Epilepsia, **46**(5):636–642, 2005
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Neocortical and Hippocampal Changes after Multiple Pilocarpine-induced Status Epilepticus in Rats

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Summary: *Purpose:* Multiple episodes of pilocarpine-induced status epilepticus (SE) in developing rats (P7–P9) lead to progressive epileptiform activity and severe cognitive impairment in adulthood. The present work studied possible underlying abnormalities in the neocortex and hippocampus of pilocarpine-treated animals.

Methods: Wistar rats were submitted to pilocarpine-induced SE at P7, P8, and P9, and were killed at P35. Immunocytochemistry was performed on 50-μm vibratome sections, by using antibodies against nonphosphorylated neurofilament (SMI-311), parvalbumin (PV), calbindin (CB), calretinin (CR), and glutamate decarboxylase (GAD-65). Ten-micron cryostat sections were processed for immunohistoblot by using antibodies against GluR1, GluR2/3, and GluR4 α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor subunits and NR2ab *N*-methyl-D-aspartate (NMDA) receptor subunit.

Results: Adult rats submitted to SE at P7–9 showed: (a) altered distribution of neocortical interneurons; (b) increased cortical and reduced hippocampal GAD-65 expression; and (c) altered expression of hippocampal AMPA and NMDA receptors.

Conclusions: We conclude that multiple SE episodes during P7–9 generate long-lasting disturbances that underlie behavioral and electrographic abnormalities later in life. **Key Words:** Immature brain—Early insult—Immunocytochemistry—Immunohistoblot.

The incidence of seizures is high in the first year of life, being most prevalent in the neonatal period, with an incidence of 1–3.5 per 1,000 live births (1). Particularly, early-life symptomatic febrile or hypoxic seizures are believed to be related to a progressive epileptogenic process and can be found in the histories of 40–50% of patients with temporal lobe epilepsy (TLE) (2). Animal studies have shown that the immature brain is more resistant to seizure-induced hippocampal neuronal cell loss (3–5) and synaptic reorganization (4,6,7). Nevertheless, neonatal seizures can induce a range of hippocampal responses such as glia activation and cytokine increase (8), altered glutamate and γ-aminobutyric acid (GABA)(A)-receptor gene expression (9), and persistent changes in electrophysiologic properties of CA1 pyramidal cells (10). Accordingly, Santos et al. (11,12) recently demonstrated that multiple episodes of pilocarpine-induced status epilepticus (SE) in developing rats (P7–P9) lead to progressive neocortical/hippocampal epileptiform activity, persistent in vitro hyperexcitability, increased hippocampal/thalamic apoptosis, and severe cognitive impairment in adulthood. Because these rats develop generalized electrographic seizures without marked hippocampal damage in the long term (11,12), the present study used immunocytochemical methods to investigate whether these findings could be related to morphologic changes involving principal cells and interneuron subtypes in the hippocampus and sensorimotor cortex.

Accepted November 16, 2004.

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METHODS

Induction of status epilepticus

Newly born male Wistar rats (UNIFESP-EPM, São Paulo, Brazil) were maintained in controlled environmental conditions (light/dark cycle of 12 h, 07:00–19:00 h; room temperature of 22–24°C) with their respective mothers. The age of the animals was determined starting from the day of birth (P0). Rats used in different groups were randomly selected in each colony. For SE induction, rats (N = 45) were isolated from their mothers and received intraperitoneal injections of pilocarpine hydrochloride, 2% (Merck, 380 mg/kg), in the seventh (P7), eighth (P8) and ninth (P9) days of postnatal life (11,12). After each injection, rats were observed for the characterization of seizures, duration of SE, occurrence of tonic episodes, and mortality. After recovery, surviving animals were returned to their respective mothers. The control group (n = 37) was constituted of rats that received saline

solution, 0.9%, instead of pilocarpine in the same volume (1 ml/kg).

