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Multiple Pilocarpine-Induced Status Epilepticus in Developing Rats: A Long-Term Behavioral and Electrophysiological Study

N. F. Santos, R. H. Marques, L. Correia, R. Sinigaglia-Coimbra, L. Calderazzo, E. R. G. Sanabria, and E. A. Cavalheiro

Laboratório de Neurologia Experimental, Universidade Federal de São Paulo-Escola Paulista de Medicina, São Paulo, Brazil

Summary: *Purpose:* Animal models are useful for the study of status epilepticus (SE)-induced epileptogenesis and neurological sequelae, especially during early brain development. Here, we show several permanent abnormalities in animals subjected to multiple SE during early development.

Methods: Wistar pup rats (7 to 9 days old) were subjected to three consecutive episodes of SE induced by systemic pilocarpine injections. To study the long-lasting consequences of early-induced SE, chronic electroencephalographic recordings were made from the hippocampus and cortex and several behavioral tests (inhibitory step-down avoidance, rota-rod, open field, elevated plus-maze, and Skinner box) were performed at postnatal days 30 to 90. We also investigated in vitro electrophysiological responses of the CA1 area using extracellular recordings in hippocampal slices. A histological analysis was done using cresyl violet staining 24 hours and several months after SE induction. Apoptotic cell death was evaluated by terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL staining) 24 hours after the last SE episode.

Results: Electroencephalographic recordings from 30- to 90-day-old rats that had been subjected to multiple SE episodes in early life showed marked changes compared with those from nontreated controls. These included frequent episodes of continuous complex spiking activity and high-voltage ictal discharges, with a small percentage of these rats presenting spontaneous behavioral seizures. These animals also presented evidence of severe cognitive deficit in adulthood. In vitro, a persistent hyperexcitability of the CA1 area was detected in experimental animals. Histological analysis of the brains did not reveal any major long-term pathological changes. Nevertheless, an increased number of TUNEL-positive nuclei were present in some animals in both the hippocampus and the thalamus.

Conclusions: These data show persistent abnormalities in animals subjected to multiple SE episodes during early postnatal development. SE may result in important plastic changes in critical periods of brain maturation leading to long-lasting epileptogenesis, as manifested by electrographic epileptiform discharges, behavioral deficits, and in vitro hyperexcitability of hippocampal networks. **Key Words:** Development—Epilepsy—Hippocampus—Pilocarpine—Rat.

The immature central nervous system has several neurobiological peculiarities that make it more sensitive for the development of abnormal electrical activity (1). Status epilepticus (SE) is a major neurological emergency and is particularly prevalent in the first years of life, a period in which SE may be more deleterious to the developing brain (2). The pathophysiological consequences of neonatal seizures are commonly an early sign of further disastrous neurological disorders. Indeed, it has been documented for many years that intellectual impairment, neurological deficits, and the development of chronic epilepsy are among the most common complications of early recurrent seizures in children (3,4). Seizures may disturb the ontogenetic pattern of excitatory and inhibitory networks in the immature brain, leading to long-lasting plastic maladaptive changes. Nevertheless, the mechanisms that underlie such persistent abnormalities are not completely understood.

Several animal models of epilepsy that attempt to mimic a condition of neonatal seizures in humans have been described in recent years (5,6). For instance, chronic seizures and brain damage after pilocarpine (PILO)-induced SE can be observed if SE is induced after the 18th day of life (7). Younger rats (younger than 18 days old) do not develop spontaneous seizures as late consequence of PILO-induced SE. Nevertheless, they present electrographic and behavioral alterations similar to those observed in rats with genetic absence epilepsy when studied 90 to 120 days later (8). In addition, animals subjected to multiple PILO-induced SE at postnatal days 7 to 9 express several electroencephalographic (EEG) disturbances but rare convulsive seizures that are not accompanied by evident neuronal loss in the limbic

Address correspondence and reprint requests to Dr. Esper A. Cavalheiro at Laboratório de Neurologia Experimental, Universidade Federal de São Paulo-Escola Paulista de Medicina, Rua Botucatu 862, 04023–900 São Paulo, SP, Brazil. E-mail: esper.nexp@epm.br

or neocortical structures (9). Here, we further investigate the effects of multiple SE episodes in early life using long-term behavioral and EEG monitoring and in vitro electrophysiological study of hippocampal slices and neuropathological abnormalities with the terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) technique and cresyl violet staining.

