

Are Acute Changes After Status Epilepticus in Immature Rats Persistent?

L. SUCHOMELOVÁ, H. KUBOVÁ, R. HAUGVICOVÁ, R. DRUGA, P. MAREŠ

Institute of Physiology, Academy of Sciences of the Czech Republic, Prague, Czech Republic

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Summary

Early consequences of lithium-pilocarpine convulsive status epilepticus (SE) were studied six days after this status had been induced in rat pups at the age of either 12 or 25 days. Studies of spontaneous EEG activity demonstrated the presence of epileptic phenomena (isolated spikes) in both hippocampus and cortex (cortical spikes were more expressed in the older group). There were no marked behavioral correlates of spikes and transition into the ictal phase was exceptional. The motor performance on a rotorod and a horizontal bar was the same in experimental and control rats of both ages. Behavior in the open field was changed in a reverse manner in the two age groups: the locomotor activity of rats with induced seizures at the age of 12 days was significantly lower than that of their control siblings, whereas animals undergoing status at the age of 25 days were hyperactive. In addition, they also exhibited increased exploratory activity (rearing) and their habituation to the open field was deranged. Nissl-stained brain sections demonstrated extensive brain damage in the older group in contrast to the negative findings in younger animals. EEG, behavioral and morphological changes induced by status epilepticus in developing rats persisted for 6 days after the status. They markedly differed according to the age of animals.

Key words

Status epilepticus • Rat • Development • EEG • Behavior • Motor performance • Histology

Introduction

There is a longlasting clinical discussion about possible damage induced by epileptic activity in the brains of infants and children (Wasterlain and Shirasaka 1994, Camfield 1997, Wasterlain 1997). Experimental studies have demonstrated that generation of epileptic seizures in the developing brain is less damaging than in the adult brain (Mareš 1991, Moshé *et al.* 1993). In contrast to this, the consequences of severe epileptic seizures in the immature brain were considered as negligible if seizures were elicited during the first three weeks of postnatal life in rats (Sperber *et al.* 1992,

Stafstrom *et al.* 1992, Holmes 1997). Only recently data have been published proving deleterious effects of severe seizures even during early stages of brain maturation. The features and time course of these effects differed from those in the mature brain (Babb *et al.* 1995, Sankar *et al.* 1998, 2000). Majority of these experiments was focused on morphological changes in the brain and data on functional changes are sparse. Liu *et al.* (1994) found that rats seized at P20 were able to master the Morris water maze, but the latency to reach the platform was longer in comparison with the controls, whereas animals receiving pilocarpine at P45 performed significantly more poorly. In contrast, de Feo *et al.* (1986) demonstrated that the

administration of KA at P10 and P25 resulted in a significantly worse performance in an active avoidance test performed on P45. In agreement with de Feo's results we found changes in behavior in the open field if rats exposed to kainic acid at P12 were tested one and two weeks later (Kubová *et al.*). We recently found that the lithium-pilocarpine model of status epilepticus (SE) induced on postnatal day 12 or 25 changes subsequent motor development of rats (Kubová *et al.* 2000). In connection with a description of the acute phase of SE (for 24 hours after pilocarpine administration) demonstrating a persistence of epileptic EEG activity in these two age groups (Suchomelová *et al.* – submitted), we decided to study a longer interval after SE (6 days). The aim of this study was to find out if changes in the EEG, behavioral and motor tests and in brain morphology are present during this time qualified as the “silent period” in adult rats (Cavalheiro 1995).

Methods

Male and female Wistar albino rats 12 and 25 days old were used (the day of birth was considered as day 0). Experiments were performed on animals from litters culled to 8-10 pups on the day of birth and maintained on a 12/12 h light/dark cycle under controlled temperature and humidity. They had free access to food and water. The experimental protocol was approved by the Animal Care Committee of the Institute of Physiology to be in agreement with the Animal Protection Law of the Czech Republic.

