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Morphological alterations of neurons and astrocytes and changes in emotional behavior in pentylenetetrazol-kindled rats

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Abstract

Changes of emotional behavior and neuronal cell loss in the hippocampus were investigated after pentylenetetrazol (PTZ) induced kindling in rats. Behavioral and morphological changes were studied in partially and fully kindled rats and after different postkindling periods comparing to the controls. The resident–intruder test indicated a diminished offensive behavior in partially and fully kindled animals. The open-field and the cat-odor exposition tests reveal changes in defensive behavioral pattern only in fully kindled rats. A decrease of exploratory locomotion and an increase in freezing were assessed in the open-field and the cat-odor exposition test, respectively, up to 10 weeks after the end of kindling. The first damaged neurons (CA4 region) were observed in the partially kindled group (PK), correlating with an increase in the glial fibrillary acidic protein (GFAP)-immunoreactivity (GFAP-IR) and hypertrophy of astrocytes. The most significant increase in the number of damaged neurons was detected 24 h after completion of kindling (selective vulnerability: CA4/CA1 > DG>CA2 + CA3). The neuronal loss went on for 10 weeks postkindling. A low correlation between the number of Stage 4 kindling seizures and the number of damaged hippocampal neurons was found 24 h after the end of kindling in individual rats. The present results demonstrate that PTZ kindling goes along with long-lasting changes in emotional behavior. The alterations of the defensive behavior after the termination of kindling can be interpreted as depression-like and are obviously associated with a characteristic pattern of neuronal loss in various hippocampal regions.

Keywords: PTZ; Kindling; Morphology; Rat; Emotional behavior; Neuronal cell loss; Hippocampus

1. Introduction

Seizure disorders can be associated with a complex mixture of psychopathology. Changes in mood with predominant depression, episodic irritability, schizophrenia-like episodes and anxiety are the prominent symptoms of the neurobehavioral disorder of epilepsy (Blumer, 1991). Thereby depression and anxiety are the most observed impairments in epileptic patients (Betts et al., 1976).

After stress which can cause depression, or after recurrent major depression, atrophy and, in severe cases, death of hippocampal neurons have been observed (for review, see Vaidya et al., 1999). There is evidence for a relationship between epileptogenic lesions in the limbic system, especially in the mesial temporal lobe, and the appearance of interictal behavioral disturbances (Babb and Brown, 1987).

The animal kindling models offer the opportunity to investigate postictal behavioral changes associated with epilepsy. Kindling is characterized by repeated administration of an initially subconvulsive electrical or chemical stimulus resulting in progressive intensification of seizure activity, culminating in generalized seizures (Goddard et al., 1969). Furthermore, it has been suggested that aspects of kindling and stimulant-induced behavioral sensitization indicate similarities with the course of recurrent affective illness (Post, 1992). Neuronal loss, which shows a remarkable parallel to the spectrum of pathological changes in human epilepsy, was described after kindling in rats (Cavazos et al., 1994).

Whereas the electrical kindling is regarded as a model of complex partial epilepsy (McNamara, 1984), the chemical kindling induced by pentylenetetrazol (PTZ) is a

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model of primary generalized epilepsy (Ono et al., 1990; Rossi, 1996). It was shown that cognitive disorders (Becker et al., 1994a) and neuronal loss in the hippocampus (Pohle et al., 1997) appear after PTZ kindling. But there exist only few and moreover inconsistent data about changes of the emotional behavior after PTZ kindling in rats (Kittner and Franke, 1996; Becker et al., 1994b). In order to evaluate the influence of different PTZ-induced kindling stages and postkindling periods, possible changes of emotional behavior were studied in the resident–intruder, the open-field and the cat-odor exposition tests. The selected tests offered the opportunity to evaluate offensive as well as defensive patterns of behavior, which may provide an analogue to human emotion and psychiatric disorders (Blanchard and Blanchard, 1988; Dixon, 1998). Following the behavioral tests, morphological alterations in the hippocampus of the same animals were studied. Neuronal changes were investigated using celestine blue/acid fuchsin staining procedure. In addition, the astrocytic reaction was studied using antibodies against the glial fibrillary acidic protein (GFAP), the major protein component of intermediate filaments in fibrous astrocytes. Furthermore, the correlation between different numbers of Stage 4 kindling seizures, size of hippocampal neuronal loss and behavioral changes in individual rats were made 24 h after the end of kindling.

2. Methods

2.1. Material

PTZ (Knoll, Ludwigshafen) used for the treatment was dissolved in 0.9% saline solution and administered intraperitoneally. All other chemicals were of analytical grade, obtained from commercial sources and used without further purification.

2.2. Animals

Male Wistar rats (Wist/Prob, 250 g) were housed in stainless steel cages with beddings of wood shavings in groups of five animals. Environmental control parameters were as follows: temperature 20 ± 2 °C, relative humidity 50–70%, and natural light–dark cycle. Lab chow (Altromin, Lage/Lippe, Germany) and water were available ad libitum.

2.3. Kindling preparation

All of the animal use procedures were approved by the committee on animal care and use of the relevant local governmental body in accordance with the law of experimental animal protection. The age of the animals at the beginning of the experiments was 8 weeks. The rats received a dose of 35 mg/kg/body weight PTZ or 1 ml physiological saline/kg/body weight intraperitoneally three times a week for a period of 5 weeks, followed by an application-free interval.

After each injection, the convulsive behavior was observed for 30 min and classified in the following stages, modified to that described by Smialowsky (1980): 0—no behavioral changes; 1—facial movements, ear twitching and tail raising; 2—myoclonic jerks of the whole body; 3—clonic convulsions with rearing; 4—clonic convulsion with falling down and loss of body control. For a more detailed evaluation of the behavior, the additional Stages 1–2, 2–3 and 3–4 were included.

The rats were killed depending on the number of PTZ injections and the period after the last PTZ stimulation: 24 h after the 6th PTZ application (partially kindled group, PK); 24 h after the 15th PTZ application (fully kindled group 1, FK1); 2 weeks postkindling (fully kindled group 2, FK2); 5 weeks postkindling (fully kindled group 3, FK3); and 10 weeks postkindling (fully kindled group 4, FK4). For further behavioral and histological analysis, after termination of kindling, only animals with at least three Stage 4 seizures in the last 2 weeks of the kindling procedure were used. The mean seizure score of the tested animals was 3.5 ± 0.3. The rats were randomly divided into six animals per group.

In order to study the relationship between different numbers of Stage 4 seizures, the number of damaged hippocampal neurons and the related behavioral changes in individual rats, animals of the FK1 group with a different expression of kindling seizures were divided according to the number of Stage 4 seizures in the following groups: controls (CON, $n = 6$), 0–2 ($n = 6$), 3–5 ($n = 9$), 6–7 ($n = 6$).