MATERIAL AND METHODS

Wistar male pups at postnatal day 7 (P7) were used in this study. The animals were bred in our laboratory and kept with the mother after birth under controlled temperature (21 ± 2°C) and light (12-hour light/dark cycle) conditions. Experiments were conducted according to the ethical rules for animal research at our university. The pups were randomly divided in two groups: a PILO-treated experimental group (n = 30) and a saline-treated control group (n = 20). Experimental animals were treated with a single intraperitoneal injection of PILO (Sigma, St. Louis, MO, U.S.A.) at 380 mg/kg for three consecutive days (P7 to P9). The PILO injection was preceded 30 minutes earlier by a subcutaneous injection of scopolamine methyl nitrate (Sigma) at 1 mg/kg to attenuate the peripheral cholinergic effects. Control rats were injected with 0.9% saline under the same conditions, including the administration of scopolamine methyl nitrate 30 minutes before the saline injections.

To analyze the effects of these three consecutive episodes of SE in this period of life (P7, P8, and P9), control (saline-injected) and experimental (PILO-injected) groups of animals were studied at P60 to P90 to test motor, learning, and memory performance through several behavioral tests (see 9 for details). After the SE period, the surviving animals were monitored continuously 24 h/d for 120 days for detection of spontaneous seizures using a video system. To monitor EEG changes, control and experimental animals at the ages of 30, 45, 60, and 90 days were anesthetized with sodium pentobarbital and stereotactically implanted with twisted bipolar electrodes of nickel-chrome wires (100 μm) in the dorsal hippocampus (10). Three stainless-steel screws were attached to the skull for surface recordings: one in the frontal bone served as an electrical ground, and the two others in the frontal region were used for EEG recording. Starting 3 days after surgery, EEG and video recordings were made continuously (24 h/d) for 30 days using a video-EEG monitoring system (Stellate System).

In vitro slice electrophysiological measurements were conducted in another group of experimental and control animals treated as described above. Transverse hippocampal slices were prepared from PILO-treated and control animals (P90) as described previously (11).

Histological analysis was performed 120 days after pilocarpine or saline administration on 40-μm sections stained with cresyl violet. For TUNEL studies, PILO- and saline-injected animals were processed for histological analysis 24 hours after the last PILO injection. Coronal sections were cut on the microtome at a thickness of 10 μm and collected in three adjacent series. Apoptotic cells were detected by end-labeling DNA fragmentation. The sections of one series were rehydrated and processed for the visualization of apoptosis using a peroxidase-based in situ apoptosis detection kit (ApoTag-Peroxidade-Plus, Oncor). They were then counterstained with 0.5% methyl green. In addition, the sections of the adjacent series were stained with cresyl violet and used for careful cytoarchitectonic study. All serial sections processed for the labeling of apoptotic cells were studied with a light microscope (Axioplan 2, Zeiss, Tokyo, Japan) by two independent investigators unaware of the animal's group assignment. Stained apoptotic cells were counted in these sections in the hippocampal formation and thalamus. The data were statistically evaluated using the Student *t* test.

RESULTS

Acute behavioral manifestation of SE during development

Immediately after the PILO injection, all animals of the experimental group began to show behavioral changes, such as intense body tremor, scratching, clonic movements of forelimbs, and head bobbing, that culminated in SE. PILO-induced SE was consistently observed in all of the animals used in the present investigation and at every session of PILO administration. Some animals experienced tonic convulsions that appeared progressively more frequently during the second and third SE inductions. All rats injected with PILO survived the three sessions.

Cognitive deficit in rats subjected to multiple SE during infancy.

Experimental animals failed to obtain water by pressing the Skinner box bar during three consecutive sessions. Additionally, it was possible to detect a significant reduction in their exploratory skills, as measured by total entries in the elevated plus-maze test. Furthermore, a significant difference was observed in the step-down inhibitory avoidance test and in open field behavior (see 12 for details). Together, such findings indicate a long-lasting cognitive deficit in adult animals subjected to multiple SE in infancy (P7 to P9).