The animals were killed at P35. After the anesthesia with chloral hydrate 4% (160 mg/kg), rats were submitted to transcardiac perfusion with paraformaldehyde, 1%, in phosphate buffer, 0.01 *M*, containing sodium chloride, 0.9% (phosphate-buffered saline; PBS), followed by paraformaldehyde, 4%, in PBS. After perfusion, the brains were carefully removed and postfixed 24–48 h in paraformaldehyde, 4%.

All experimental procedures were previously approved by the Animal Care and Use Committee of our institution.

Immunocytochemistry (ICC)

Fifty-micron vibratome sections, including the dorsal hippocampus and the sensorimotor cortex, were collected from coronal levels –3.3 mm to –4.3 mm (13) and then processed for ICC according to Silva et al. (14). In brief, free-floating sections were treated with H_2O_2, 3%, for 10 min, washed in PBS, incubated with normal horse serum (NHS), 10%, in PBS with Triton X-100, 0.4%, for 45 min, and then incubated overnight with primary antibody at 4°C. We used primary antibodies anti-nonphosphorylated neurofilament (SMI-311, monoclonal, 1:1,000; Stemberger Monoclonals Incorporated Lutherville, MD, USA) to observe the cytoarchitectural organization of the tissue, particularly of pyramidal cells (15). We also used antibodies against the calcium-binding proteins (CaBP) parvalbumin (PV, mono and polyclonal, 1:10,000; Swant Swiss Antibodies Bellinzona, Switzerland), calbindin (CB, mono and polyclonal, 1:10,000; Swant Swiss Antibodies), and calretinin (CR, polyclonal, 1:1,000; Swant Swiss Antibodies) to observe the different subtypes of interneurons. Finally, we used primary antibodies anti–glutamic acid decarboxylase (GAD-65, monoclonal, 1:300; Chemicon Temecula, CA, USA) to observe the distribution of GABAergic terminals. Sections were washed in PBS, incubated at room temperature with biotinylated anti-mouse immunoglobulin G (IgG; Vector Burlingame, CA, USA) (1:200 in NHS 1%) for 1 h, washed in PBS, incubated in avidin–biotin complex peroxidase (ABC) for 1 h, washed several times in PBS, and revealed with diaminobenzidine (DAB), 0.075%, in H_2O_2, 0.002%. Sections were finally washed in PBS, mounted on gelatin-coated slides, dehydrated, covered, and observed with light microscopy.

Immunohistoblot (IHB)

The IHB procedure was performed in P35 rats according to the protocol described by Tönnes et al. (16), with some modifications. In brief, animals were anesthetized, and the brains were carefully isolated from the skull, covered with Tissue-Tek embedding medium (Bio-Optica; Milan), frozen in 2-methhylbutane, and stored at –70°C until used. Serial cryostat-cut horizontal sections (10 μm thick) were mounted on glass slides and laid onto nitrocellulose membranes (Schleicher & Schuell; Dassel) for 5 min. The protocol described by Tönnes et al. was followed except for temperature of incubation in strip buffer (42°C) and for the preincubation of membranes in 10 m*M* Tris-base, 150 m*M* NaCl, and 0.1% Tween-20, pH 8.0 (TBST-8) plus bovine serum albumin (5% when human sera were used and 3% for commercial antibodies) (BSA; Sigma, St. Louis, MO, U.S.A.). Membranes were incubated with rabbit polyclonal antibodies against GluR1 [working dilution in TBST-8 plus 3% BSA, 1:200), GluR2/3 (1:150), GluR4 (1:200), NMDAR1 (1:50), and NMDAR2A/B (1:50)]. Alkaline phosphatase—conjugated goat anti-rabbit IgG (Jackson Immuno Research Lab, West Grove, PA, U.S.A.) were used as secondary antibodies. Wet membranes were scanned and digital images analyzed by Adobe PhotoShop 5.0.2 software. As negative control, some membranes were incubated, omitting the primary antibody. One section of every six processed onto nitrocellulose membranes was mounted on a slide and stained with thionin for histologic identification of brain structures.