2.4. Behavioral analysis

Each animal was used only once in all three behavioral experiments (in the following order: resident–intruder test, open-field test, cat-odor exposition test) with 3 h test-free interval between the experiments. For the correlation analysis, the elevated plus-maze test was added a day later for further validation of the open-field data. The experiments were done between 8:00 a.m. and 6:00 p.m.. The behavior was recorded and later evaluated using a video activity measurement system (TSE, Bad Homburg, Germany).

2.4.1. Resident–intruder test

Changes in offensive behavior were assessed in a home cage intruder test. This test based on the social interactions between rats which are defending their home cage against unfamiliar intruding conspecifics.

The experiment was performed after a 24-h period of single housing of the resident rats in standard laboratory cages with food and water ad libitum. After placing of the intruder into the home cage of the resident rat, the behavior was recorded for 10 min. The pairs were chosen on the basis

of weight (±5 g). According to other studies (Mitchell and Fletcher, 1993; Haller et al., 1998), aggressive grooming, lateral threat, standing on-top-of, keep down, chase and biting were summed up as offensive behaviors.

2.4.2. Open-field test

The open-field test is based on the conflict between exploration of a new environment and the aversion to open spaces from which escape is prevented by a surrounding wall. The stimulus of the novel environment might be seen as simultaneously induced anxiety and exploratory behavior. It has been shown that novelty is a critical factor for the expression of kindling-induced emotionality (Kalynchuk et al., 1998a).

A square, open-field cage with a side length of 1 m surrounded by a 0.4-m-high wooden wall was used. The dark floor of the apparatus was cleaned thoroughly between the tests. The locomotor activity, the numbers of rears and the time spent in the inner area of the open field (exploratory locomotion) were recorded for 5 min.

2.4.3. Cat-odor exposition test

The results of a number of previous studies have shown that exposure to cat-odor generates a state of anxiety in rodents (Blanchard and Blanchard, 1988; Blanchard et al., 1990; File et al., 1993; Kavaliers et al., 1994). Laboratory rats without previous exposure to a cat or to its odor expressed behavioral avoidance (freezing), which is characterized by immobility and scanning movements of the head and vibrissae. When escape is impossible, freezing is the only effective method of avoiding risk.

Furthermore, it was shown that it is possible to detect corticosterone response to cat odor (File et al., 1993). Other studies have demonstrated that laboratory rats have a hereditary behavioral and stress response to predator odor such as that of a fox or weasel (Cattarelli and Chanel, 1979; Heale et al., 1994; Perrot-Sinal et al., 1999). As distinct from the elevated plus-maze test, the cat-odor exposition test is suggested as a model of more phobic anxiety because the test is relatively insensitive to benzodiazepines (Zangrossi and File, 1992; for review, see File, 1995). It was also demonstrated that a lack of between-session habituation of phobic responses to cat-odor exists (Zangrossi and File, 1994).

In accordance to Hogg and File (1993), the cat-odor was obtained by rubbing a damp cloth on a domestic cat (female, 5 years of age) for 5 min one hour before the experiment. The cat-odor cloth was kept in a sealed plastic bag. Damp pieces from the same cloth were used as a neutral odor. Immediately before starting the experiment, the cloth was divided into single pieces (6 × 6 cm) with equal size using a pair of scissors and rubber gloves. Each cloth was used for one exposure only. The rats were exposed to cat-odor cloths in a separate dimly lit room in their home cages. The behavior of the rat was observed for 10 min to analyze the duration of freezing and exploration behavior (risk assessment).

2.4.4. Elevated plus maze

The elevated plus maze is a widely used and extensive validated animal model of anxiety based on the natural aversion of rodents for open spaces and on the elevation of the maze (Handley and Mithani, 1984; Pellow et al., 1985; File, 1995).

The apparatus was made of wood with black rubber flow. It consisted of two open arms (50 × 10 cm) and two enclosed arms (50 × 10 cm) with walls 40-cm high, elevated 50 cm above the ground. The arms of the same type were opposite to each other, connected by an open central area (10 × 10 cm). A camera was mounted vertically above the maze, and the behavior was scored from a monitor in an adjacent room. At the beginning of the experiment, rats were placed in the center of the maze, facing on the enclosed arms and observed for 10 min. An increase in the percentage of time spent on the open arms (open × 100/open + enclosed) and in the percentage of open arms entries (open × 100/total entries) is interpreted as an anxiolytic response, whereas the number of entries into enclosed arms provide a measure of general activity.

2.5. Tissue fixation

At the end of the experiments, the rats were anaesthetized with thiopental-natrium (Trapanal) and perfused transcardially with solution 1 (2% paraformaldehyde in 0.1 M sodium acetate buffer, pH 6.5) and solution 2 (2% paraformaldehyde/0.1% glutaraldehyde in 0.1 M sodium borate puffer, pH 8.5). Following perfusion, the brains were dissected out and postfixed at 4 °C in solution 2 (without glutaraldehyde).

2.6. Histological staining

After the postfixation, the brains were prepared for embedding in paraffin. Microtome coronal serial sections (Jung, Heidelberg, Germany) of 8 μm throughout different brain regions were cut and stained with celestine blue (Sigma, Deisenhoven, Germany)/acidic fuchsin (Fluka Chemie, Buchs, Switzerland) according to standard procedures. The stained sections were dehydrated in a series of graded ethanol, processed through *n*-butylacetate and embedded in entellan (Merck, Darmstadt, Germany).

The brains were analyzed qualitatively and quantitatively. Qualitatively in the nucleus accumbens (bregma + 1.70 mm), hippocampal formation (level 1, bregma − 2.00 mm), amygdala (bregma − 2.00 mm), piriform area (bregma + 1.70 mm, − 2.00 mm, − 3.70 mm), red nucleus (bregma − 6.06 mm), substantia nigra (bregma − 6.06 mm). Quantitatively in the dorsal hippocampal formation (level 4, bregma − 3.70 mm) (for stereological data, see Swanson, 1992).

The quantitative analysis method was performed as previously described (Franke et al., 1997). The neurons were characterized by different staining qualities. Intact neurons were equivalent to clearly defined blue-stained round neurons. A sign of irreversible nerve cell injury is acidophilia — visible in densely red or red-violet staining of the cytoplasm (Smith et al., 1988).

The cell counts in the celestine blue/acid fuchsin-stained sections were performed within the dorsal hippocampus per section, by direct visual counting (magnification $\times 450$) throughout the entire striatum pyramidale and stratum granulosum in equivalent hippocampal levels, using a laboratory cell counter (UZG1, Medical Academy, Magdeburg). Neurons of the CA4 region in this study were defined as those pyramidal cells located strictly within the hilus of dentate gyrus (hilus of DG).

2.7. Immunocytochemistry

The GFAP-staining procedure was performed as previously described (Franke, 1995).