Electroencephalographic findings in animals subjected to multiple SE

EEG recordings from animals subjected to multiple SE during development were clearly abnormal. Rats re-

corded at P30 expressed a wide spectrum of electrographic epileptiform discharges characterized by frequent and continuous interictal spiking and polyspiking activity in the hippocampal area or the neocortex and in some cases by the emergence of sudden rhythmic high-amplitude sustained discharges resembling electrographic seizures. Such abnormalities were also observed in animals recorded at other postnatal ages (e.g., P60 to P90) (Fig. 1). Animals that presented tonic convulsions during SE episodes were more prone to develop such EEG abnormalities. Interestingly, a clear correlation between electrographic seizures and behavioral manifestations could not be detected. Electrographic epileptiform alterations were usually accompanied by behavioral arrest and were followed in some cases by masticatory and orofacial stereotyped movements. As reported previously (12), some animals presented clear manifestations of spontaneous partial seizures, characterized by forelimb clonus and masticatory movements, concomitant with epileptiform discharges in both hippocampus and cortex.

In vitro epileptiform abnormalities in the hippocampal CA1 area

Extracellular CA1 recordings from PILO-injected rats (n = 5) revealed a chronic hyperexcitability characterized by polyspikes at different stimulus intensities (Fig. 2). Similar epileptiform responses were detected previously in adult models of localization-related epilepsy, such as mesial temporal lobe epilepsy models (13,14).

Neuropathological features

In all experimental animals, the cresyl violet–stained sections did not show obvious degenerative features (such as marked cell loss) in the hippocampal formation compared with those derived from control animals (Fig. 3).

In the series of sections processed for the staining of apoptotic phenomena, labeled cells were evident in both the control and experimental groups. Apoptotic cells exhibited staining in their nuclei and were surrounded in most cases by a halo of cytoplasm, indicating that they were neurons and not glial cells (Fig. 4). At the microscopic level, apoptotic neurons exhibited primarily the

