

Effects of a Free Radical Scavenger N-tert-butyl-alpha-phenylnitron (PBN) on Short-Term Recovery of Immature Rats after Status Epilepticus

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Summary

The present study examined the effects of a free radical scavenger, N-tert-butyl-alfa-phenylnitron (PBN) on lithium-pilocarpine-induced status epilepticus (SE) and its short-term consequences in rats 12 (P12) or 25 (P25) days old. PBN (2 x 100 mg/kg i.p.) was injected according to the following schedules: 1) PBN-pretreated animals received the first dose 30 min prior to pilocarpine, the second dose was given 1 min after SE onset, and 2) PBN-treated animals received the first dose of PBN 1 min after SE onset and the second one 60 min later. Paraldehyde was administered to decrease mortality. Effects of PBN were highly age-dependent. In P25 group, PBN-pretreatment increased latency to SE onset and significantly suppressed the severity of motor manifestation of SE. Both PBN pretreatment and treatment improved recovery after SE. In contrast, administration of PBN in P12 animals did not affect SE pattern or recovery after SE. Administration of PBN had no effects on the motor performance of animals 3 and 6 days after SE. Neuronal damage was examined 24 h and 7 days after SE using Fluoro-Jade B staining. Mild neuroprotective effects of PBN in hippocampal fields CA1 and CA3 occurred in P25 rats in both experimental schedules. In contrast, administration of PBN aggravated neuronal injury in the hippocampus in P12 rats. Administration of PBN to intact rats did not induce neurodegeneration in either age group.

Key words

Free radical scavenger • Status epilepticus • Neuroprotection • Behavior • Ontogeny

Introduction

Free radicals are normal intermediates in the aerobic metabolism of all cells. They are formed in increased amounts whenever cells are exposed to hyperoxia, or to various pathologic conditions (Thomas 1997, Hensley *et al.* 1997). Endogenous antioxidants, including glutathione, ascorbic acid and vitamin E, are

likely involved in the detoxification of free radicals. These protective mechanisms can, however, be overwhelmed under the conditions of increased formation of free radicals, which then can induce oxidative damage observed under various pathological states (Hall 1997).

Nitron-based free radical scavengers, such as PBN (N-tert-butyl-alfa-phenylnitron), react readily with many different types of free radicals, forming nitroxide

spin adducts. The spin adduct is generally much more stable and less reactive than the original free radicals (Hensley *et al.* 1997). The neuroprotective effect of PBN has recently been tested in various models. PBN reduces substantially infarct volume following experimentally induced ischemia (Cao and Phillis 1994, Zhao *et al.* 1994) and improves recovery of brain energy state following transient focal ischemia (Folbergrová *et al.* 1995). PBN and 2-sulfo-PBN significantly attenuate striatal excitotoxic lesions in rats, elicited by intrastriatal injections of malonate (Schulz *et al.* 1995) or by infusion of glutamate (Lancelot *et al.* 1997). In adult rats subjected to flurothyl-induced status epilepticus (SE), histopathological lesions can be ameliorated by PBN (He *et al.* 1997) and PBN treatment also improved the bioenergetic state in substantia nigra in the same model (Folbergrová *et al.* 1999). However, PBN treatment has never been tested in epileptic seizures induced in immature animals.

It is well known that in both adult and immature rats SE leads to long-lasting consequences, such as neuronal loss, functional impairment, cognitive deficits, and potential development of chronic epilepsy (Dubé *et al.* 2001, Sankar *et al.* 1998, 2000, Suchomelová *et al.* 2002, Kubová *et al.* 2004). For this study, experiments were performed using Li-pilocarpine-induced status epilepticus (SE) according to the established protocol (Hirsch *et al.* 1992, Sankar *et al.* 1998, Kubová *et al.* 2000, Druga *et al.* 2003). The primary goal of this study was to explore whether PBN influences recovery and has a neuroprotective effect in temporal lobe structures of the immature brain at short intervals following Li-pilocarpine-induced SE. Recovery of rat pups in the first week following SE was determined by exposing the pups to a battery of motor tests, whereas neuronal degeneration was evaluated using Fluoro-Jade B staining.

Methods

Animals

Experiments were performed in male Wistar rats of two age groups: 12 and 25 days old. The day of birth was defined as P0. Number of pups in the nest was reduced to ten. Animals were housed under controlled conditions (temperature 22±1 °C, humidity 50-60 %, lights at 06:00 h to 18:00 h) with free access to food and water. Animal care and experimental procedures were conducted in accordance with the guidelines of the European Community Council directives 86/609/EEC.

Experiments were approved by the Animal Care and Use Committee of the Institute of Physiology of the Academy of Sciences of the Czech Republic.