Tissue sections (50 μm) of the dorsal hippocampus in the frontal plane (level 4) were cut on a vibratome (TSE) and collected as free-floating slices in 0.1 M Tris (pH 7.6). The sections were incubated with polyclonal rabbit anti-cow GFAP (1:600, Dako, Glostrup, Denmark), biotinylated protein A (1:400, Calbiochem, La Jolla, CA, USA) and streptavidin/avidin complex (1:1000, StreptABComplex/HRP, Dako). 3,3′-Diaminobenzidin (DAB, Sigma) was used as chromogen. The stained sections were dehydrated in a series of graded ethanol, processed through *n*-butylacetate, and embedded in entellan.

2.8. Statistics

The behavioral data of the various partially and fully kindled groups were analyzed using the Mann–Whitney test. The behavioral data in relation to different numbers of Stage 4 seizures were statistically treated by one-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls test for multiple comparisons.

The sum of neurons for each animal, both the right and left hemispheres, was used for the calculation of means ± S.E.M. Group means for each subdivision were compared with the equivalent region in the controls and the changes observed in the PTZ-treated animals were expressed in terms of percentage.

For determination of differences between the controls and the different kindling groups, the Mann–Whitney test was used. Comparison among the partially kindled and the fully kindled groups at different timepoints after completion of kindling were carried out with the one-way ANOVA followed by the Bonferroni *t*-test. The relation between the number of Stage 4 seizures and the number of damaged neurons in the hippocampus was determined using Pearson's correlation coefficient.

3. Results

3.1. Behavioral analysis of partially and fully kindled animals

The behavior was assessed after 2 weeks (PK group), 24 h (FK1 group) and 10 weeks (FK 4 group) after the end of kindling in the resident–intruder, the open-field and the cat-odor exposition test. For the behavioral analysis of the fully kindled rats (FK1 and FK4 group), only animals with at least three Stage 4 seizures in the last 2 weeks of the kindling procedure were used.

3.1.1. Resident–intruder test

The resident–intruder test indicated significant changes in offensive behavior already after 2 weeks of kindling (PK group). Only two animals without at least Stage 2 seizures showed no inhibition of offensive behavior. The rats tested after the end of the kindling injections (FK1 and FK4 groups) exhibited also a significant diminished fighting behavior. The number of offensive interactions was reduced by about 60% (Fig. 1).

3.1.2. Open-field test

The test of the open-field behavior of the PK group revealed no significant changes. However, the time spent in the inner areas of the open field (Fig. 2) and the number of rears tended to be increased. The open-field behavior of the FK1 and FK4 groups was characterized by a significant decrease of exploratory locomotion expressed as the time spent in the inner areas of the open field by about 50–30%

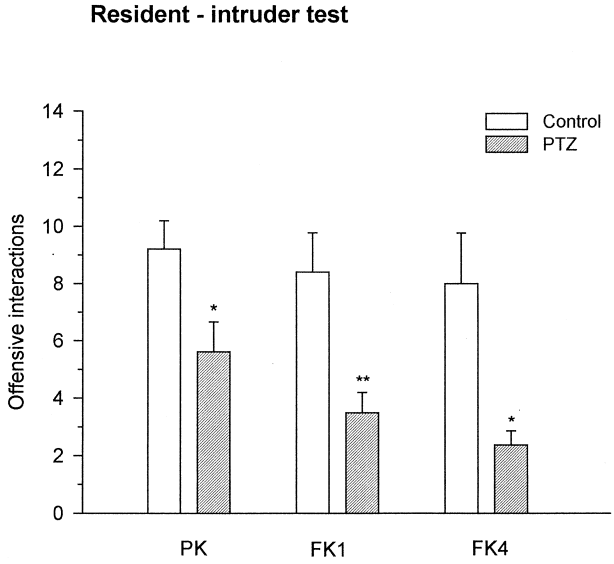

Fig. 1. Effects of PTZ kindling on the offensive behavior assessed in a resident–intruder test in the rat. The number of offensive interactions (means ± S.E.M.) was recorded in partially kindled rats (PK group) after 2 weeks kindling procedure and in fully kindled rats 24 h (FK1 group) and 10 weeks (FK4 group) after the end of the kindling procedure ($n = 6$; per group). * $P < .05$ (Mann–Whitney test).

(Fig. 2). There were no significant changes of the horizontal locomotion and the number of rears (data not shown).

3.1.3. Cat-odor exposition test

In the cat-odor exposition test, the time of freezing as a parameter for anxiety tended to decrease after 2 weeks of kindling procedure (PK group). However, after the completion of kindling both groups (FK1 and FK4) showed an increase in freezing, about 55–75%, indicating enhanced level of anxiety (Fig. 3). The exploration behavior was inversely changed (data not shown).

3.2. Histology

3.2.1. Quantitative study

Criteria for cellular abnormality included the appearance of clearly visible red- or red-violet-stained cytoplasm of neurons (in Figs. 4 and 5 as dark-stained cells, examples indicated by thick arrows) in contrast to clearly defined blue-stained intact neurons (in Figs. 4 and 5 as slight-stained, round cells, examples indicated by thin arrows). The observed neuronal alterations reflect the temporal evolution of most neurons after injury. Condensed, dark blue-stained neurons ("basophilic," reversible process) or stained dark-purple neurons ("pre-acidophilic"), developed later into bright red-stained neurons ("acidophilic") followed by nerve cell decline (Smith et al., 1988; Auer et al., 1984). Other observed morphological alterations were dark blue-stained cells with triangular shape, microvacuolation or shrinkage of the cell body (Fig. 4). Aberrant staining of the nucleus (e.g., dark blue) will be considered as potentially reversible and were not taken into account in the described counting method.

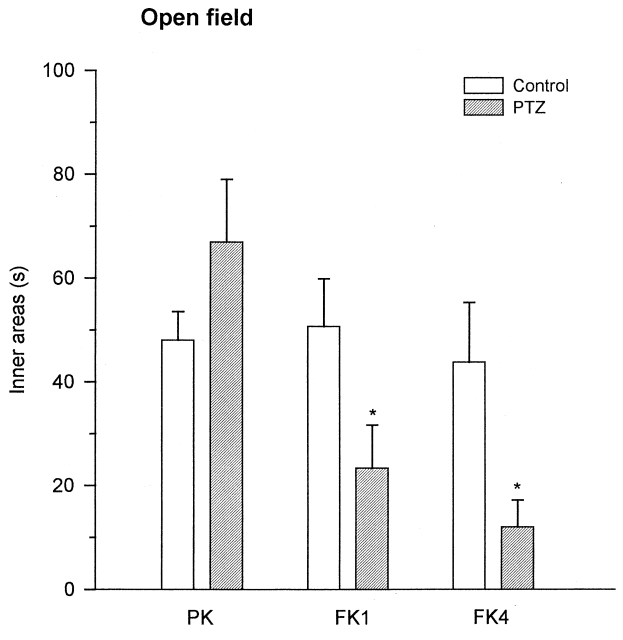

Fig. 2. Effects of PTZ kindling on the exploratory locomotion in the open field expressed as the time the rats spent in the inner areas of the open field. The open-field behavior was assessed (means ± S.E.M.) in partially kindled rats (PK group) after 2 weeks kindling procedure and in fully kindled rats 24 h (FK1 group) and 10 weeks (FK4 group) after the end of the kindling procedure ($n = 6$; per group). * $P < .05$ (Mann–Whitney test).