Status epilepticus was induced using the lithium pilocarpine model. An aqueous solution of lithium chloride (3 meq/kg) was injected intraperitoneally 24 hours prior to an intraperitoneal injection of pilocarpine (40 mg/kg). After pilocarpine administration, animals were observed in isolation for at least 3 hours. The incidence and latency of behavioral changes (automatisms), first seizures (clonic seizures of head and forelimbs) and continuous convulsions, i.e. SE, were registered. After 120 min (12D) and/or 90 min (25D) of continuous SE, paraldehyde was administered in doses of 0.3 (12D) and/or 0.6 (25D) ml/kg to prevent mortality and, in 25-day-old rats, also to prevent transition into generalized tonic-clonic seizures. Control rats received lithium chloride and paraldehyde in the same dose and time schedule. After approximately 5 hours, the animals received an injection of saline and were returned to their mothers (weaning takes place at the age of 28 days). As far as 12-day-old rats are concerned, the mothers took full care of these pups. The older group required special care (extra hydration – s.c.injections of physiological saline

immediately after SE and feeding with soft food prepared from ground pellets and a sucrose solution). All experiments were performed 6 days after SE, hence the two groups were designated as PD 12+6 and PD 25+6. The animals were handled and their body weight was monitored till the end of the experiment. In spite of a tendency to a decrease in body weight 3 days after SE there was no difference between the experimental and control rats 6 days after SE.

1. EEG study

Preliminary surgery was performed under ether anesthesia. Two flat silver epidural recording electrodes were placed symmetrically over the right and left sensorimotor area at coordinates AP 0; L 2 mm in relation to the bregma; two stainless steel recording electrodes isolated up to their tips were implanted symmetrically into the dorsal hippocampus. The coordinates used for young rats were calculated from those for adult animals (AP 4.0; L 3.0; V 4.5 mm) in relation to the bregma-lambda distance. An indifferent electrode was localized in the nasal bone. All electrodes were fixed to the skull by means of fast curing dental acrylic. The animals were allowed to recover for at least one hour, their reflexes (righting, placing and suckling) were checked and then the recording was started. EEG was monitored for 5 hours. Each age group consisted of 10 experimental and 3 control animals.

2. Motor and behavioral tests

These tests were performed in another group of animals without implanted electrodes (N=16;14 and 14;14 for the two experimental and control age groups, respectively).

A) Behavioral test

An open field was used to check the behavioral responses to a novel environment, motor abilities and habituation. Special room with a constant temperature, light and sound conditions was used for this test. The home cage with animals was transferred into the testing room two hours before beginning of the experiment. The floor of a square plastic board (50x50 cm) with plastic sides (30 cm high) was divided into 16 squares. Animals were exposed to the open field for 5 min. The rat was placed into the central area and latency necessary to leave this area was recorded. Motor activity was expressed as the number of squares crossed; distribution of locomotor activity during the five-minute testing interval was used to evaluate the level of habituation. Exploratory activity was expressed as the total number of rearings.

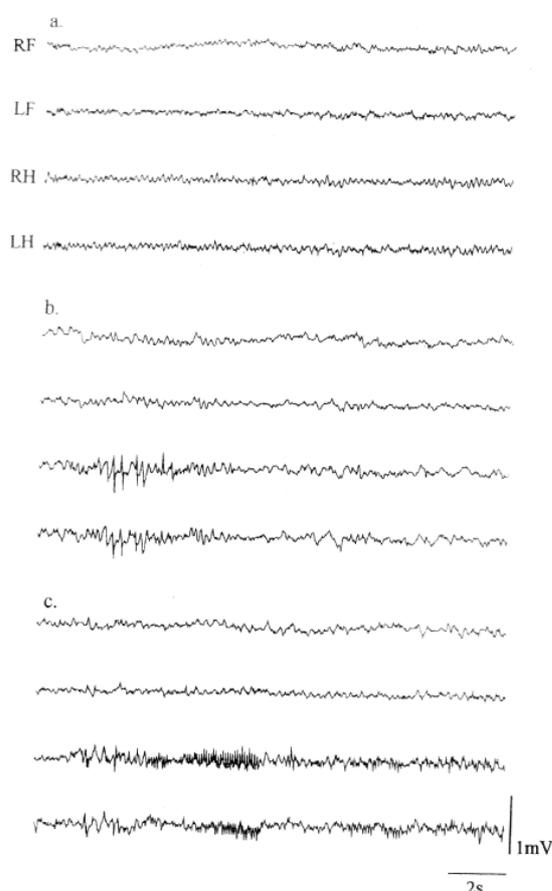


Fig. 1. EEG activity of a PD 12+6 rat. a. section without any abnormality; b. and c. episodes of spikes in both hippocampi. Individual leads from top to bottom: RF – right and LF – left frontal neocortex, RH – right and LH – left dorsal hippocampus always in reference connection. Time mark 2 s, amplitude calibration 1 mV.