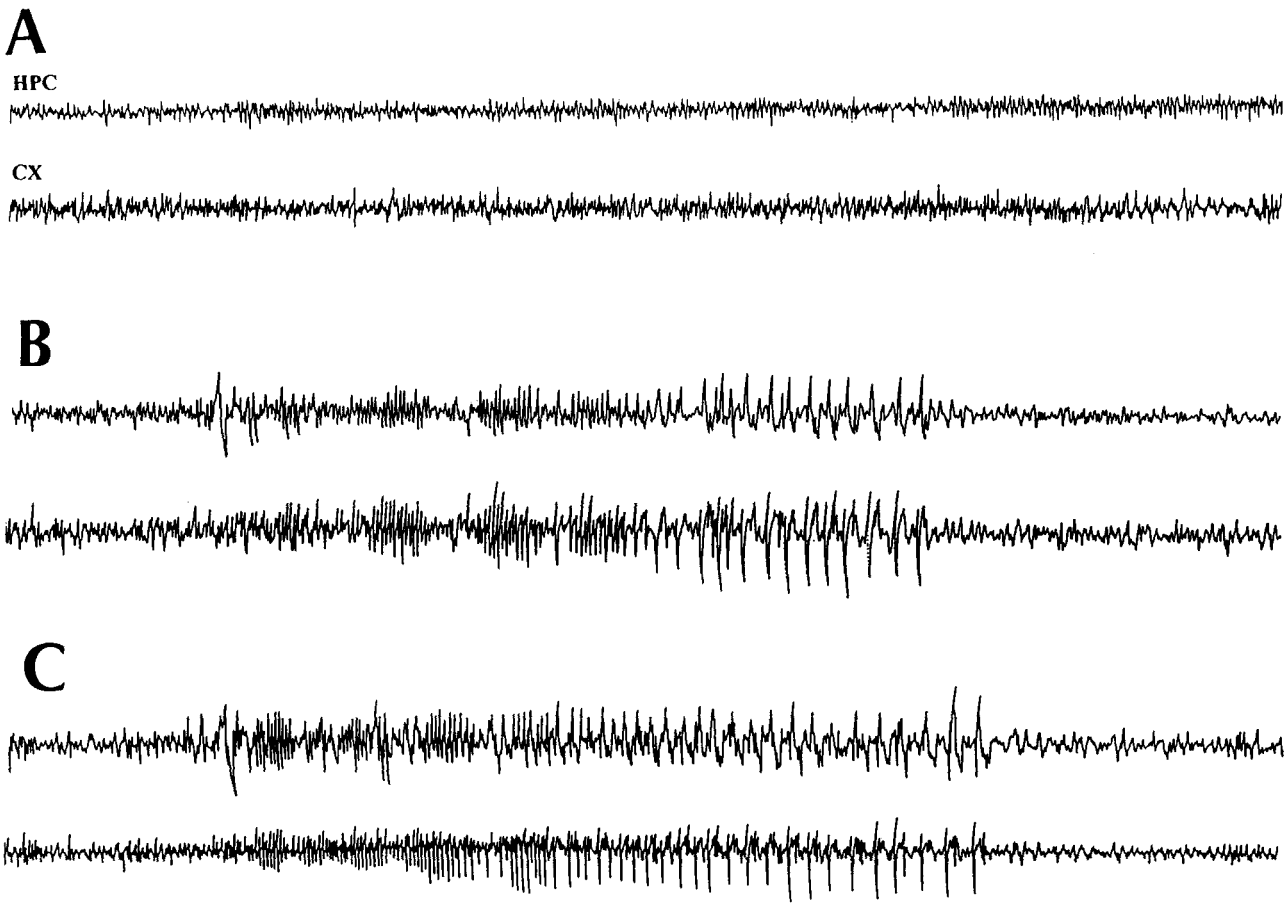
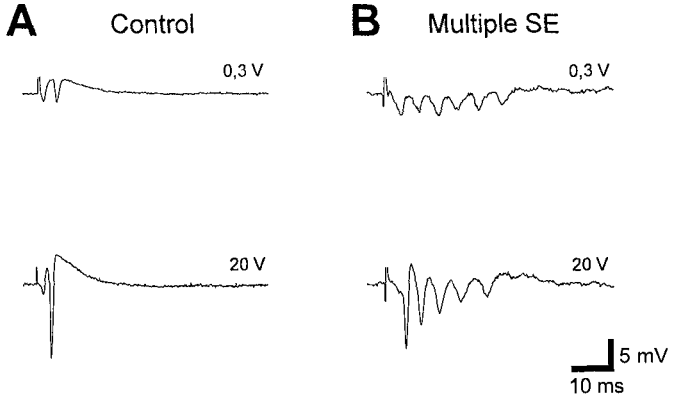


FIG. 1. Representative EEG traces from control adult rat (**A**) and from different experimental rats subjected to multiple SE during early development (P63 in **B** and P97 in **C**). Note the synchronized fast high-amplitude continuous spiking activity typical of ictal electrographic discharges. HPC, hippocampus; CX, cortex.



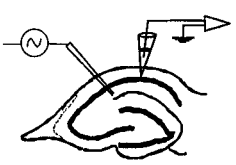


FIG. 2. **A:** Extracellular field potentials from the CA1 area showing a single population spike in a slice from a control animal. **B:** Persistent hyperexcitability characterized by polyspikes in the CA1 area of an adult animal subjected to multiple PILO-induced SE at P7 to P9.

features of hippocampal interneurons located mainly in the hilus and also in granule cells in the dentate gyrus. More stained apoptotic neurons were observed consistently in the hippocampal formation (Fig. 4, B and B1) and in certain thalamic nuclei of experimental compared with control animals. Interindividual quantitative variability was observed in the experimental cases. Quantitatively, the increase in the density of apoptotic cells in the hippocampal formation of PILO-treated animals ($n = 5$) compared with control animals ($n = 4$) was highly significant ($p < 0.001$) (Fig. 4C). Moreover, TUNEL-stained neurons were present in the thalamus of PILO-treated animals (not shown), whereas they were not found in control animals, in agreement with the scarcity of apoptotic cells in the rat thalamus at P10 (15).