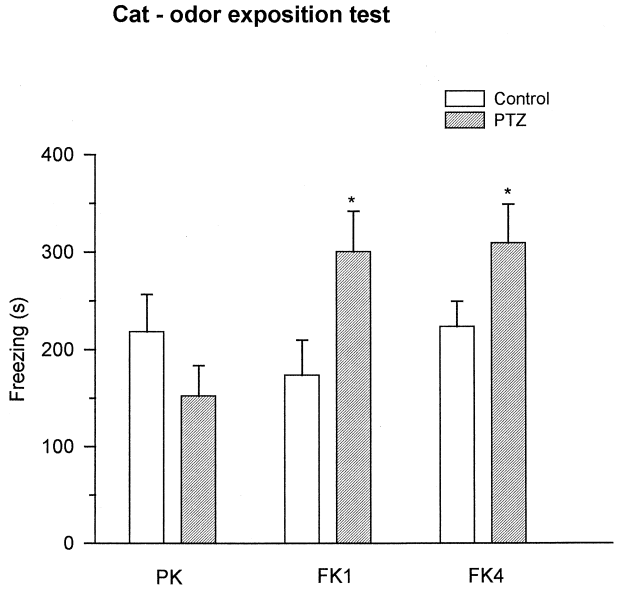

Fig. 3. Effects of PTZ kindling on the defensive behavior assessed in a cat-odor exposition test in the rat. The time of freezing (means ± S.E.M.) was recorded in partially kindled rats (PK group) after 2 weeks kindling procedure and in fully kindled rats 24 h (FK1 group) and 10 weeks (FK4 group) after the end of the kindling procedure ($n = 6$; per group). * $P < .05$ (Mann–Whitney test).

Neurons with altered morphology and signs of degeneration were present in the whole hippocampal formation. Morphological changes occurred at different degrees, depending on kindling state and time after completion of kindling. The total number of neurons per slice was gradually reduced in all regions with time, when compared to the controls (Fig. 6A). Only in the 2 weeks group (PK group) did the number of neurons not differ strongly from the controls in the investigated regions. Dark blue-stained neurons (hyperchromatic) were observed, which may indicate early stages of degeneration. First signs of irreversibly damaged cells (red staining) were found. At the end of the kindling procedure (FK1 group), the total number of neurons per slice decreased in all regions (CA4/CA1 > DG > CA2 + CA3). Simultaneously, the highest number of visible damaged neurons (e.g., ~17% of the control value in the CA4 region and in the granule cell layer of the DG) was found (Fig. 6A,B). A high number of dark blue-stained neurons in the CA1, CA4 regions and the DG are noticeable.

After 2 weeks postkindling (FK2 group), the kindling-associated high number of damaged neurons resulted in a decreased total number of neurons. The number of red-stained cells was reduced in all regions.

Prolonging the survival time of the fully kindled rats to 5 or 10 weeks (FK3 and FK4 groups), the neuronal

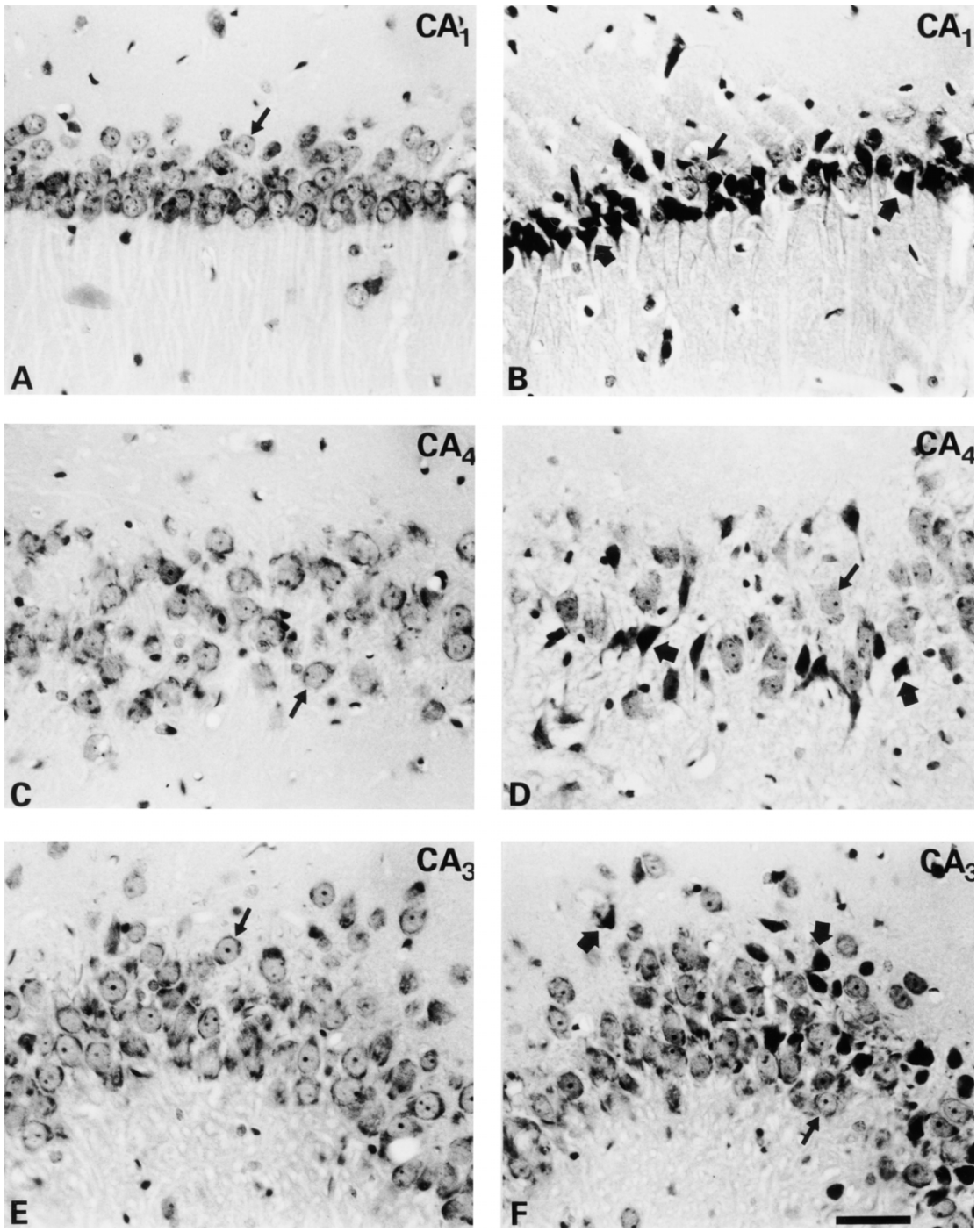

Fig. 4. Photomicrographs of celestine blue/acid fuchsin-stained 8-μm coronal sections from the dorsal hippocampal formation of rats (−3.70 mm to bregma) after repeated PTZ applications (B,D,F) in comparison to control animals (A,C,E). Photomicrographs illustrate examples of the CA1 pyramidal cell layer (A,B) after 15 PTZ applications (FK1 group), the hilus fascia dentate (C,D) after 6 PTZ applications (PK group) and the CA3 pyramidal cells after 5 weeks postkindling (FK3 group). Changes in staining quality of damaged neurons [dark-stained neurons, often with triangular shape and shrunken cytoplasm, surrounded by perineuronal vacuoles (B,D); examples indicated by thick arrow] are clearly visible compared to normal appearing round cells (slight-stained; thin arrow) (bar = 29 μm).