RESULTS

Behavioral features of PILO-induced seizures were similar to those reported previously (11,12). A few minutes after pilocarpine administration, all animals showed continuous scratching, strong body tremor, mastication, clonic movements of forelimbs, and head bobbing, culminating in SE. The latency for the first motor signs after pilocarpine administration increased progressively until the third injection (P7, 108 ± 27.37 s; P9, 324 ± 24.97 s). The severity of SE was higher at P9, and most of the animals had one or more tonic seizures often accompanied by breathing spasms and cyanosis. Behavioral signs lasted 3–4 h, and four of 45 experimental rats died during the three SE sessions, most after the first injection. Surviving animals did not show, with rare exceptions, any behavioral manifestation that could be directly associated with epileptic activity. Behavioral alterations were not observed in control rats.

ICC

Hippocampus

In control rats, SMI-311 expression was observed in neuronal processes of CA1-3 pyramidal layer and in the hilus and granular layer of dentate gyrus (DG; Fig. 1a). Neurons had an intense cytoplasmic reactivity outlining the nucleus and the dendritic processes; PV expression was observed in cellular cell bodies and neuropil of CA1-3 interneurons, and in the hilus and granular layer of DG (Fig. 1c); CB expression was more evident in the molecular layer of the DG and in the stratum lucidum of CA3 (Fig. 1e); CR-positive cells were observed in the hilus of the DG, and CR-positive neuropil was observed in the granular layer of the DG and in the stratum