Induction of status epilepticus (SE)

SE was induced in P12 (n=104) and P25 (n=102) rats. Part of these animals (n=29 and 30 for P12 and P25, respectively) was left to survive up to adulthood for examining the long-term effects of the study (Rejchrtová *et al.*, data to be published). Classification of their SE was supplemented to the data from rats forming groups for the present behavioral and morphological study. The previously published protocol (Hirsch *et al.* 1992) was used. Briefly, rats were injected with an aqueous solution of LiCl (3 mmol/ml/kg, i.p.; # L-0505, Sigma Chemical Co., St. Louis, MO) 24 h prior to the pilocarpine (40 mg/ml/kg i.p.; # P-6503, Sigma Chemical Co., St. Louis, MO; dissolved in saline). After pilocarpine injection, motor manifestations of seizure activity were monitored by an experienced observer for 3 h. The appearance of motor seizure manifestation continuous for more than 1 min was considered to be the beginning of SE. Latency to SE onset was also recorded. In order to quantify motor phenomena, a 5-point scale was used for dividing the rats according to the development of the motor pattern:

- (1) Automatism (e.g. scratching, wet dog shakes)
- (2) Head nodding
- (3) Clonic convulsions of forelimbs (with rearing in P25; P12 rat pups are not able to rear)
- (4) Wild running
- (5) Generalized tonic-clonic seizures accompanied with loss of righting reflexes

To calculate the mean seizure severity the rats were scored according to the most severe phenomenon present. Animals reaching only stage 2, i.e. non-convulsive seizures (n=10 and 12 in P12 and P25 groups, respectively), were not used in further behavioral and morphological testing.

In order to decrease mortality of animals, an aqueous solution of paraldehyde (# 76260 Fluka Chemie AG, Buchs, Switzerland) was administered intraperitoneally 120 min (P12) or 90 min (P25) after SE onset in a dose chosen according to the age (0.07 ml/kg in P12 and 0.3 ml/kg in P25 groups due to age-related differences in sensitivity – Kubová, unpublished data). In spite of this treatment, mortality was high during the acute stage of SE (13 and 6 rats in the P12 and P25 groups, respectively).

Control siblings received saline instead of pilocarpine; all other treatments were identical to experimental animals.

During the entire period of separation from their mothers, body temperature of P12 animals was maintained at +34 °C with an electric heating pad connected to a digital thermometer. Approximately 5 h after pilocarpine injection, the pups were injected subcutaneously with 0.9 % NaCl (up to 3 % of the body weight divided into 2-3 doses) to restore volume loss, and then returned to their mothers (the duration of isolation from mothers in the control and SE groups was the same). The pups were weaned at the age of 28 days (Babický *et al.* 1970). The animals with SE at P25 were not able to ingest normal rat chow, therefore they were fed with a paste made of ground rat chow (Bergman; Czech Republic). In addition, they were given water from a pipette. Because of this intensive care during the recovery period they could not be returned to their mothers. All animals were regularly handled and their body weight was checked daily. In order to compare the growth of animals among various treatment groups, relative body weight for each animal was calculated for every day of the experiment (body weight at P12 and/or P25 was taken as 100 %) and used to construct weight curves.

Treated groups

N-tert-butyl- α -phenylnitron (PBN; # B/7263-5G, Sigma) was administered in a total dose of 200 mg/kg i.p. (freshly dissolved in saline, 10 mg/ml) divided into two consecutive doses. Five different treatment groups were used for both ages in this study:

1) *CONTROL (lithium-paraldehyde) groups*: Animals (n=14 for either age group) were treated with LiCl, saline and paraldehyde according to the protocol mentioned above.

2) *PBN-C groups*: In order to test possible effects of PBN on the motor performance in animals without SE, two additional groups (n=12 in both P12 and P25) were added. Animals received two injections of PBN (100 mg/kg i.p. each) separated by an interval of 1 h.

3) *PILO groups*: control rats with SE (14 and 15 animals in P12 and P25 groups, respectively) received saline instead of PBN.

4) *PBN/PILO groups*: PBN pretreated animals (19 and 20 rats in P12 and P25 groups, respectively) received two doses of PBN. The first one 30 min prior to pilocarpine, the second dose 1 min after SE onset.

5) *PILO/PBN groups*: The first dose of PBN was given 1 min after SE onset, the second 60 min later to 19 rats in either age group.

After SE, animals were randomly divided into two groups, one for motor tests and the other for histology.

Motor tests

In order to check the motor performance, animals were examined twice 3 and 6 days after SE. Because of incomparable level of maturation, specific age-related tests (Altman and Sudarshan 1975) were used for the two age groups. The negative geotaxis test, wire mesh ascending test and bar holding test were used in this order in P12 groups. Animals of the P25 group were tested by the bar holding test and then by the rotarod test. Some animals were not able even to start an experiment due to hyperactivity or aggressivity towards the observer. Hence, the percentage of animals able to be tested was also registered. All these tests were made in a room used only for the behavioral tests. Rat pups with their mothers were allowed to adapt to the room for at least 2 h before testing. The rats were tested between 09:00 and 14:00 h.