number was further decreased in all regions but the differences between the two groups were small (without the CA2 + CA3 region). The order of the regional vulnerability was identical CA1 > DG > CA4 > CA2 + CA3. To the late timepoint of 15 weeks (FK4 group), the total number of neurons in the CA2 + CA3 region was noticeably decreased in comparison to the control and the FK1 group. Significant differences between the kindling and the post-

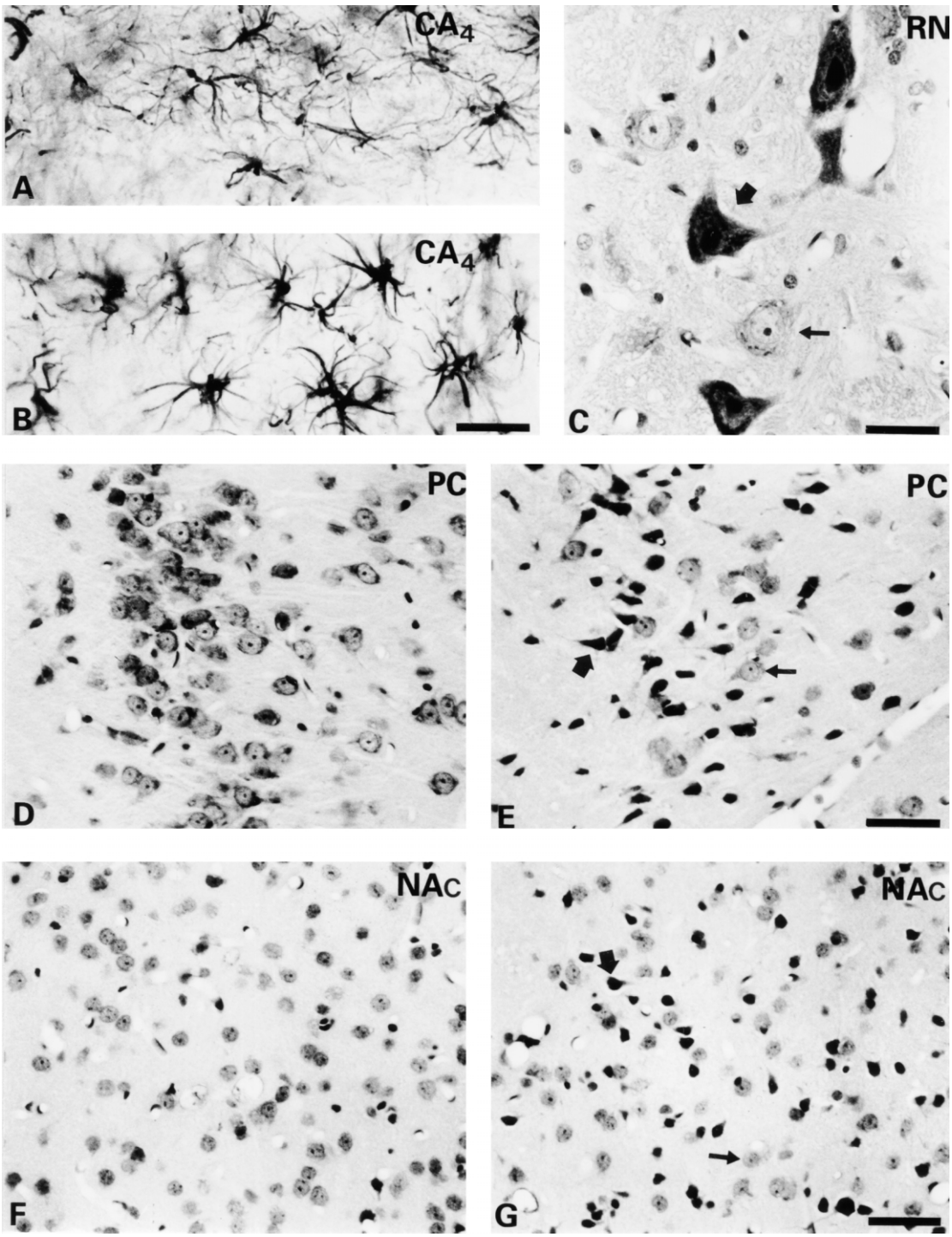

Fig. 5. GFAP-positive, hypertrophic astrocytes in the hilus fascia dentate in coronal sections of the dorsal hippocampus of rats (− 3.70 mm to bregma; A,B) after six PTZ applications (PK group) (B) in comparison to a control animal (A) (bar = 29 μm). (C–G) Distribution of celestine blue/acid fuchsin-stained neurons after 15 PTZ applications (FK1 group) (C,E,G) in comparison to controls in different brain areas (D,F). (C) Dark-stained neurons (shrunken, triangular shape and perineuronal vacuoles; thick arrow) are interspaced among normal appearing neurons (round, slight-stained; thin arrow) in the red nucleus (RN; bregma − 6.06 mm; bar = 21 μm). (D,E) Neuropathological alterations in the piriform cortex (PC; bregma + 1.70 mm; bar = 29 μm) and (F,G) in the nucleus accumbens (NAc; bregma + 1.70 mm; bar = 65 μm).

kindling groups were calculated (Fig. 6A). The pyramidal cells in the CA1 subfield showed the greatest alterations during the time after completion of kindling. The number of acidophilic neurons reached a minimum 5 weeks post-kindling (FK4 group) and increased again. The loss of granular cells and CA4 pyramidal cells in the hilus of the DG sometimes were accompanied by vacuolization among the pericarya.