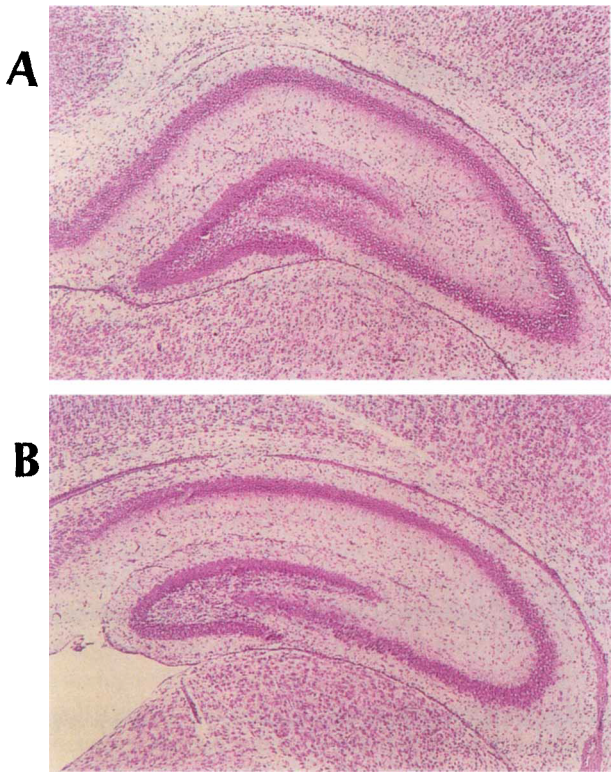


FIG. 3. Example of cresyl violet–stained sections of hippocampi from control (**A**) and experimental (multiple SE; **B**) animals. Original magnification, 40.

DISCUSSION

The rapid development of SE after PILO injections at P7 to P9 confirms previous data on the susceptibility of immature brain to epileptogenesis (5,16–18). Despite its seizure-prone condition, the immature brain is less vulnerable to seizure-triggered brain injury and synaptic reorganization (19,20). Indeed, a previous study from our group demonstrated that PILO-induced SE led to adult-type neuropathology and behavioral and EEG features only when animals were injected after P15 to P21 (7). Here, we show that multiple SE at P7 to P9 can induce harmful plastic changes that are expressed by electrographic and behavioral alterations and that can be observed several months after the triggering initial insult.

Long-lasting EEG and behavioral disturbances

Animals subjected to three consecutive episodes of PILO-induced SE in early life experienced a wide spectrum of long-lasting electrographic alterations, including frequent and continuous spiking activity. Such alterations were detected in almost all animals recorded at P30 to P90. The qualitative analysis of EEG recordings revealed that epileptiform discharges were more complex and prolonged in animals evaluated later (P90). Such animals exhibited several patterns of ictal electrographic discharges followed in some cases by discrete but constant subconvulsive disturbances (behavioral arrest, stereotyped movements, and rarely clonus). Previous studies have focused on the acute electrographic abnormalities related to stimuli-induced SE during early life (9,21,22). Our findings suggest that early recurrent seizures may lead to a persistent epileptogenic condition that gradually evolves until adulthood. Nevertheless, these EEG changes are not accompanied by major behavioral seizures, but some minor manifestations could be observed (masticatory movements, forelimb-localized clonus, and head tremors), although we did notice that behavioral manifestations worsened (clonic and tonic