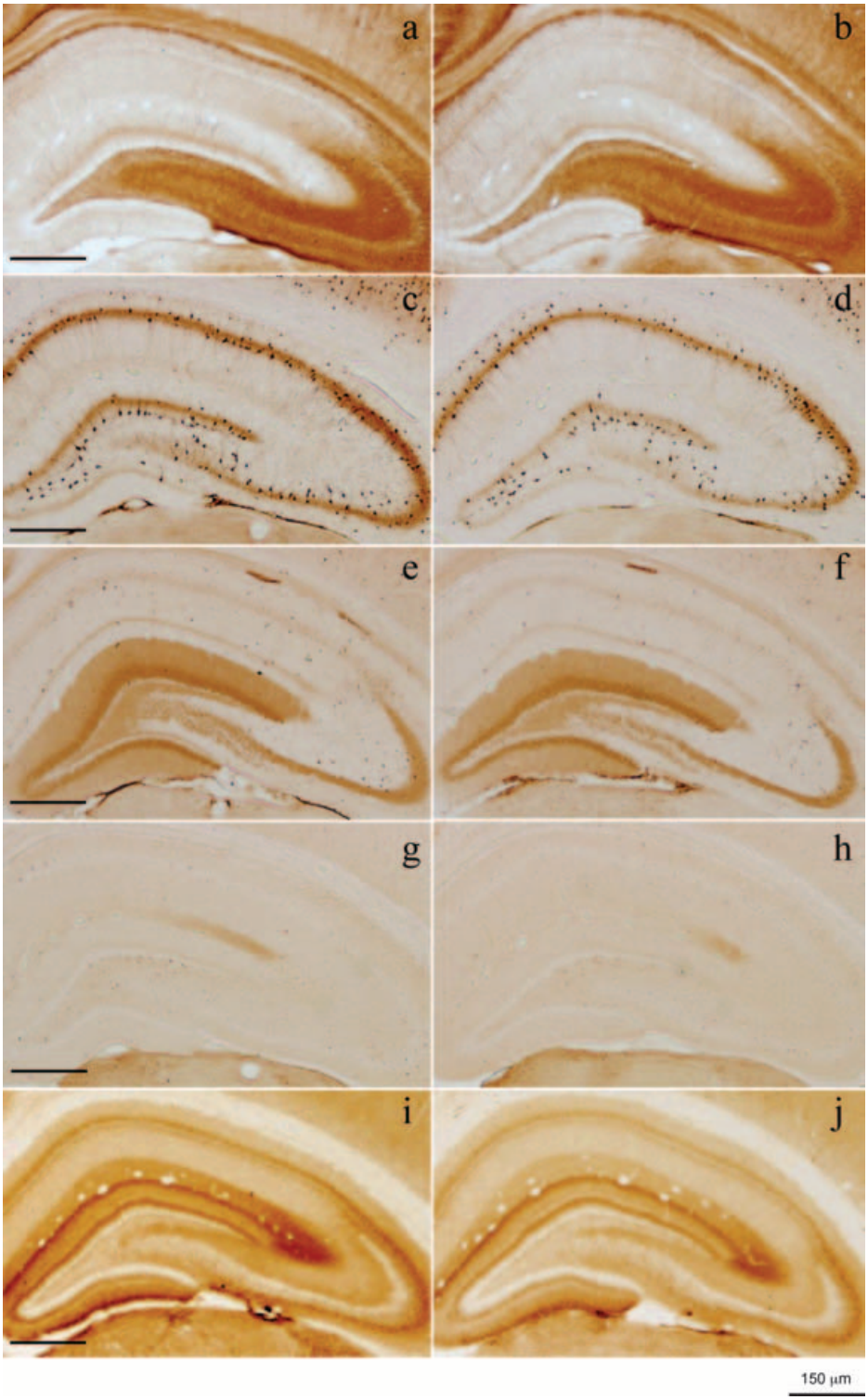

FIG. 1. Coronal sections of the hippocampus from control (left side: **a–i**) and experimental (right side: **b–j**) rats at P35 immunostained with antibodies against neurofilaments **(a/b)**, parvalbumin (PV) **(c/d)**, calbindin (CB) **(e/f)**, calretinin (CR) **(g/h)**, and glutamate decarboxylase (GAD65) **(i/j)**. Note, in treated rats, a reduced PV expression in CA1 and in dentate gyrus, and a reduced GAD-65 immunostaining, when compared with control sections. Calibration bar, 150 μm.

lacunosum-moleculare (Fig. 1g); GAD-65 expression was more intense in the molecular layer of the DG, in the external board of the CA3 pyramidal layer, and in the stratum lacunosum-moleculare (Fig. 1i).

Treated rats showed a normal SMI-311 expression when compared with controls (Fig. 1b); PV expression was reduced in CA1 and in the DG (Fig. 1d); CB and CR expression was similar to that in control rats (Fig. 1f and h);