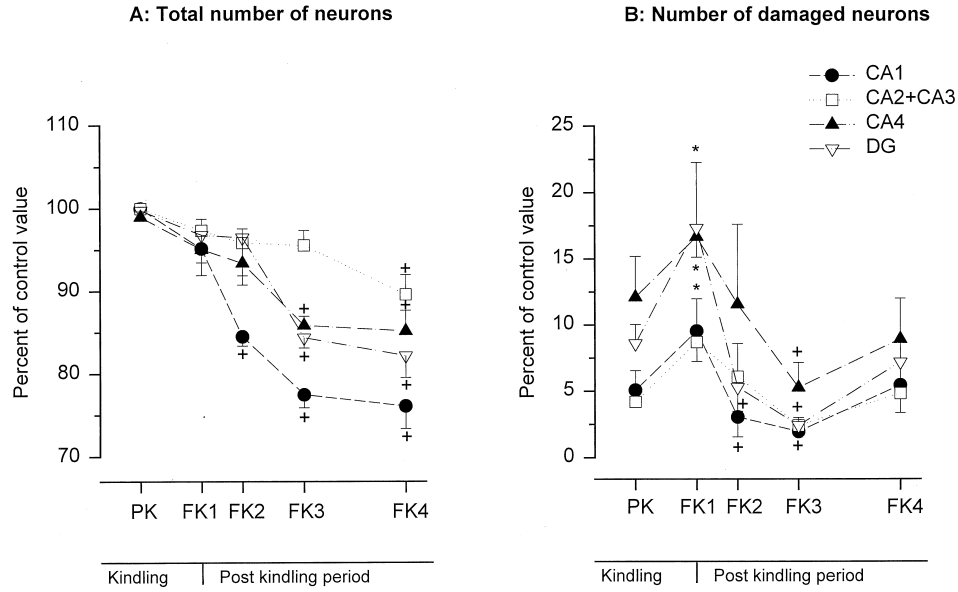

Fig. 6. Mean values of the total number (A) and number of damaged (B) neurons of pyramidal (CA1–CA4) and dentate gyrus granule cells (DG) ±S.E.M. in the dorsal hippocampus of rats (−3.70 mm to bregma; 8 μm coronal sections) from PTZ-treated rats expressed as percentage of controls. Significant differences between PTZ-treated groups and the controls ($n=6$; per group) were found (A) in the fully kindled group (FK1) (CA2+CA3, CA4) and in the fully kindled groups FK2, FK3, FK4 (all regions) ($P<.05$); (B) * $P<.05$. Significant differences between the FK1 group and FK2, FK3, FK4 are indicated by the cross ($^{+}$ $P<.05$).

Finally, the sum of all counted cells in all subfields of the pyramidal cell layer (CA1+CA2+CA3+CA4) clearly reflects the described cell loss. The mean value of the total number of cells of the PTZ-treated animals (and the control animals) in the PK, the FK1 and the FK4 groups were: 1813±11 (1814±17), 1742±42 (1795±28), 1427±46 (1709±32). In the granular cell layer, the following mean values were found: 2580±28 (2581±22), 2503±44 (2580±28), 2024±67 (2438±39). A comparison of the results of the control groups (beginning of the experiments till the end of it) showed no significant differences.

3.2.2. Qualitative study

The distribution of neuronal necrosis in a number of other brain structures was observed at all timepoints. The greatest number of affected neurons was found in the kindling group. In the majority of the investigated structures, a higher number of darkly basophilic neurons than clearly red-stained neurons were observed. The extent of the damage of this quality decreased in the postkindling periods.

Acidophilic neurons were distributed throughout the nucleus accumbens in the most animals in comparison to the controls. Many red-stained and condensed dark basophilic neurons were found in the piriform cortex (PC) in all investigated layers. In the hippocampal formation (level 1), degeneration after the completion of kindling procedure was observed within the subfields CA3 and DG. The amygdaloid nuclear complex was particularly sensitive to PTZ. The basolateral and the basomedial nucleus amygdala, the anterior and posterior parts were most consistently affected, the central nucleus amygdala and medial and lateral part and the medial nucleus amygdala anteroventral part and anterodorsal part being affected less severely. Intensive red-stained and strongly basophilic (dark blue-stained) neurons were observed in the red nucleus (RN) in most of the animals, partly surrounded by marked perineuronal vacuolization and are triangular in shape (Fig. 5). The cell damage was rare in the ventral tegmental area and the substantia nigra at all timepoints studied.

3.3. Immunocytochemistry

GFAP-positive stained astrocytic cell bodies and processes were visualized in all regions and layers of the hippocampal formation, with the layer-specific differences in GFAP-immunoreactivity (GFAP-IR).

An increase of the GFAP-IR was found in partially kindled animals (PK) 24 h after the 6th PTZ application. Differences in GFAP-IR were slightly stronger in the hilus of the fascia dentate (Fig. 5B) and the stratum lacunosum–moleculare in the CA1 region than in other regions compared to the control animals. These astrocytes were characterized by an enhanced immunostaining of the cell body and of the fibrous processes (Fig. 5B). The number of processes appeared increased. A swelling of these cells around the capillaries was observed. Hypertrophy of astrocytes was found only in few animals, especially in the hilus, in the CA3 region and in the basolateral amygdala. GFAP-positive cell bodies and processes rarely disturbed the continuity of the pyramidal cell layer of the hippocampal formation.

In contrast to the PK group, an up-regulation of the GFAP-IR in fully kindled animals (FK1 group) was not

Table 1
Behavioral analysis in relation to the number of Stage 4 seizures

	Number of Stage 4 seizures			
Test	CON	0–2	3–5	6–7
Resident–intruder				
Offensive interactions	8.2 ± 0.6	4.6 ± 1.2*	3.3 ± 0.9*	2.8 ± 0.8*
Open-field				
Locomotion (cm)	840.3 ± 58.0	997.6 ± 108.5	733.4 ± 69.0	695.8 ± 85.7
Inner areas (s)	29.8 ± 3.8	45.4 ± 3.7*	16.4 ± 2.4*,+	15.1 ± 2.9*,+
Cat-odor exposition				
Freezing (s)	208.3 ± 24.4	146.3 ± 54.5	344.0 ± 102.8*	351.3 ± 39.0*
Elevated plus maze				
Time on open arms (%)	19.3 ± 1.5	31.6 ± 4.1*	10.9 ± 2.1*,+	9.7 ± 1.9*,+
Open arm entries (%)	21.3 ± 2.7	28.4 ± 3.5	14.9 ± 2.9*	13.6 ± 3.7*
Enclosed arm entries	22.9 ± 2.9	22.2 ± 3.4	22.1 ± 2.9	21.6 ± 3.5

Data of the behavioral analysis (mean ± S.E.M.) in relation to the number of Stage 4 seizures. The animals were assigned to four groups regarding to the number of Stage 4 seizures 24 h after the end of the kindling. Control group (CON, $n=6$), 0–2 ($n=6$), 3–5 ($n=9$), 6–7 ($n=6$). The two numbers connected by a hyphen indicate the minimum and the maximum number of Stage 4 seizures in each group.

* $P<.05$ vs. CON group (one-way ANOVA followed by the Student–Newman–Keuls test).