B) Motor tests

Bar holding. The rats were held so that their forelimbs touched a 25 cm long wooden bar extended between two poles 50 cm high. The time spent on the bar as well as grasping with forelimbs and hindlimbs were registered for up to 120 seconds.

Rotarod. This test was used in P25+6 group only because even control younger animals exhibited a very poor performance. Animals were placed on the rotating rod with a diameter of 10 cm (speed 5 rpm). The time spent on the rod was measured for up to 180 seconds.

Specific age-related tests were used for animals of the PD 12+6 group:

Negative geotaxis. Pups were placed on an inclined (30°) surface with their heads facing downward. The time necessary for turning by 90° and consequently

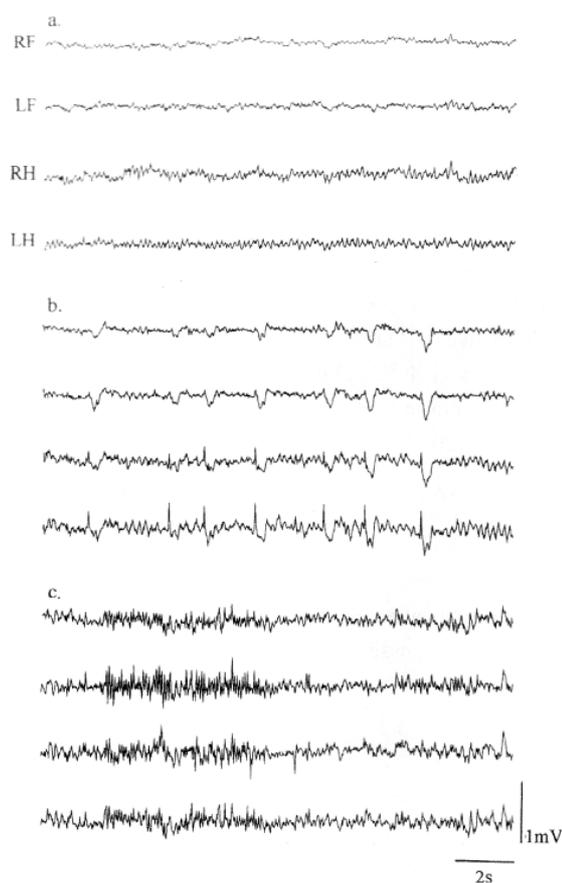


Fig. 2. EEG activity of a PD 25+6 rat. a. section without any abnormality; b. isolated spikes in hippocampus with a propagation to neocortex; c. sections of generalized spikes. Details as in Fig.1

by 180° was recorded. The animals were tested for 90 seconds.

Wire-mesh climbing. A 10-mm wire mesh, 45 cm high and 15 cm wide, was placed at an angle of 70° in contact with a platform on the top and with an edge of laboratory desk at the bottom. To motivate a pup placed on the bottom of the wire mesh to ascend, its littermates were placed on the top platform. The time necessary to rejoin the siblings was measured for up to 120 seconds.

Incidence was statistically evaluated by means of Fisher's exact test, time differences between control and experimental groups were compared using the t-test, and differences among the one-minute periods in an open field in the same group by one-way repeated measures ANOVA with subsequent multiple comparison according

the Dunn's method (SigmaStat®, SPSS). The level of significance was set at 5 %.

3. Histology

After the end of EEG monitoring, the animals were perfused after an overdose of urethane anesthesia (2 g/kg i.p.) with ice cold 0.01 M phosphate buffered saline (PBS, pH 7.4) followed by a fixative solution (4 % formaldehyde). The brains were removed and postfixed for 5 days at 4 °C. The brains were washed in 0.1 M phosphate buffer (PB, pH 7.4) and then soaked gradually in 10 %, 20 % and 30 % sucrose. Coronal sections (30µm) were cut through whole brain on a cryostat and every fifth section was mounted onto gelatin-coated slides and stained with cresylviolet (Nissl staining). Then the slides were coverslipped. The brains were used to control the localization of hippocampal electrodes as well as to examine possible brain damage.