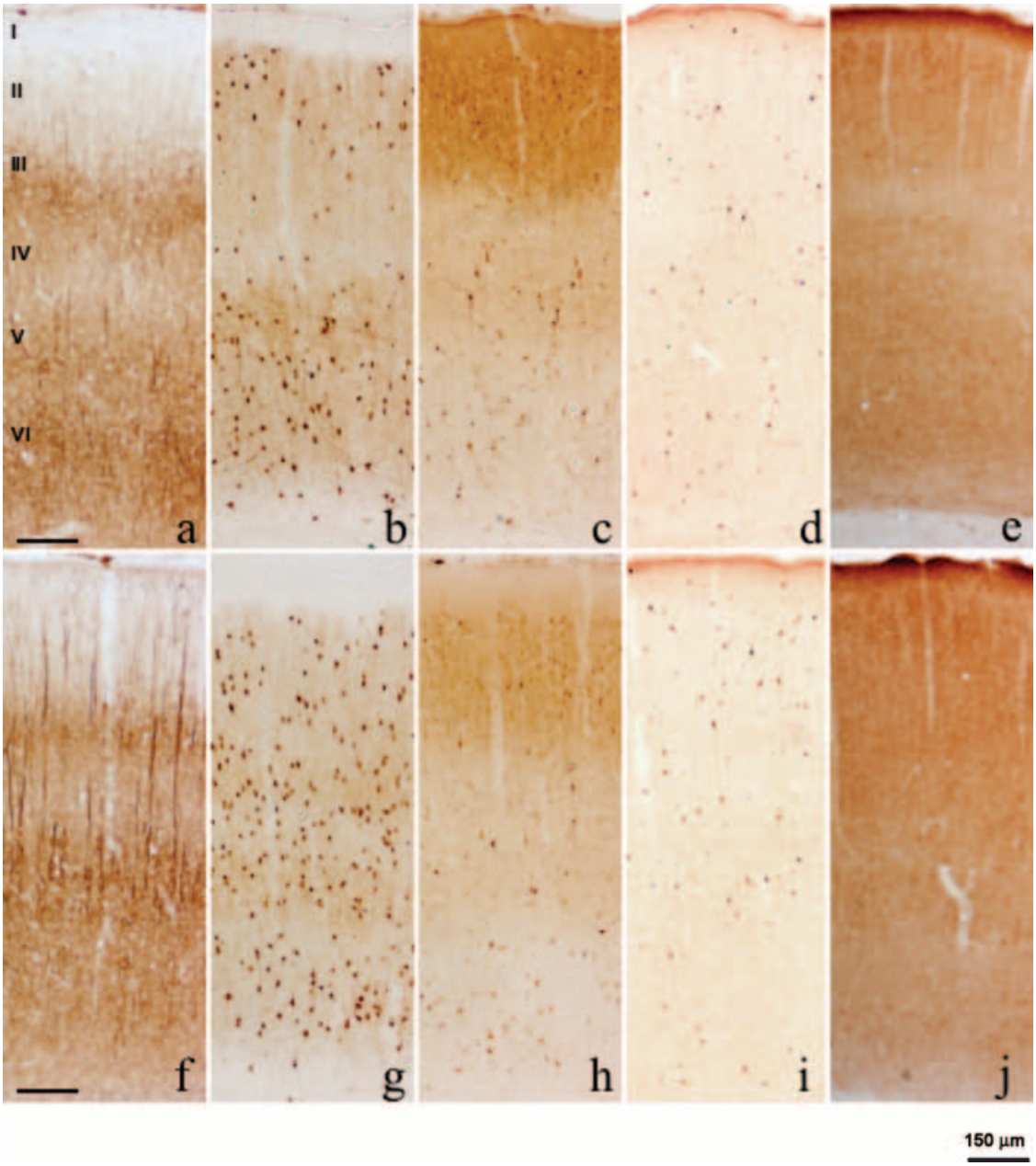

FIG. 2. Coronal sections of the sensorimotor cortex from control (above: **a–e**) and experimental (below: **f–j**) rats at P35 immunostained with antibodies against neurofilaments **(a/f)**, parvalbumin (PV) **(b/g)**, calbindin (CB) **(c/h)**, calretinin (CR) **(d/i)**, and glutamate decarboxylase (GAD65) **(e/j)**. Note, in treated rats, darkly stained pyramidal soma and apical dendrites with extended superficial ramifications. Treated rats also showed an unlayered PV distribution associated with reduced CB and increased GAD-65 immunostaining when compared with control sections. Calibration bar, 150 μm.

GAD-65 immunostaining was reduced, particularly in the DG (Fig. 1j).

Sensorimotor cortex

Control rats showed SMI 311–positive cells in cortical layers III, V, and VI (Fig. 2a); PV-positive neurons located mainly in layers II, V, and VI (Fig. 2b); CB-positive cells distributed throughout the cortex and the CB-positive neuropils in layers II and III (Fig. 2c); CR-positive cells had a diffuse and scattered distribution (Fig. 2d); and GAD-65 expression was restricted to neuropil and was observed in all cortical layers (Fig. 2e).

By contrast, pyramidal soma and apical dendrites in cortical sections of treated rats exhibited increased neurofilament-ir with extended superficial ramifications frequently reaching the layer I (Fig. 2f). In these animals, PV-positive neurons were diffusely distributed without a clear division among different layers (Fig. 2g); in superficial cortical layers, CB-ir was reduced, whereas GAD-65-ir was increased (Fig. 2h and j). No evident changes