Negative geotaxis test: Rats were placed on an inclined (30°) surface with their heads facing downward. Time necessary to turn to 90° and to 180° was recorded for a maximum of 90 s.

Wire mesh ascending test: Wired mesh, 45 cm high and 15 cm wide, was placed at an angle of 70° in contact with a platform on the top. To motivate a pup to ascend, its littermates were placed on the top platform. The latency to reach the platform was recorded up to 120 s.

Bar holding test: A wooden bar 25 cm long with a diameter of 1 cm was suspended 40 cm above a padded soft surface. The rat was held so that its forelimbs touched a bar. Successful trials, total time spent on a bar as well as the strategy of the movement (grasping with forelimbs and hind limbs) was recorded for 120 s.

Rotarod test: Animals were placed on the rotating rod with a diameter of 10 cm (speed 10 rpm). Time spent on the rod was measured up to 180 s. Percentage of animals able to pass this test was registered. To calculate the mean time spent on the rod only data from animals suitable for testing were used. In addition, the strategy to solve this task and incidence of other behavioral parameters (rearing and changing direction of running) were also recorded.

Morphological techniques

Six to eight rats from each SE group and four animals from CONTROL and PBN-C groups were used at 24 h or 1 week after SE. The animals were deeply anesthetized with 20 % urethane (2 g/kg i.p.; U-2500, Sigma) and perfused with 0.01 M phosphate buffered saline (PBS), pH 7.4, followed by a fixative solution (4 % paraformaldehyde in PBS). The brains were removed from the skull and postfixed in a fixative solution for 3 h at 4 °C, and then cryoprotected in a solution containing increasing concentration of saccharose (10, 20 and 30 %,

respectively) in 0.1 M potassium-buffered saline (KPBS) for 24 h. Then the brains were frozen in dry ice and stored at -70 °C until cut. The brains were sectioned in the coronal plane (50 µm, 1-in-5 series) with a cryocut Leica CM 1900 and sections were stored in a cryoprotectant tissue-collecting solution (30 % ethylene glycol, 25 % glycerol in 0.05 M sodium phosphate buffer) at -20 °C until processed. Adjacent series of sections were used for cresyl violet staining and Fluoro-Jade B (FJB) staining.

Table 1. Distribution of FJB-positive cells in the hippocampus of rat pups 24 h and seven days after SE in different groups.

	P12/24h			P12/week		
	PILO	PBN/PILO	PILO/PBN	PILO	PBN/PILO	PILO/PBN
SEPTAL PART						
CA 1	±	+	++	0	0	0
CA 3	±	±	++	0	0	0
DG	±	+	±	0	0	0
Hilus	0	±	±	0	0	0
TEMPORAL PART						
CA 1	+	+	++	0	0	0
CA 3	0	+	+	0	0	0
DG	0	+	0	0	0	0
Hilus	0	±	±	0	0	0

	P25/24h			P25/week		
	PILO	PBN/PILO	PILO/PBN	PILO	PBN/PILO	PILO/PBN
SEPTAL PART						
CA 1	+++	+	+	+++	±	±
CA 3	+++	++	+	+++	++	++
DG	0 → ±	0 → ±	±	+	0	0
Hilus	++	++	+	++	++	++
TEMPORAL PART						
CA 1	+++	+++	+++	+++	+++	+++
CA 3	+++	+++	+++	+++	+++	+++
DG	±	±	±	+	0	0
Hilus	++	++	++	++	++	++

Density of FJB-positive neurons was scored as follows: 0 = negative finding, ± = 1–5 FJB-positive neurons, + = 5–10 neurons, ++ = 10–20 neurons, +++ = more than 20 neurons. CA1, CA3 – hippocampal fields, DG – dentate gyrus. For further details see Methods.

Cresyl violet staining (Nissl staining) was mainly used to identify the cytoarchitectonic boundaries of brain structures. FJB staining (Schmued and Hopkins 2000) according to the protocol described by Schmued *et al.* (1997) was used to detect degenerating neurons. The sections were analyzed under Olympus AX 70 fluorescent microscope. For evaluation, the hippocampus was divided into the septal and temporal parts. In the septal part, analysis was started at the level at which the suprapyramidal and infrapyramidal blades of the dentate gyrus become fused together (AP -2.3 in relation to the bregma according to Paxinos and Watson 1998), and continued caudally to AP -3.8 where the septal hippocampus fuses with the more temporal hippocampus. The temporal part was analyzed between levels -4.8 and -5.8 from the bregma. For comparison of results in the hippocampal formation a semiquantitative method was used: FJB-positive neurons were counted in a field from three sections and classified according to the scale in Table 1.

Photography

Photomicrographs were taken with a digital camera Olympus DP 70 connected to microscope Olympus AX 70.