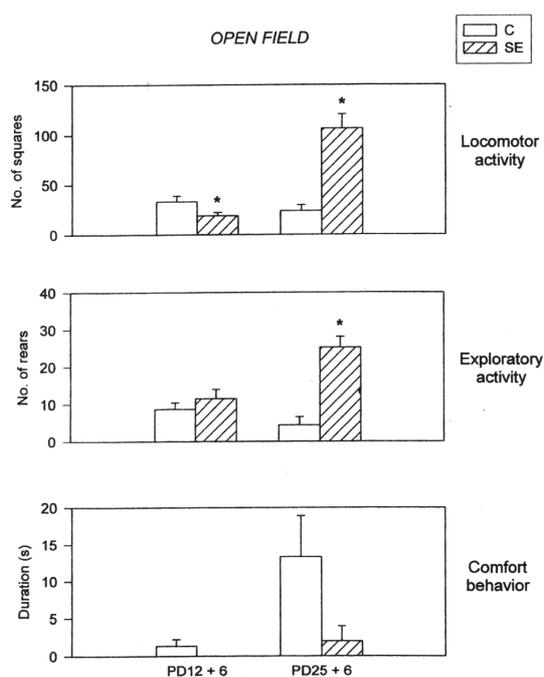


Fig. 3. Behavior of rats in the open field. From top to bottom: Locomotor activity expressed as the average number of squares crossed; Exploratory activity expressed as the average number of rearings; Comfort behavior (washing) expressed as average duration. Left pair of columns – PD 12+6 rats, right columns – PD 25+6 rats; white columns – controls, hatched columns – animals after status epilepticus. Asterisks denote significant difference between control and seized rats.

Results

1. EEG

Younger group (PD 12+6): Control animals exhibited a background rhythm formed by theta waves of low amplitude. Active wakefulness was characterized by desynchronization, whereas a mixture of slow waves was recorded during sleep. Isolated spikes might occur but they were rather exceptional. Clear-cut theta activity was recorded in the hippocampus in connection with active behavior. On the contrary, nine out of ten rats 6 days after SE exhibited frequent spikes both isolated as well as in series (Fig. 1). The spikes appear in the hippocampus (unilaterally or bilaterally), a cortical projection was infrequent if any. Ictal activity (episodes of spikes longer than 5 s) was registered in two of these rats.

Older group (PD 25+6): Control rats did not markedly differ from the control PD 12+6 animals, only their EEG was more organized – segments of regular rhythm were more common than in younger rats. Among rats undergoing SE again, nine out of ten exhibited spikes – isolated and/or in series (with a frequency of 11-12 Hz) mainly in the hippocampus (Fig. 2). In contrast to the younger group, cortical projection was more common. Ictal activity appeared in one rat only and was restricted to the hippocampus.

2. Behavioral and motor tests

Younger group (PD 12+6): Open field: There was no significant difference between experimental and control animals in the latency before leaving the central area at the beginning of the test (2.1 ± 2.8 vs. 3.6 ± 1.1 s, mean \pm S.E.M.), in exploratory activity (number of rearings were 12 ± 3 and 9 ± 2 , respectively) and in the time spent with comfort behavior – the value for control animals (1.3 ± 0.9 s) was not different from the experimental rats in spite of the fact that seized rats did not exhibit comfort behavior at all. The only significant difference concerned locomotor activity. Control rats crossed 33 ± 6 squares on the average in contrast to 19 ± 3 squares crossed by the experimental rats (Fig. 3). Activity was equally distributed during the 5-min observation period in both control and experimental groups, i.e. there were no signs of habituation (Fig. 4).

Motor tests: no significant difference was found in any test used. Surprisingly, experimental animals tended to perform better on the horizontal bar (47.8 ± 7.8 s on the average) than control rats (30.6 ± 4.2 s) but this difference was also not significant.