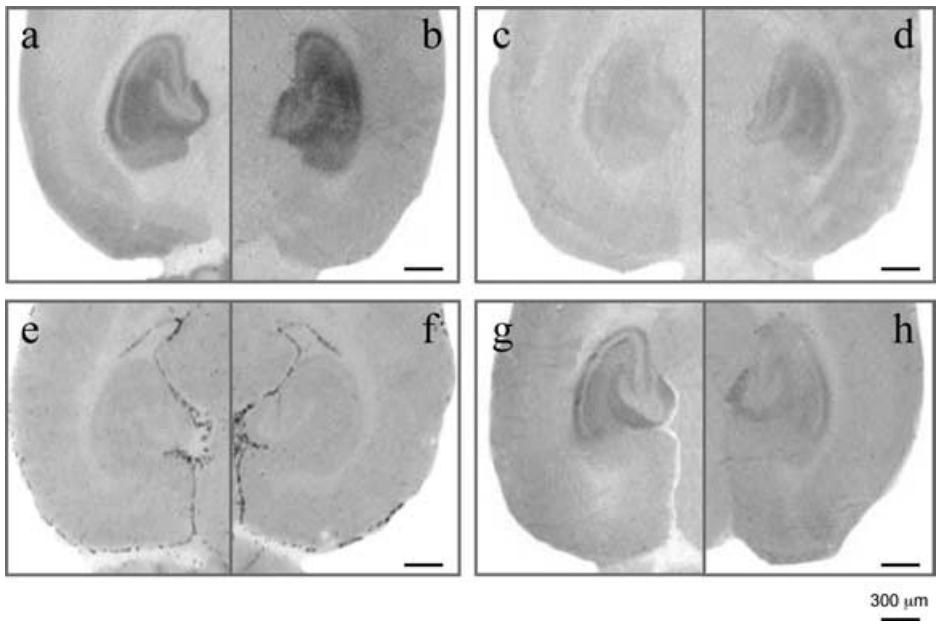

FIG. 3. Horizontal sections of the hippocampus from control **(a, c, e, g)** and experimental **(b, d, f, h)** rats at P35 immunostained with antibodies against α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-receptor subunits GluR1 **(a/b)**, GluR2-3 **(c/d)**, GluR4 **(e/f)**, and NMDA-receptor subunit NR2ab **(g/h)**. Note, in treated rats, an increased immunoreactivity of AMPA-receptor subunits (GluR1 and GluR2/3), and a reduced immunoreactivity of NMDA-receptor subunit NR2ab, when compared with control sections. Calibration bar, 300 μm.

in CR-ir were observed as compared with control rats (Fig. 2i).

IHB

In treated rats, an increased hippocampal expression of GluR1 and GluR2/3 subunits of AMPA receptors was observed (Fig. 3b and d), whereas GluR4 expression was similar to that in control (Fig. 3f). A reduced expression of the NR2ab subunit of NMDA receptors was also observed in treated rats (Fig. 3h) when compared with the control group.

DISCUSSION

As shown in previous reports from our laboratory (11,12,17), no epileptic activity, hippocampal damage, or supragranular sprouting were observed in P7–11 rats after a single dose of pilocarpine, but three consecutive pilocarpine-induced SE episodes during P7–9 induce an epileptic condition later in life. It includes generalized EEG alterations in both the hippocampus and neocortex, characterized by high-amplitude spiking or polyspiking activity that appeared mainly during behavioral arrest. The severity of the electrographic epileptiform features increased with age (i.e., they were longer and more pronounced when recording was carried out in older animals).

Accordingly, in the present study, we show that rats submitted to three consecutive episodes of pilocarpine-induced status epilepticus in P7, P8, and P9 have several neocortical and hippocampal changes later in life. The most prominent changes observed were (a) altered distribution of CaBP-positive neocortical cells; (b) increased cortical and reduced hippocampal GAD-65 expression; and (c) altered expression of hippocampal AMPA and NMDA receptors. Altogether, our results demonstrate that the occurrence of SE episodes during development induces complex cellular changes that probably alter the maturation of neocortical and hippocampal circuits.

