). Despite this, our study clearly demonstrated TUNEL positivity in the hippocampus after SE, and that this correlates with ceramide increases.

We demonstrated in experiment II that NMDA-receptor blockade prevented both increases in ceramide levels and TUNEL positivity, whether seizure severity was reduced (with the use of high-dose MK801) or not (with the use of low-dose MK801). Thus NMDA-receptor activation is essential for the induction of ceramide increases and of DNA fragmentation in this model. Previous studies had shown apparently contradictory results. Some showed, as we did, that MK801 pretreatment prevented PCD (6,10). Others, however, failed to show such an effect (5). The explanation for this discrepancy, we believe, is that none of those studies, within the same experiment, used multiple doses of MK801 to achieve different degrees of blockade of NMDA receptors. Additionally, none of those studies controlled for the duration of the seizures. Large doses of MK801 may prevent or markedly attenuate the occurrence of SE, whereas small doses relative to KA may not result in significant NMDA-receptor blockade. In our study, we corrected for those potential limitations by using two doses of MK801 and by documenting and comparing seizure duration among the groups. Hughes et al. (35) described the low dose of MK801-KA model that we used. In this model, the injection of 1 mg/kg of MK801 resulted in NMDA-receptor blockade that did not reduce the severity of the seizures as compared with KA alone, but did alter the expression of certain immediate-early

genes and growth factors (35). We were able to demonstrate, by using the same model, that increases in ceramide levels are NMDA-receptor dependent, and that ceramide does not increase nonspecifically after any type of SE. Part of the differences seen between the two MK801 groups we observed may be related to variable effects on body temperature secondary to alternate doses of MK801. However, this effect is probably not very significant because prevention of hypothermia while using 5 mg/kg MK801 did not modify the neuroprotective effects of this dose of MK801 in the gerbil brain subjected to an ischemic insult (36).

Increases in endogenous ceramide levels have been shown to mediate apoptosis in several neuronal cell lines and under several experimental conditions such as hypoxia/ischemia (37,38). In experiment III, we showed that the injection of the active ceramide analogue C2-ceramide into the hippocampus of adult rats caused massive and widespread DNA fragmentation. This constitutes, in our opinion, compelling evidence that the increases in ceramide levels we observed are not simply an unrelated epiphenomenon, but are instrumental in inducing PCD. Our findings are also consistent with prior studies that showed that C2-ceramide exposure induces apoptosis in cortical neurons and cerebellar granule cell cultures (39,40).

In experiment IV we found that ceramide levels increased in a two-peak pattern. This pattern was previously observed in several cell lines undergoing PCD, but had not been investigated in neural tissues or in vivo (41–46). We plan further studies to investigate the mechanisms leading to these increases, which, potentially, could be due to the activation, or inhibition, of several enzymes in the sphingomyelin cycle (17,44).

Our experiment V demonstrated that the increases in ceramide levels in the hemisphere are largely, if not exclusively, attributable to the increases occurring in the hippocampus.

The mechanisms by which NMDA-receptor activation leads to increases in ceramide are possibly related to the alteration of calcium homeostasis that occurs after activation of those receptors. It has been shown that in low serum-induced apoptosis, increases in intracellular calcium induce calcium release from the endoplasmic reticulum (ER) through the activation of the ER membrane ryanodine receptors, and that this later release results in ceramide increases (47). Similar mechanisms may be active in SE. Moreover, it has been shown that, in the KA model, SE induces a sustained increase in inositol triphosphate (IP<sub>3</sub>) levels in the adult, but not in the immature rat brain (48). IP<sub>3</sub> binds to IP<sub>3</sub> receptors on the ER membrane (calcium channels), leading to the opening of those channels and to the release of calcium from the ER stores (48). Thus the difference that we observed in the ceramide and in the PCD responses between the P7 and



adult brains may be secondary to differences in the response to IP<sub>3</sub> and its subsequent effects on ER calcium release. It also is possible that ceramide can have different effects at different developmental stages such as reducing apoptosis in the immature animal (18).

Several mechanisms exist by which ceramide can lead to PCD after SE. Ceramide can directly induce the opening of the mitochondrial permeability transition pores (PTP), which disrupts the transmembrane potential, thus causing the release of cytochrome *c* and the generation of hydrogen peroxide. These molecules are able to induce APAF-1 and caspase-3 activation and subsequent poly-ADP-ribose polymerase (PARP) cleavage and DNA fragmentation (49–51). Ceramide also can induce changes in the expression of the Bcl-2 family of proteins by activating specific transcription factors such as NF- $\kappa$ B and c-jun (52,53). Downregulation of the antiapoptotic members, or upregulation of the proapoptotic members, of this family can subsequently, in itself, lead to the opening of the mitochondrial PTP (54,55). Another factor that could possibly contribute to PCD induced by ceramide is tumor necrosis factor (TNF) (56).

We monitored, in this study, clinical seizures and not EEG. It is likely that EEG monitoring in experiment II would have revealed persistence of EEG seizure activity in the 5-mg/kg MK801 dose group, as has been demonstrated with NMDA-receptor blockade, with MK801 and with other agents, in several previous studies (57–59). This would have supported our conclusion that increases in ceramide and DNA fragmentation are dependent on NMDA-receptor activation and not on the occurrence of seizure activity. Our demonstration of a neuroprotective effect of MK801 in KA-induced SE is consistent with prior literature demonstrating such effects after NMDA-receptor blockade with MK801 and with other agents (57–60). We also note that, as emphasized and clarified in recent literature, it is important to distinguish the morphologic changes of apoptosis from those of necrosis (61–65). Our study was not a morphologic study, and we did not use electron microscopy. However, in our study, despite the demonstration of DNA fragmentation, as evidenced by TUNEL and Hoechst stains, histologic changes seen on H&E were often consistent with those seen in necrosis. This is consistent with recent observations that in neuronal tissue, PCD may be accompanied by histologic changes of necrosis rather than apoptosis (26,54,61–65). It also supports the increasing trend to use the term *apoptosis* as an indication of specific morphologic changes, and not necessarily exchangeably with the term PCD. As defined in the introduction section of this article, PCD implies the activation of specific genes, factors, and pathways involved in the process of cell death.

In brief, we believe that our data provide strong evidence for the hypothesis that PCD after SE is an NMDA receptor-dependent process, and that ceramide is a me-

diator of this process. Understanding further the mechanisms of neuronal injury after SE could, we hope, lead to promising therapeutic strategies and interventions.

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