\+ $P<.05$ vs. 0–2 group (one-way ANOVA followed by the Student–Newman–Keuls test).

observed. In the other fully kindled groups, only single hypertrophic astrocytes were found in the hilus region without further astrocytic changes in other regions. An increase in numerical density of astrocytes (proliferation) relative to controls or glial scar formation was not observed.

3.4. Correlation between the number of Stage 4 kindling seizures and the number of damaged hippocampal neurons in individual rats

For the investigation of a possible correlation between the number of Stage 4 seizures and the number of damaged hippocampal neurons, the timepoint of 24 h after completion of the kindling procedure (FK1 group) was selected. The correlative analysis revealed a relationship ($r=.86$, $P<.01$) between both parameters. However, with regard to the animals with at least three Stage 4 seizures, the correlation between the number of fully expressed kindled seizures and the number of damaged hippocampal neurons was only low ($r=.62$, $P<.05$).

3.5. Relationship between the number of Stage 4 kindling seizures and changes of emotional behavior

The behavioral data in relation to the number of Stage 4 seizures are summarized in Table 1. The number of offensive interactions was reduced in all groups independently from the number of Stage 4 seizures. Compared with this, the open-field and the cat-odor exposition test revealed two phases in the change of defensive behavioral pattern according to the number of Stage 4 seizures. Whereas the animals with none or only one (two) fully expressed kindling seizure showed an increase of the time spent in the inner area of the open field and a tendency to reduced freezing, the behavior of the animals with at least three Stage 4 seizures (3–5 and 6–7 groups) was characterized by diminished exploration of the inner areas of the open field and enhanced freezing. No significant differences in the locomotor activity were registered.

For a further validation of the open-field data regarding the anxiety level of the animals, the behavioral analysis was completed by an elevated plus-maze test. The rats of the 0–1 seizure group spent more time on the open arms of the elevated plus-maze, whereas the animals of the 3–5 and 6–7 seizure groups showed a reduced exploration of the open arms (time on open arms and open arm entries). The number of enclosed arm entries indicated no significant differences in the locomotor activity between the various groups. Regarding the animals with three or more Stage 4 seizures, there was no correlation between the number of fully expressed kindling seizures and the changes in emotional behavior assessed in the open-field and the elevated plus-maze test.

4. Discussion

In the partially kindled group, six of eight animals exhibited at least Stage 2 seizures (three of them showed already Stage 3 seizures). The offensive behavior of these animals was significantly reduced. A previous electroencephalographical study has also shown the expression of first permanent changes at this state of PTZ kindling (Schramek et al., 1995).

A decreased number of offensive interactions in rats was also described after the recovery of epilepsy induced by injection of tetanus toxin into the hippocampi (Mellanby et al., 1995) and after the expression of chronic mild stress (D'Aquila et al., 1994). Furthermore, intermale aggression

has been reported to decrease by single or repeated exposure to restraint stress (Albonetti and Farabollini, 1993). The diminished offensive behavior, exhibiting in a reduction of competitiveness and aggressiveness as a part of symptoms observed in laboratory rodents after various forms of uncontrollable stress, appears similar to the symptoms observed in human depression (Weiss et al., 1994). On the other hand, no significant changes were registered in the open-field and the cat-odor exposition test. However, some of the assessed parameters such as the exploratory locomotion (tended to be increased) and the duration of freezing (tended to be decreased) indicate anxiolytic behavioral changes. Differences between the changes in the offensive and the defensive behavior are in accordance with the assumption that both kinds of behavior are based on different neuronal systems (Blanchard and Blanchard, 1988). Thereby, offensive behavior appears to have a greater sensitivity to aversive stimuli and a lower threshold for stress-induced changes than other behavioral systems (Albonetti and Farabollini, 1994). The observed neuronal and astroglial reactions in selected brain regions in present study occurred depending on the kindling state and the time after completion of kindling. In the PK group, first damaged neurons were found but the detectable neuronal loss was slow. The appearance of damaged neurons in the CA4 region correlated well with the observed phenotypic changes of astrocytes (increase in GFAP-IR, hypertrophy). The transformation of astrocytes into ''reactive astrocytes'' in epileptic diseases is known (Malhotra et al., 1992). Electrically induced seizures in rat hippocampus led to an increase in GFAP mRNA (Steward et al., 1991), GFAP-content (Hansen et al., 1990) or GFAP-IR (Dalby et al., 1995). An increase of hilar GFAP immunostaining was also registered after hippocampus and amygdala kindling in rats (Adams et al., 1998; Khurgel et al., 1992). The morphological responsiveness of astrocytes in kindling could be associated with adaptive processes and seems to be an early event which precedes any neuronal degeneration (Hansen et al., 1990; Hawrylak et al., 1993; Niquet et al., 1994; Tiffany-Castiglioni and Castiglioni, 1986). An increased GFAP-IR is also discussed as one of the adaptation mechanisms in the mesolimbic–mesocortical dopamine (DA) system, which was found after chronic treatment with morphine, cocaine and ethanol (Berhow et al., 1995; Ortiz et al., 1995). A long-lasting elevation of Met-enkephalin occurred in several brain regions after PTZ kindling (Vindrola et al., 1984). In this view, it appears of interest that opioids tend to suppress offensive behavior while either reducing the defensive behavior or having no effect on it (Miczek et al., 1984).

The increased GFAP-IR in various limbic structures could be discussed in line with the possible adaptation to repeated PTZ-induced seizures as stressful experiences. Repeated exposure to the same stressor (e.g., psychostimulant, aversive experiences) promotes enhanced response of the dopaminergic neurons in the mesolimbic–mesocortical projection system (for review, see Cabib and Puglisi-Allegra, 1996). It has been shown that the basal activity of dopaminergic neurons was also increased after PTZ kindling (Dazzi et al., 1997). The tendency to enhanced exploratory locomotion in the open-field of the PK group may be related to these adaptive mechanisms. The described depressive symptoms occurring after completion of the PTZ kindling procedure could be at least partly connected to an inhibitory phase of mesolimbic-dopaminergic response. These findings suggest that endogenous opioid- and DA-related mechanisms contribute to the PTZ kindling-associated behavioral changes.

In the fully kindled rats (FK1 and FK4 groups), also a reduced offensive behavior was observed. Furthermore, the behavioral analysis reveals an increase in freezing in the cat-odor exposition test and a decrease in the exploratory locomotion indicating an increase in anxiety.

A decrease in open-field exploration was also observed for at least 2 months after long-term amygdala kindling (Kalynchuk et al., 1998b). Furthermore, the open-field behavior with lowered exploratory activity showed similarities with the situation after chronic stress (Katz et al., 1981).