Hirsch et al. (18) reported no cellular injury, but very high mortality due to lithium-pilocarpine treatment in developing rats. Nevertheless, because very few animals survived in that study, it is possible that the lack of injury may have represented those animals that did not experience the same intensity of seizures. Because most of the animals in our study survived, we believe that such a bias can be excluded from our results. In another study, Sarkisian et al. (19) performed kainic acid (KA) injections on P20, P22, P24, and P26 developing rats. They found that serial episodes of SE in this period result in a progressive decrease in seizure severity and cause no long-term behavioral consequences. We analyzed the effect of three consecutive administrations of pilocarpine in a different time window (P7–9). We previously demonstrated that the susceptibility to and the effect of pilocarpine-induced seizures during P7–11 is different from that observed after P18 (17). Therefore our results differed from those of the study of Sarkisian et al. probably because we used younger animals and because of the different effects of cholinergic versus glutamatergic stimulation during development. In addition, findings from our laboratory (not yet published)

with three consecutive episodes of KA-induced SE (instead of pilocarpine) in developing rats reproduced the same EEG and behavioral pattern as observed when the triggering agent was pilocarpine.

Some evidence exists that specific populations of cortical GABAergic neurons, particularly the PV-containing ones, could be particularly involved in the establishment of epilepsy in humans (20) and rats (14). In the present work, neocortical PV expression was increased, and PV-positive cells were abnormally distributed and exhibited extensive ramifications in treated rats. One explanation for these changes could be that SE episodes altered the physiologic apoptotic process (21) during the cortical development. PV-positive neurons programmed to die would be erroneously preserved, occupying atypical positions and establishing abnormal connections. Furthermore, the abnormal PV expression could be alternatively explained by a de novo PV expression, induced by treatment, in other cortical layers. In any case, the function of abnormal PV-positive neurons in pilocarpine-treated animals remains an open question.

Recently, Zhang et al. (22) examined the effect of SE in P10 rat pups on subsequent GABAR development in dentate granular neurons and found increased α-1 subunit expression and type I benzodiazepine augmentation when the rats reached adulthood. The authors suggested that the increased α-1 subunit expression after P10 SE could be an "overmaturation" of GABARs in dentate granular neurons because of the excessive neuronal activity associated with SE.

The role that the alterations of the GABA system may play in epileptogenesis continues to be a topic of considerable debate. We recently reported a reduction of neocortical GAD-65 immunoreactivity in chronic epileptic rats (14). In the present work, we observed an increased neocortical GAD-65 expression in rats treated during development, for which two possible explanations exist. First, it is likely that increased levels of GAD occur within axon terminals. It also is possible that GABAergic neurons exhibit reorganization of their axons. However, because of multiple sources and widespread distribution of neocortical GABAergic terminals, it is presently impossible to distinguish axonal reorganization from increased GAD levels in terminal fields.

In rats, NMDAR density peaks late in the first postnatal week in many forebrain structures, including hippocampus and neocortex, whereas AMPAR density peaks in the second postnatal week at around P10 (23). Jensen and Wang (24) reported that hippocampal slices prepared from adult rats that underwent hypoxia at P10 show significantly lower thresholds to convulsant conditions compared with slices from controls. Similarly, previous electrophysiological data from our laboratory showed that rats submitted to SE at P7–9 had hippocampal hyperexcitability in the later in life (11,12). Accordingly, in the present study, the imbalance between glutamatergic (GluR1 and GluR2/3) and GABAergic markers (PV and GAD-65) could explain the hippocampal hyperexcitability in treated rats. Additionally, the abnormal expression of NMDA receptors could be associated with memory and learning deficits previously observed in this model (11,12). Our results, however, are in contrast to those of Zhang et al. (25), who recently demonstrated that a single SE episode at P10 reduces the expression of the GluR2 subunit of glutamate receptors later in life. This discrepancy could be due to a difference in methods, as Zhang et al. used a different model. It is possible that consecutive SE episodes have a different impact on glutamatergic system development, inducing different modifications in glutamate receptor composition.

In conclusion, consecutive episodes of SE in P7–P9 rats alter the cortical maturation. The resultant abnormal circuitry development could be responsible for permanent impairment of neocortical–hippocampal function, leading to cognitive deficits and electrographic epileptiform abnormalities observed later in life.

Acknowledgment: This work was supported by CNPq, CAPES, and FAPESP from Brazil, and Associazione Paolo Zorzi per le Neuroscienze from Italy. Alexandre Valotta da Silva received Ph.D. grants from CAPES and CNPq.

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