To illustrate the distribution of neuronal damage in different brain areas, Fluoro Jade B-labeled cells were plotted from selected sections (corresponding to the level AP-3.6 according to Paxinos and Watson, 1998) with a computer-aided digitizing system MDPlotTM (AccuStage, Minnesota Datametrics, St. Paul, MN). Anatomic boundaries were drawn from adjacent cresyl violet stained sections using a stereomicroscope equipped with a drawing tube, and then superimposed on scanned plots by using CorrelDraw (version 9).

Statistics

Differences in latencies to SE onset were compared using Mann-Whitney's U-test. Seizure severity (scored according to the most severe phenomenon present) and the incidence of successful trials and strategy differences in motor tests were evaluated by means of the Chi-square test, followed by Fisher's exact test. Relative body weights were analyzed by two-way ANOVA with one grouping factor (experimental groups) and one repeated measures factor (measurement on days after SE). Repeated measures ANOVA followed by the Student-Newman-Keuls method was used for evaluating the latencies in motor tests (SigmaStat, SPSS, Chicago,

IL, USA). The level of significance was set at 5 %.

Results

Status epilepticus and body weight

P12 groups

Majority of animals had clonic seizures of forelimbs (stage 3) as the most severe phenomenon, part of rats exhibited running fits. The incidence of wild running was not different in the three SE groups (20, 17 and 25 % in PILO, PBN/PILO and PILO/PBN groups, respectively). Transition into generalized tonic-clonic seizures was never observed in this age group. Administration of PBN before or after SE onset did not affect the severity of motor manifestations (Fig. 1). A tendency to prolonged latency of SE onset was observed in the PBN/PILO group (1854 ± 190 s in PBN/PILO group vs. 1597 ± 126 s in animals receiving only pilocarpine or PBN after SE onset). There was no difference in mortality among the three groups.

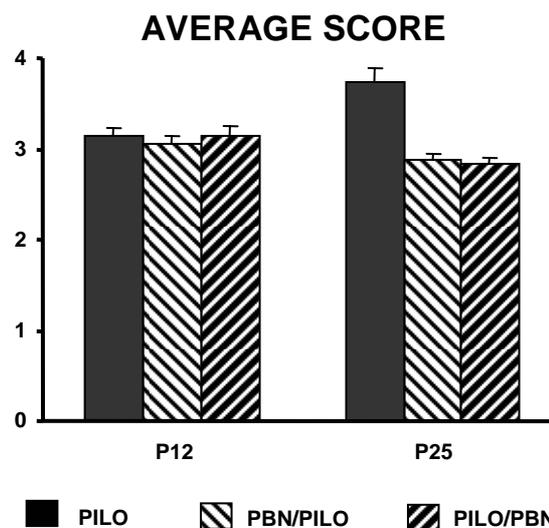


Fig. 1. Severity of status epilepticus expressed as an average score in P12 (left three columns) and P25 (right three columns) rats. Abscissa: the two age groups, individual columns – see explanation under the graph. Ordinate: score (see Methods). # denotes significant difference in comparison with PILO group.

Body weight decreased 24 h after SE onset in all animals experienced SE, whereas controls increased their weight by 7 ± 3 %. Since the second day after SE (P13) there was a daily increase of body weight in the range from 4 to 7 % in all animals. No difference was observed among individual treatment groups and PILO group. Relative body weight of animals exposed to SE (PILO,

PBN/PILO and PILO/PBN) remained, however, significantly lower compared to both CONTROL and PBN-C groups during the whole experiment (Fig. 2). An the increase of body weight was significantly lower in the PBN-C group than in CONTROL animals. Both PBN-treated SE groups exhibited a tendency to increase body weight more rapidly than the PILO group (Fig. 2).