The present study identified an increase in the quantity of damaged neurons after completion of the kindling procedure. The hierarchy of selective vulnerability to seizure-induced damage (neurons in the CA4 were most vulnerable, followed by CA1 neurons) corresponds with results from electrical kindling models (Cavazos et al., 1994), the elevation of steroid hormones associated with stress (Levy et al., 1994) or neuropathologic studies of humans with temporal lobe epilepsy (Meldrum, 1993). It has been demonstrated that the correlation between acid fuchsin staining and subsequent neuronal death can be recognized clearly 2 weeks later in the decrease in the total number of neurons. The neuronal loss continued for 5 weeks postkindling (FK3 group) and could still be recognized at a low degree 10 weeks after kindling (FK4 group). This ''delayed neuronal death'' is also known from results obtained in ischemia (for reference, see Kogure et al., 1988). Final cell damage ''matures'' over hours and days [acute neuronal change (up to 3 h), maturational death (3 to 24 h) and delayed neuronal death (up to 7 days)]. Despite identical morphological pictures of injured neurons, the hypothesis that neuronal damage in the hippocampus after epilepsy could be a result of cerebral ischemia and hypoxia accompanying generalized seizures exists (Dam, 1982; De Vasconcelos et al., 1992; Nevander et al., 1985). However, it seems unlikely that the neuronal loss after PTZ kindling is exclusively a cause of seizure-induced ischemia and the following neuronal death. It appears that the process of the delayed neuronal death was considerably accelerated after the end of kindling and continued up to 10 weeks postkindling. An explanation of the prolonged neuronal death after the end of the kindling procedure could be a different time course of endogenous anticonvulsant and kindling-related excitatory mechanisms. Whereas the state of hypersensitivity was permanent after termination of kindling, the endogenous

anticonvulsant mechanisms, which were induced by the occurrence of seizures, were terminated after some days (Shandra et al., 1996). One of the mechanisms responsible for the enhanced seizure susceptibility may be the kindling-induced death of hippocampal neurons per se, which could contribute to enhanced seizure susceptibility via associated sprouting as well as reactive synaptogenesis and reorganization (Cavazos et al., 1994; Hawrylak et al., 1993; Hosokawa et al., 1995). Another reason for long-lasting increased seizure susceptibility after kindling could be the enhanced expression of NMDA receptors causing a greater potency of NMDA in kindled rats (Martin et al., 1992). Long-term sequel (1–3 month) after parenteral application of kainic acid animals displayed spontaneous seizures notably when handled and morphological changes were described. This behavior reflects the ongoing nature of hyperexcitability and brain damage (Ben-Ari, 1985). In the present study, such late seizures after the end of kindling stimulations were not observed. The effects of secondary systemic responses could not be separated from primary responses.

The correlation between the number of Stage 4 seizures and the number of damaged hippocampal neurons in individual rats was low (Fig. 7). It was found that neuronal damage occurred only after at least three Stage 4 seizures. However, it seems unlikely that the difference of one or two Stage 4 seizures is responsible for the expression of the neuronal loss. A possible explanation could be a different time course of the kindling development. The 1–2 seizure group displayed a markedly delayed kindling development and the rats expressed the first Stage 4 seizure not until the last week of the kindling procedure. In comparison, the animals with three or more seizures displayed a more rapid kindling development with the first Stage 3 or 4 seizures already after 2 weeks of kindling stimulations.

The analysis of the relationship between morphological alterations and changes of behavioral parameters with regard to the number of fully expressed kindling seizures indicated a suppressed offensive behavior in all groups independently from the number of Stage 4 seizures. On the other hand, two different phases of behavioral changes were observed. Animals with not more than one (or in one case two) Stage 4 seizures showed behavioral changes which were characterized by enhanced exploratory activity in the open-field test and by reduced freezing in the cat-odor exposition test indicating a lower anxiety level. On the other hand, in the groups with at least three Stage 4 seizures, reduced exploratory activity in the open field and enhanced freezing in response to cat-odor were noticed. Thus, the results may suggest a relationship between hippocampal neuronal cell damage and an increase of defensive behavior. In this view, it should be noted that destruction of the DG in rats enhances fearfulness in novel situations (Takahashi, 1996).

Additionally performed investigations in the elevated plus-maze supported the interpretation of the open-field findings. The time which the animals spent in the inner areas of the open-field and in the open arms of the elevated plus-maze was altered in the same manner in all tested groups. Both tests are basing, at least partly, on the animals' conflict between aversion to open spaces and the inquisitiveness to explore a new environment. The decreased open arm activity indicates an increased level of anxiety in the fully kindled animals 5 weeks after the last stimulation, which causes a more passive response to the elevated plus-maze situation. Comparable results were found after amygdala kindling if the behavioral test was done at least 1 week after the final kindling stimulation (Kalynchuk et al., 1998b).

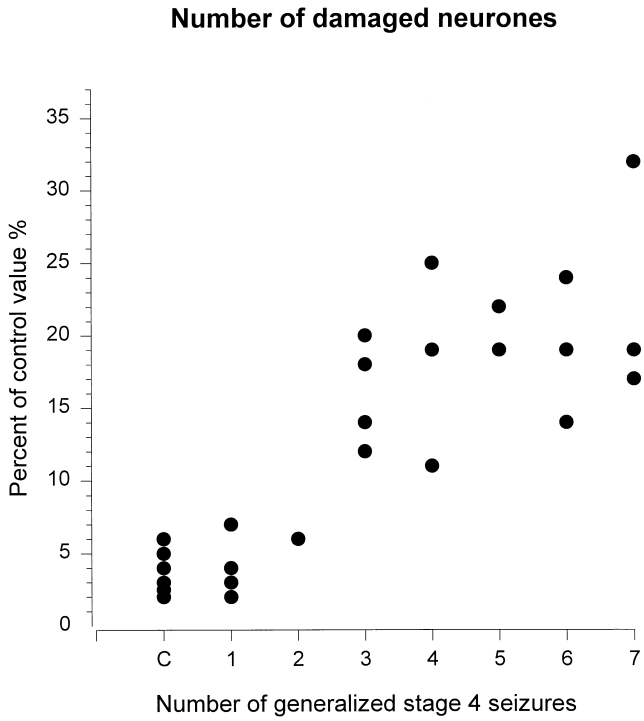

Fig. 7. Correlation between seizure frequency (number of generalized Stage 4 seizures) and the hippocampal neuronal cell damage (percentage of control value %) of individual rats 24 h after the end of the kindling (Pearson's test).

The present results demonstrate that PTZ kindling goes along with long-lasting changes in emotional behavior, which can be interpreted as depression-like and are obviously associated with a characteristic pattern of neuronal loss in various hippocampal regions. Whereas a reduced offensive behavior was found already in partly kindled animals, changes in defensive behavioral pattern occurred only in fully kindled animals.

The reduced offensive behavior, the changes of the defensive behavior, as well as the hippocampal neuronal loss, indicate a strong similarity between the long-lasting adaptive changes induced by PTZ kindling and various stress models.

The investigation of long-lasting changes after PTZ kindling seems to be an approach to understand the underlying basic mechanisms between the occurrence of repeated seizures and the development of psychiatric disorders such as depression and anxiety.

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