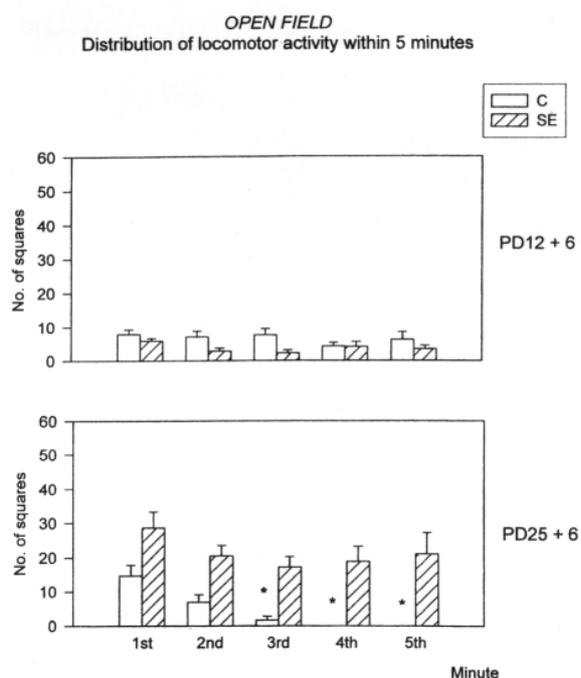


Fig. 4. Time profile of locomotor activity in the open field. Upper graph – PD 12+6 rats, lower graph – PD 25+6 animals. Abscissa – first to fifth minute of exposure, ordinates – number of squares crossed. Other details as in Fig. 3.

Older group (PD 25+6): Open field: control and experimental animals did not differ only in the latency to leave the central area. As concerns locomotor activity, the experimental animals were more active (106 ± 14 crossed squares vs. 24 ± 6 in controls). In addition, control animals exhibited the highest activity in the first minute and then their locomotion decreased (a level of statistical significance was reached in the third to fifth minute) whereas experimental rats were equally active during the whole 5-min observation period. Exploratory activity was also higher in experimental rats (25.1 ± 2.9 rearings vs. 4.4 ± 2.2 in the controls). Experimental animals spent less time with comfort behavior (2 ± 2 s) than their control siblings (13.3 ± 5.5 s).

Motor tests: The tendency was opposite to that in younger rats, i.e. seized PD 25+6 animals spent a shorter time on the horizontal bar (89.5 ± 11.7 s in the control group vs. 51.5 ± 13.1 s in the experimental animals) but the difference did not attain the level of statistical significance. There was also no significant difference in the time spent on the rotating rod (controls – 160.3 ± 19.8 ; experimental rats – 132.3 ± 17.2 s) but animals surviving SE exhibited a different strategy. All 15 experimental rats only moved against rotation, 6 out of 14

controls turned in the opposite direction and then fell down. The incidence of this behavior was significantly different ($p=0.0063$, Fisher's exact test).

3. Histology

Younger group (PD 12+6): There was no obvious difference in the Nissl-stained sections between the brains of experimental animals and those from the control siblings.

Older group (PD 25+6): Rats undergoing SE exhibited a constant damage in the piriform cortex from AP level 9.7 (Fig. 5B) in the field Pp1 and lateral half of the Pp2 and in the ventral claustrum (areas DEn and VEn). In addition, degeneration was observed in cortical amygdalar nuclei (posterolateral and lateral half of posteromedial cortical ncc.). In contrast to these reliable changes, damage of the hippocampus was very variable and even absent in two out of ten animals. If present, hippocampal damage was formed by dramatic cell loss and conspicuous gliosis in the caudal part of the hippocampus (Fig. 5F). Furthermore, in addition, an evident decrease of large hilar neurons was observed in the septal part of the hippocampus. According to their morphology and localization these large cells may belong to the category of mossy hilar neurons.

Discussion

Pilocarpine as well as lithium/pilocarpine-induced status epilepticus was also described in immature rats (Cavalheiro *et al.* 1987, Hirsch *et al.* 1992). Recently, we performed video EEG monitoring for 24 (in 12-day-old rat pups) and 48 hours after pilocarpine administration (in 25-day-old animals), respectively (Suchomelová *et al.* – submitted). The epileptic EEG activity was registered in all these animals. The present results demonstrated that nearly all animals exhibited specific epileptic graphoelements in the EEG 6 days after the SE. To decide if epileptic activity persisted for 6 days after SE (which seems more probable) or if there is a real silent period after SE in immature rats it is necessary to make recordings at intervals shorter than those used in the present study.