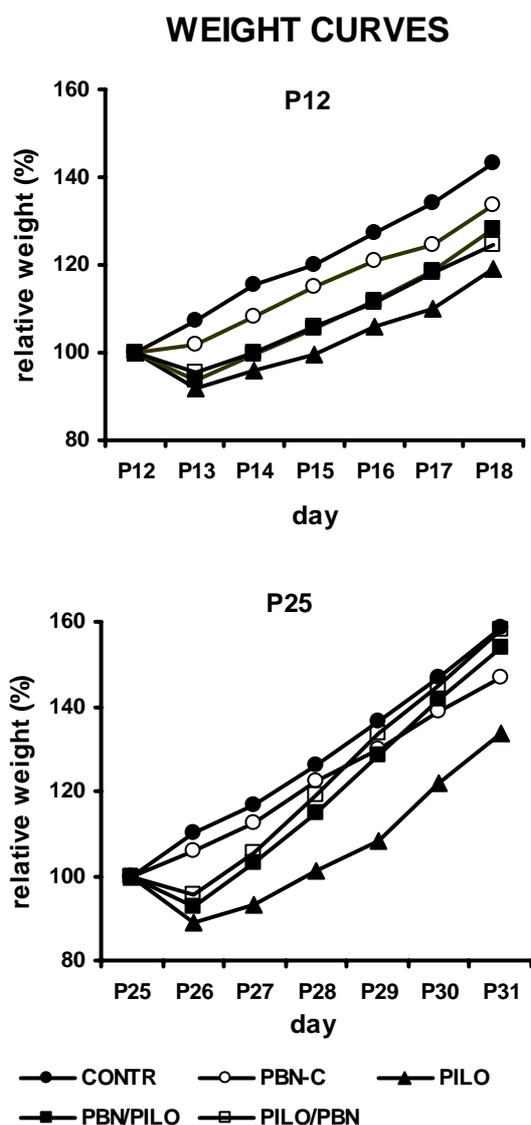


Fig. 2. Relative body weight of animals exposed to status epilepticus at the age of 12 (upper graph) and 25 (lower graph) days. The weight at the day of SE was taken as 100 %. Abscissa: postnatal days; ordinate: relative body weight. Symbols for individual groups are explained at the bottom. Statistically significant difference (not marked in the graphs) in P12 rats was among the three groups exposed to status and the CONTROL and PBN-C groups during all six days; the same was true only for PILO group in P25 rats. Both PBN/PILO and PILO/PBN animals exhibited significantly lower weight in this comparison only at P26, then their body weight was comparable to CONTROL and PBN-C rats.

P25 groups

Convulsive status epilepticus (i.e. at least stage 3) was induced in all rats in the PILO group. In addition, wild running represented the most severe phenomenon in 23 % of rats; generalized tonic-clonic seizures were observed in 26 % of animals. The two PBN groups had a significantly less severe SE because 15 % and 19 % of rats in the PBN/PILO and PILO/PBN groups, respectively, exhibited only head nodding (stage 2) and no generalized seizures (Fig. 1). Pretreatment with PBN significantly increased the latency of SE (2051 ± 229 s vs. 1164 ± 73 s in PBN/PILO and PILO groups, respectively). The tendency to a lower mortality in both PBN-treated groups did not reach the level of significance.

Body weight of control animals increased by 10 ± 1 % during the first 24 h, the increase in PBN-C group was 6 ± 1 %. In contrast, all animals exposed to SE lost weight during this time period. Rats in the PILO group remained significantly lighter than rats in all other groups. Animals of both PBN/PILO and PILO/PBN groups, however, gained weight more quickly during the following days and therefore their weights were comparable with controls (Fig. 2).

Motor performance

P12 animals

Animals of all five experimental groups were able to pass all tests within the time limit in both sessions. No difference was observed between the PBN/PILO and PILO/PBN group in any motor test used in the present study. In the negative geotaxis test, animals of PBN-C group needed a significantly shorter time to turn to 90° (3.6 ± 0.3 s vs. 5.9 ± 0.7 s) as well as to 180° (5.3 ± 0.2 s vs. 8.3 ± 0.8 s) compared to the CONTROL group in the first session. No difference in latencies occurred between the CONTROL group and SE animals, i.e. PILO, PBN/PILO and PILO/PBN group. A significant decrease of latency between the 1st and 2nd sessions was observed in the CONTROL group as well as in both PBN-treated groups, but not in PILO and PBN-C groups. In the wire mesh test, PBN-C rats exhibited significantly a longer latency to reach the platform than CONTROL rats in the first session (51.8 ± 5.6 s vs. 29.3 ± 4.9 s). This difference was no longer present three days later. No difference in the time necessary to reach the elevated platform was observed between CONTROL rats and SE animals. In the bar holding test, no difference in latency was found between CONTROL and PBN-C rats at any interval. Animals of the PILO group spent significantly longer

P25 animals

Animals of both CONTROL and PBN-C groups were able to pass all tests during the chosen time limit in both sessions. Rats in PBN-C group exhibited a normal reaction to manipulation in both sessions. In contrast, rats with SE were hypersensitive to manipulation and difficult to handle. No difference occurred between PBN/PILO and PILO/PBN group in any motor test used in the present study. The number of animals able to pass the bar holding test in the 1st session was significantly lower in the three groups with SE than in the CONTROL and PBN-C groups. This difference was no longer observed 6 days after SE. The PBN administration schedules did not change the time spent on the horizontal bar (Fig. 3). Significantly less animals of PILO group were able to pass the rotarod test 3 days after SE compared to the controls (5 of 9 vs. 14 of 14). No such difference was found between the controls and either PBN group (10 of 12 in PBN/PILO group and 10 of 11 in PILO/PBN group were suitable for testing). In both sessions, animals of the PILO group tended to spend a shorter time on the rod than the CONTROL or PBN-C animals, but this difference did not reach the level of significance (Fig. 3).