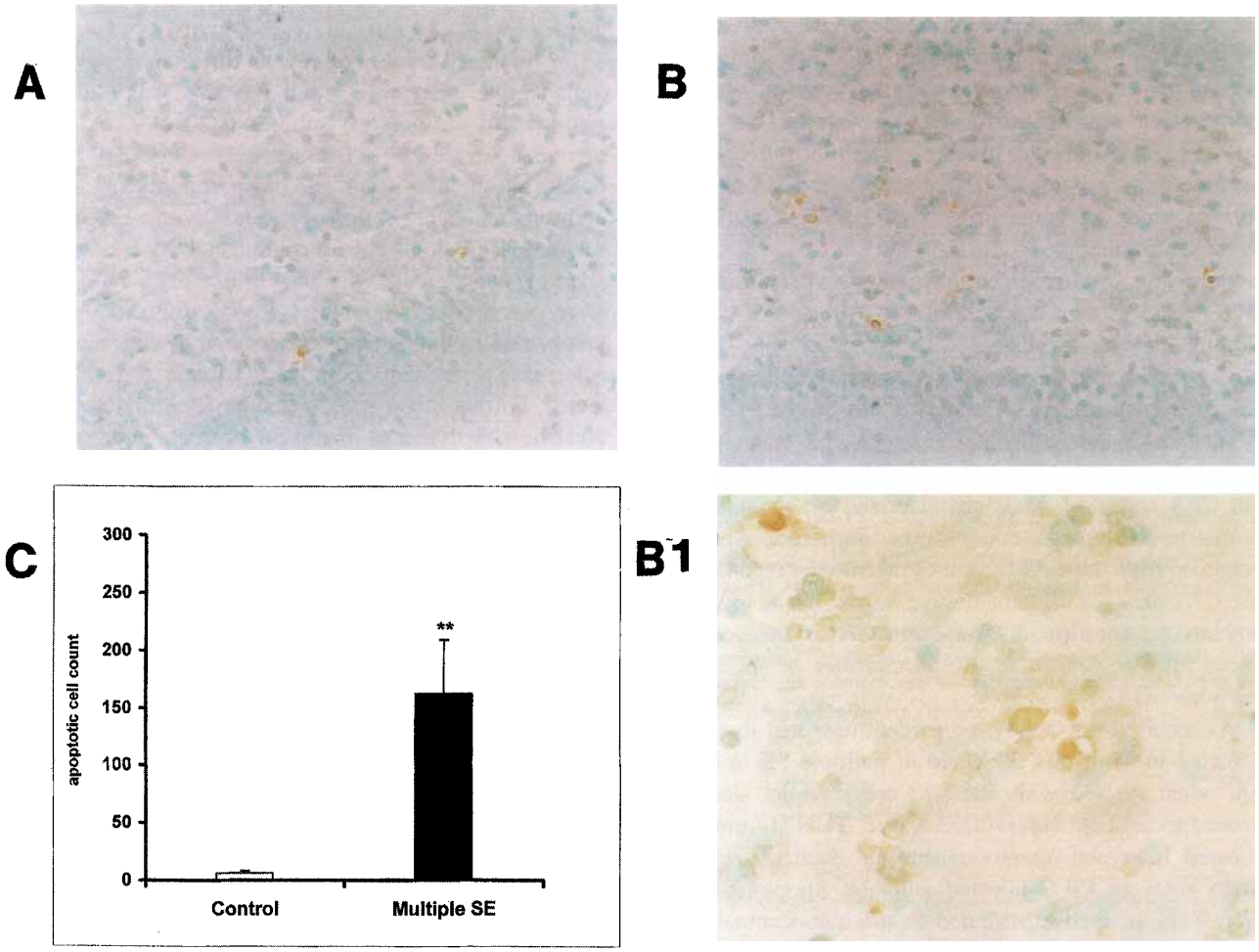


FIG. 4. In situ TUNEL staining 24 hours after the last PILO-induced SE in the immature rat. **A:** Sparsely stained neurons in the dentate gyrus were observed in control sections. **B:** Labeled nuclei were seen consistently in neurons located mainly in the hilus and in the granule cells of the dentate gyrus of rats subjected to multiple SE. **B1:** Apoptotic figures observed at higher magnification. **C:** Statistical graph showing a significant (*$p < 0.001$) increase in apoptotic cell counts in epileptic animals. Magnification in **A** and **B**, 400, in **B1**, 1000 (immersion oil microphotograph).

components) with longer survival periods after SE induction (12). Possibly, subconvulsive electrographic epileptogenesis may act as an endogenous kindling effect on brain excitability, as reported in the original description of the pilocarpine model of epilepsy in adult animals (23).