A marked predominance of pathologic activity in the hippocampus of the younger group and nearly equal representation of this activity in the hippocampus and neocortex in the older group was surprising. According to literature (kainic acid-induced SE – Cavalheiro *et al.* 1983) as well as our acute recordings (Suchomelová *et al.* – submitted) epileptic EEG activity is strongly expressed in cerebral cortex of 12-day-old rats

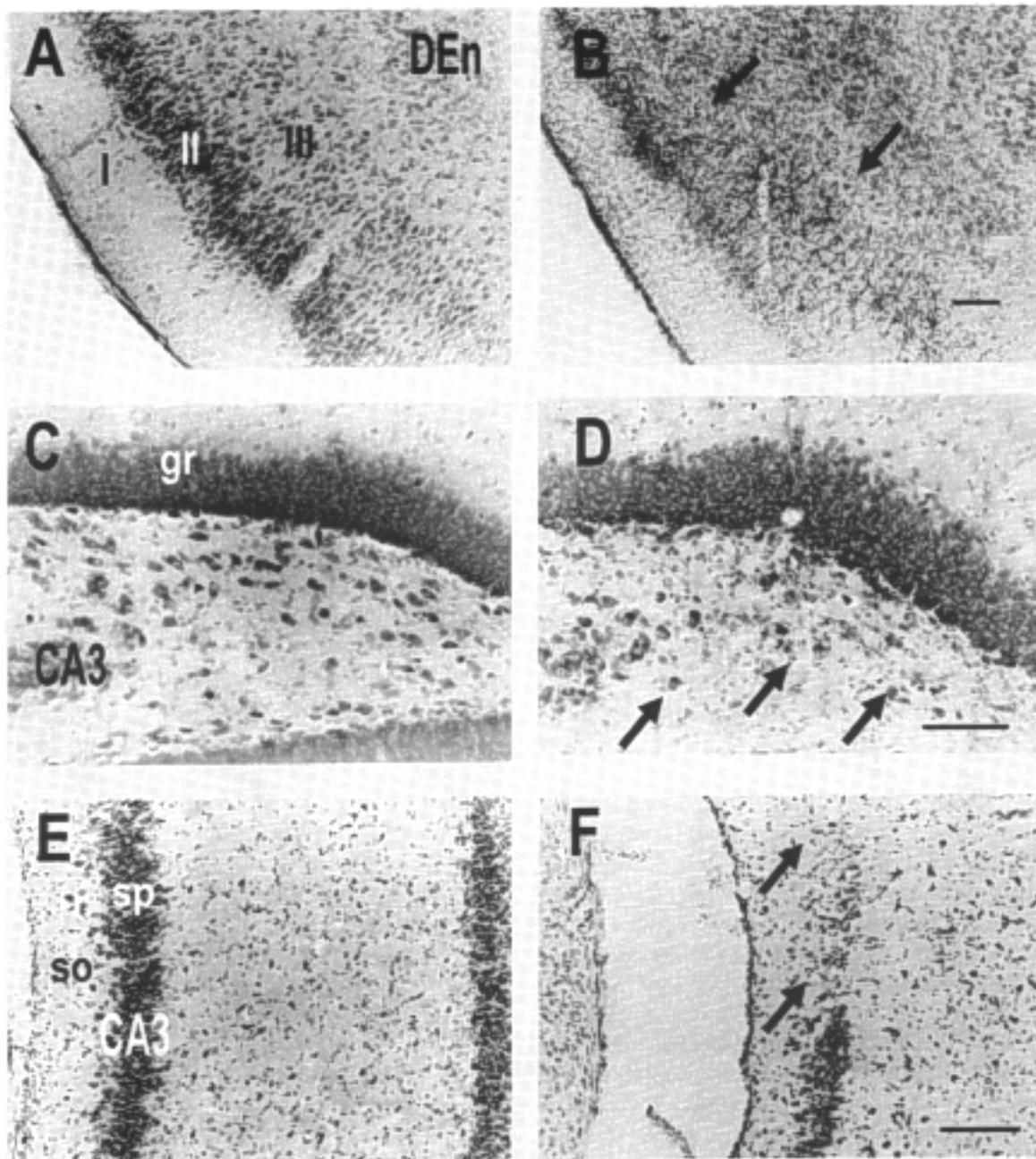


Fig. 5. Photomicrographs of piriform cortex at AP plane 9.7 mm (A and B), dentate gyrus of the septal part of hippocampus (C and D) and field CA3 of the caudal part of the dorsal hippocampus (E and F) from PD 25+6 control rats (A, C and E) and seized animals (B, D and F). Seized animals exhibit destruction of tissue, cell loss and a decrease of staining in the piriform cortex, a lower number of large bipolar neurons in the hilus and total CA3 cell loss and decrease in staining in the hippocampus. A and B - piriform cortex: layers I to III, arrows indicate a boundary of the heavily damaged tissue, DEn – dorsal endopiriform nucleus. C and D – septal part of the dentate gyrus: gr – granule cell layer, CA3 – end of the pyramidal cell layer; arrows indicate a part of the hilus with a decreased number of large bipolar cells. E and F - dorsal hippocampus: CA3 – hippocampal field, sp - pyramidal layer, so - stratum oriens; arrows indicate marked loss of pyramidal cells. Scale bar = 100 μ m.

whereas rats 3 and more weeks old exhibited epileptic activity mostly in the hippocampus. In addition, the epileptic EEG activity was never accompanied by motor seizures in either age group. This again is in agreement with the short-term recordings demonstrating even ictal EEG activity without any obvious motor correlate.

Motor functions were not compromised at the interval studied – performance on the bar did not differ between control and experimental rats, the time spent on the rotorod is the same. A peculiar behavior of older animals on rotorod has nothing to do with motor performance; it is a question of behavioral strategy. In contrast to a good motor performance there is a marked hypoactivity in the open field in younger animals.