Histology*P12 animals**Twenty-four hours after SE*

There were no FJB-positive cells in control animals (CONTROL and PBN-C groups) and those sacrificed 24 h later. Single cells or a small number of FJB-positive neurons were found in PILO rats in all studied structures, but there was a moderate number of positive neurons in the medial amygdala nucleus. As far as hippocampal formation is concerned (Fig. 4), isolated positive cells (with characteristics of interneurons) were found in CA1 and CA3 of the septal part and in the granular layer of the dentate gyrus (DG). The temporal part of the hippocampus exhibited only a small number of FJB-positive neurons in CA1. The same results were found in the PBN/PILO group with the exception of CA1 field and granular layer of the DG in the septal part and CA3 and DG of the temporal part where the number of positive neurons was higher than in PILO rats. Similarly, the PILO/PBN group exhibited differences only in the hippocampal formation – a marked increase of the number of FJB-positive neurons in CA1 and CA3 fields and isolated positive cells in the hilus of both septal and temporal parts (Table 1)

One week after SE

There were practically no positive cells in PILO animals. The same was true for both PBN/PILO and PILO/PBN groups (Table 1)

*P25 animals**Twenty-four hours after SE*

Again, PBN-C group did not exhibit positive cells. A small to moderate number of FJB-positive neurons was found in nearly all brain structures of rats from the PILO group. A high number of positive cells was present in the septum and amygdalar nuclei. The medial half of CA1 in the septal part of hippocampal formation exhibited a small number of degenerating neurons in stratum oriens and pyramidale (Fig. 4). These cells with small round bodies corresponded by their morphology and localization to interneurons. A continuous line of degenerating pyramidal cells together with degenerating interneurons was observed in the lateral half of the septal CA1. A similar finding was observed in CA3 whereas nearly no positive cells were found in DG and only a moderate number of them in the hilus. The temporal part of the hippocampal formation exhibited plenty of FJB-positive neurons (mostly pyramidal cells) in CA1 as well as CA3. A large number of positive cells was also present in the hilus, whereas DG was nearly without degenerating neurons.

Majority of structures in both PBN groups exhibited the same results as in animals from the PILO group. The differences were found in the septal (but not temporal) part of hippocampal formation (Fig. 4). There was a marked decrease in the number of FJB-positive cells in CA1 field of both PBN groups; the remaining FJB-positive cells were interneurons. In addition, a clear protective action in CA3 and hilus appeared in the PILO/PBN group (Table 1)

One week after SE

PILO rats exhibited a small to moderate number of FJB-positive neurons in the same brain structures as 24 h after SE. The exceptions were formed by cortical nuclei of the amygdala with plenty of degenerating and disintegrated neurons. Septal and temporal parts of the hippocampal formation exhibited identical changes: a high number of positive cells with a predominance of pyramidal neurons in both CA1 and CA3 fields, a moderate number in the hilus and a small number of degenerating neurons in the granular layer of the upper blade of DG.