Seizures during early postnatal life have been considered as a factor in the generation of late brain dysfunction (24,25). It was formerly described that rats subjected to kainic acid–induced seizures during the first weeks of life (P22 to P26) learned at a slower rate in the water maze and T maze, had evidence of impaired memory during spatial bias testing in the water maze, and were significantly more active than control animals in the open field test (26). Conversely, it was also shown that kainic acid–treated P5 and P10 rats had no demonstrable cognitive deficits on any test when tested on P80 (27). In contrast to these data, our behavioral findings certainly indicate a chronic cognitive deterioration, because animals subjected to multiple PILO-induced SE in early life (before P10) exhibited evident learning impairment and increased spontaneous exploratory activity. The apparent disagreement between these age-matched samples may be related to the fact that in the kainic acid experiments animals were subjected to a single SE episode, whereas in the present work we provoked multiple PILO-induced SE in P7 to P9 rats. It is probable that before P10, neuronal networks may recover their functional and structural normal properties after an isolated acute insult. Thus, multiple SE episodes after this age lead to catastrophic and lasting effects in neuronal circuits, producing subsequent late cognitive impairment and chronic hyperexcitability. It is important to recall that childhood epileptic encephalopathies are also characterized by progressive seizure-dependent cognitive deterioration (28). In particular, epileptic syndromes as Lennox-Gastaut and West syndromes are commonly associated with progressive mental retardation, which seems to be related to the persistence and severity of epileptic manifestations (29,30). Multiple SE during early development in

rats can partially reproduce some aspects of childhood epileptic encephalopathies (electrographic and behavioral data). Regardless of the lack of specific convulsive manifestations (tonic, atonic, or even spasms), we believe that the methodological approach used here could be a useful experimental tool for the study of some aspects of Lennox-Gastaut or West syndrome.

Persistent in vitro electrophysiological abnormalities were found in the CA1 area of PILO-treated rats. This finding may be associated with the propensity of the hippocampus to generate in vivo electrographic epileptiform discharges, as observed in this study. Previous studies on the developmental aspects of the tetanus toxin model also revealed a local network hyperexcitability in the CA3 and CA1 areas characterized by spontaneous discharges. Although epileptiform discharges seem to originate from the CA3 area, they can also be recorded in the CA1 area (31). Interestingly, we did not note spontaneous epileptiform discharges in CA1. These differences may be related to the peculiarities of such epilepsy models.

As reported previously, no evident neuronal loss was detected in adult rats subjected to multiple SE in early life when studied with standard cresyl violet staining procedures (12). Nevertheless, the TUNEL method showed increased apoptotic neuronal death in several brain areas of PILO-injected animals. Apoptotic cells were very sparsely distributed in the hippocampal formation of control animals. Their occurrence indicates phenomena of programmed cell death in the hippocampal formation at P10, which is a physiological phenomenon (32). On the other hand, the density of these labeled cells in the hippocampus and dentate gyrus was markedly enhanced in all of the PILO-treated animals compared with controls, indicating a pathological phenomenon of cell death subsequent to repeated SE (Fig. 4). The pathological implications of augmented apoptosis in PILO-treated animals are not clear. It is possible that multiple SE episodes in the immature brain could activate developmentally regulated mechanisms for neuronal death in susceptible neuronal populations. Moreover, increased apoptosis was not active enough to induce severe neuron loss in such animals, which might have alternative compensatory mechanisms (e.g., increased neurogenesis) (33). It is attractive to speculate that local deafferentation dependent on microscopic cell loss (neurons undergoing SE-induced apoptosis) may also produce a slight abnormal synaptic rearrangement of neuron-to-neuron connectivity in different structures (e.g., dentate gyrus and thalamus). Such circuit rearrangement may account for the emergence of aberrant electrical activity and persistent epileptogenesis.

CONCLUSIONS

In summary, our data reveal different long-lasting maladaptive changes in adult rats that had undergone multiple SE during early infancy (P7 to P9). Although there is no evidence for late neuronal injury (cell loss) or degeneration in this model, these animals did exhibit enduring and latent epileptogenesis. Diverse EEG abnormalities (including frequent spiking activity and ictal discharges) concomitant with seizure-like behavioral events were observed. Additionally, chronic hippocampal hyperexcitability in the CA1 area as well as cues that characterize a permanent cognitive detriment were also detected. Further elucidation of the basic mechanisms that underlie the dynamic evolution of such abnormalities after early SE may contribute to the understanding of the pathophysiology and late consequences of recurrent SE in the human immature brain.

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