Spontaneous locomotor activity was changed in an opposite manner in the two age groups – decreased activity in the younger animals vs. hyperactivity in the older group. The simplest explanation of hypoactivity in the younger group may be a developmental retardation – activity in the open field steeply increased with age (Nadel *et al.* 1993, Mikulecká and Mareš – in preparation). This explanation is supported by the finding that rats seized at P12 exhibited hyperactivity in adulthood (Kubová *et al.* 2000). Hyperactivity observed in the older group might be the cause of failure of habituation in the open field. An alternative explanation may be based on compromised perception or elaboration of external stimuli.

In contrast to behavioral changes in both age groups a marked difference was found in brain morphology. Younger animals did not exhibit obvious changes in Nissl stained sections whereas extensive brain damage could be demonstrated in animals seized at postnatal day 25. The lack of changes in the younger group might be taken as a confirmation of older data claiming that seizures in the immature brain do not result

in permanent brain damage (Sperber *et al.* 1992). On the contrary, Nissl-stained sections exhibit a low sensitivity to a loss of neurons – without cell counting a substantial part of cells must be lost to be sure that there is a deficit. Recent literature confirmed the findings of Babb *et al.* (1995) that even in immature rats seizures led to neuronal loss (Sankar *et al.* 1998) but that the distribution and time course of damage differs from those in the mature brain. A selectivity of cell death in the immature brain was also proved in our experiments demonstrating a decrease in the number of neurons containing parvalbumin in rats seized at postnatal day 12 (Kubová *et al.* 1997).

It is impossible to make a direct comparison between electrophysiological and morphological findings in the present study. Our data from the study of the acute phase of SE where we performed videoEEG monitoring and histological control in the same animals speak in favor of such correlation – 25-day-old rats with poor control of SE by paraldehyde exhibited more severe brain damage than their littermates with better effect of paraldehyde (Suchomelová *et al.* – submitted). Detailed analysis at different time intervals after SE remains to be done.

Our data speak rather in favor of the possibility that epileptogenesis in the developing rats after severe status epilepticus is a continuous process and that the “silent period” is silent only from the point of view of motor seizures. This hypothesis has to be proved by further investigation.

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Reprint requests

L. Suchomelová, PhD., Institute of Physiology, Academy of Sciences of the Czech Republic, Vídeňská 1083, CZ-14220 Prague 4, Czech Republic