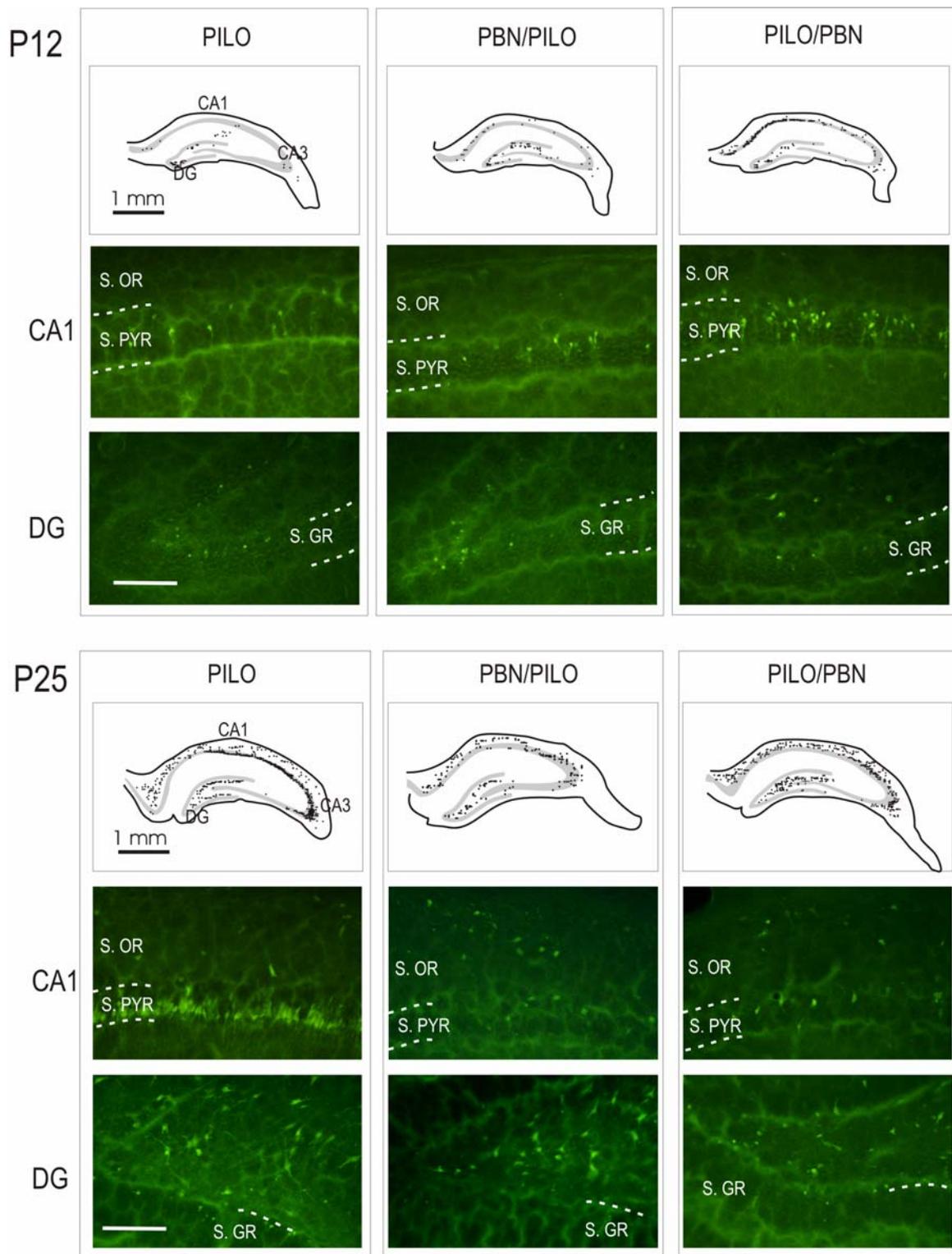


Fig. 4. Distribution of Fluoro-Jade B (FJB)-labeled neurons in the hippocampus 24 h after SE. The upper panel demonstrates pattern of neurodegeneration in P12 rats. In the first row, computer-generated plots show the distribution and density of FJB-positive cells in the hippocampus. From left to right: PILO, PBN/PILO and PILO/PBN animal. Each dot corresponds to one labeled cell. High-magnification microphotographs in the second (CA1) and third (DG) rows show detailed pictures of degenerating neurons. Only scattered degenerating neurons were observed in the pyramidal layer (S. PYR) in P12 animals treated only with paraldehyde in a dose of 0.07 ml/kg (PILO group). Note markedly increased number of labeled neurons in the CA1 field in both stratum pyramidale and stratum oriens (S. OR) in both PBN-treated groups (PBN/PILO and PILO/PBN). Note also the presence of FJB-labeled neurons in the infragranular region of the dentate gyrus (DG) in all groups exposed to SE. In contrast, FJB-positive neurons occurred in the hilus in both PBN/PILO and PILO/PBN groups, but not in PILO group. Panel at the bottom illustrates distribution of FJB-labeled neurons in the hippocampus of P25 rats. Note a decreased number of degenerating neurons in the CA1 field in septal part of hippocampal formation in stratum oriens and pyramidale in both PBN-treated groups (PBN/PILO and PILO/PBN) compared to PILO group treated only with paraldehyde (0.3 ml/kg). Clear protection of the hilar neurons appeared in the PILO/PBN group. Details as in upper panel. Scale bars equal to 1 mm in plots and 100 μm in microphotographs (rows CA1 and DG)

Both PBN/PILO and PILO/PBN rats differed from PILO animals by a less severe degeneration in the septal part of the hippocampal formation. There were only isolated positive interneurons in the stratum oriens of CA1 and a moderate number of degenerating cells (both interneurons and pyramidal cells) in CA3. DG did not contain FJB-positive cells; the finding in the hilus was the same as in PILO animals. The temporal part exhibited a single difference: neurons in the DG were protected; other structures did not differ from PILO rats. No differences were found between the two groups treated with PBN (Table 1)

Discussion

The present study demonstrates data concerning the effect of free radical scavenger PBN on SE induced in immature rats with lithium-pilocarpine. Pilocarpine as well as lithium/pilocarpine-induced status epilepticus in immature rats was already described in previous studies (Cavalheiro *et al.* 1987, Hirsch *et al.* 1992, Sankar *et al.* 1998, Kubová *et al.* 2000, Druga *et al.* 2003). Our experiments were performed in two age groups of developing animals, 12-day-old rats (P12) – which is the age that corresponds to human newborns and 25-day-old rats (P25) – corresponding to early school age in humans. Our results confirmed that the severity of SE (expressed as a score) is significantly lower in P12 than in P25 group.

Free radical detection, particularly *in vivo*, is difficult due to their short half-life and low concentrations. Therefore, studies have attempted to demonstrate evidence of free radical damage by using free radical scavengers. We chose PBN for our experiments because its neuroprotective effect has already been proven in various pathological models (Carney and Floyd 1991, Hall 1997, Hensley *et al.* 1997, Thomas 1997). PBN was also selected because of its high blood brain barrier penetration (Chen *et al.* 1990). The protective effect of PBN, concerning both amelioration of brain damage and improvement of energy status, was found in flurothyl-induced SE in adult rats (He *et al.* 1997, Folbergrová *et al.* 1999). It was thus of interest to examine whether its protective effects could also be observed when SE is induced in immature rats. Doses of PBN (twice 100 mg/kg) and time schedule for i.p. application, i.e. one dose before and the other after the beginning of SE, were used according to the previous study in adult rats with SE (Folbergrová *et al.* 1999). In

addition, we have also used the second schedule under which both doses of PBN were applied after SE, starting shortly after its onset. However, as far as the present results are concerned, no significant difference could be observed using the two mentioned schedules.

The results of the present study showed that the severity of seizures in P12 group was not affected. There was a tendency to normalize body weight after PBN treatments in the P12 groups, as compared to PILO group. However, no effect of PBN was found in motor tests. Contrary to P12, the effect of PBN in P25 rats was evident in the longer latency to SE and in lower severity of seizures. There was also significant improvement in the weight curves. Therefore, we expected the same protective influence of PBN in behavioral tests. However, the time spent on the bar or on the rod was not prolonged by PBN treatment. Only mild amelioration was evident in the ability to pass the rotarod test (in the second session, all animals from both PBN groups were able to pass this test). This is the first study of PBN effect on motor skills of animals. It is thus not clear whether the lack of protection may be due to the age of animals or whether this type of deficit is resistant to free radical scavenger treatment.

A decrease in neuronal degeneration was found in PBN-treated P25 rats in agreement with the lower intensity of seizures. The PILO group exhibited the same extensive damage as described in previous papers (Sankar *et al.* 1998, Kubová *et al.* 2001). Both our schedules of PBN treatment obviously protected the septal part of the hippocampus; especially pyramidal neurons (positive in CA1 as well as CA3 fields of PILO rats) did not exhibit FJB positivity. No similar changes were found in the temporal part of the hippocampal formation and in other studied structures. The difference between the septal and temporal part may be at least partly due to their different inputs. Afferent fibers from the amygdala, a structure involved in SE, terminate preferentially in the temporal part (Krettek and Price 1977). It thus has more intense synaptic activation that is difficult to be overcome. Protection against morphological consequences of SE specific for only some brain structures was described in several studies, e.g. in adult rats treated with topiramate (Rigoulot *et al.* 2004). The incomplete protection in the present study may suggest that only a part of neuronal degeneration is due to free radical action and that apparently other mechanisms may also be involved. The protective action in P25 rats is in agreement with the data from adult rats. PBN (or S-PBN) substantially

ameliorated lesions of substantia nigra pars reticulata after flurothyl-induced SE (He *et al.* 1997) and excitotoxic striatal lesions elicited by malonate (Schulz *et al.* 1995) or glutamate (Lancelot *et al.* 1997). Our study is the first to report effects of free radical scavenger on SE-induced neuronal damage in immature rats. Due to a lack of literary data the unexpected results in P12 rats – aggravation of damage in some structures in spite of unchanged severity of convulsions – cannot be explained at present.

Our findings indicate that the PBN effect is age-dependent. It is well established that PBN is capable of scavenging many types of free radicals. In addition, the literary data suggest that the mechanism of action may be more complex than just simple free radical scavenging. Thus, many effects of PBN (and of other nitrones) have been described, such as reversible Ca²⁺ channel blockade (Anderson *et al.* 1993), effect on acetylcholinesterase (Milivojevic *et al.* 2001), on inducible nitric oxide synthase, or on immediate early gene induction (Hensley *et al.* 1997), to mention at least some of them. It is likely that many of the mentioned processes are undergoing developmental changes. However, which of them may be responsible for the age-dependent effect of PBN observed in our study remains unknown at present.

It is worth to mention some other data indicating that PBN administration in immature rats with SE can

afford protection as revealed in adulthood. Our preliminary results suggest that PBN treatment of immature rats with lithium-pilocarpine SE could positively influence a cognitive deficit observed in adulthood (Rejchrtová *et al.* – unpublished results). Recent studies of Marklund *et al.* (2001) and Lewén *et al.* (2001) have demonstrated a protective effect of PBN against cognitive deficits observed after traumatic brain injury.

The severity of seizures in the present study was evaluated only from the behavioral manifestations of seizures. It will thus be of interest to characterize the pattern also by EEG recordings. These studies, as well as those on the chronic effect of PBN, are in progress.

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