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Antiepileptogenic and neuroprotective effects of losartan in kainate model of temporal lobe epilepsy

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ABSTRACT

Recently, we have shown that the blockade of AT_1 receptor might be useful as an adjuvant treatment strategy for the prevention of oxidative stress and neurotoxicity caused by status epilepticus (SE) in rats. The purpose of the present study was to further assess the efficacy of long-term treatment with losartan (10 mg/kg), the selective AT_1 receptor antagonist, during kainate (KA)-induced epileptogenesis in Wistar rats. Losartan treatment started after onset of SE and continued for 4 weeks. The rats were video- and EEG-recorded for 3 months. Locomotor activity, anxiety and depressive-like behavior were evaluated 9 weeks after SE, when all rats had developed chronic epileptic state. Neuronal damage in hippocampus was analyzed by hematoxylin while serotonin (5-HT) levels in hippocampus by HPLC. AT_1 receptor antagonism increased the latent seizure-free period and decreased the frequency of spontaneous motor seizures. Losartan positively affected epilepsy-provoked behavioral changes, including impulsivity, low anxiety level and depression in a phase-dependent manner and restored the changes in diurnal fluctuation of motor activity. Losartan exerted neuroprotection selectively in the CA1 area of the hippocampus in the KA-treated rats and lowered the 5-HT levels both in normal and abnormal conditions. Our findings suggest that the AT_1 receptor antagonist exerts disease-modifying effects during KA-induced epileptogenesis and neuronal damage in CA1 hippocampal area, attenuated some of the behavioral changes and restored diurnal variability in locomotor activity.

1. Introduction

Status epilepticus (SE) is an example of a brain injury that causes epileptogenesis and leads to the development of acquired epilepsy with spontaneous recurrent seizures (SRS) (Delorenzo et al., 2005). One of the most deleterious forms of epilepsy is temporal lobe epilepsy (TLE), which is often refractory to antiepileptic drugs (Semah et al., 1998). Kainic acid (KA) model has been established to reproduce the pathophysiological events of epileptogenesis and chronic behavioral state in TLE (Hellier et al., 1998). The neuropathological changes detected in CA3 and CA1 subfields, the hilus of the dentate gyrus, amygdala, and perirhinal and enthorhinal cortices after KA-induced seizures (Chen and Buckmaster, 2005) are strikingly similar to those in human TLE. These structures are involved in emotionality/anxiety-related behaviors as well (Jarrard, 1993). The optimal treatment strategy for epilepsy should include intervention during epileptogenesis to suppress seizure onset and improve long-term outcome.

The putative role of the renin–angiotensin system (RAS) in the regulation of certain behavioral functions including stress responses, cognitive processes and depression has been reviewed in detail by Wright and Harding (1994, 2013) and Wright et al. (2008). Moreover, cerebro-protection and regulation of seizure susceptibility have been attributed to this system (von Bohlen und Halbach and Albrecht, 1998; Gard, 2002; Łukawski et al., 2010; Tchekalarova and Georgiev, 2005; Wright et al., 2008). Angiotensin AT_1 receptors are highly expressed in brain structures associated with hormonal, cerebrovascular and behavioral regulation (reviewed in: Wright and Harding, 1994). Accumulated evidence suggests that the inhibitory control of neuronal firing rate and long-term potentiation are mediated by AT_1 receptor subtype (reviewed in Wright et al., 2008). Although AT_1 receptors are mostly localized in the anterior pituitary, hypothalamus and circumventricular organs (CVOs), they are also expressed in areas that control brain excitability, including the piriform cortex, hippocampus, lateral geniculate, caudate putamen, amygdala and septum (reviewed in: Wright et al., 2008). The selective AT_1 receptor antagonists were

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reported to attenuate the pentylenetetrazol (PTZ) kindling in mice (Georgiev et al., 1996), potentiate the anticonvulsant effect of valproate in mice (Łukawski et al., 2010) and decrease the seizure severity in epileptic rats (Pereira et al., 2010). Both experimental and clinical studies suggest that AT_1 receptors might be an interesting target for treatment of TLE. Losartan has been demonstrated to be an effective treatment strategy against acquired epilepsy via TGF-β signaling suppression (Bar-Klein et al., 2014). The repetitive seizures induced in an experimental model of TLE caused an up-regulation of the AT_1 receptor expression in the hippocampus of Wistar audiogenic rats (Pereira et al., 2010). Clinical data have also demonstrated an up-regulation of AT_1 receptors and their mRNA expression in cortex and hippocampus of patients diagnosed with TLE (Argañaraz et al., 2008). Recently, we have demonstrated that repetitive treatment with the selective AT_1 receptor antagonist losartan is able to attenuate PTZ seizures in rats (Pechlivanova et al., 2011), while its chronic infusion suppresses KA-induced oxidative stress and neurotoxicity in the hippocampus during SE (Tchekalarova et al., 2014).

In view of the facts that AT_1 receptor antagonists are broadly used in clinics as a treatment for hypertension and accumulated evidence about their effects on seizure susceptibility including SE (Argañaraz et al., 2008; Pereira et al., 2010), the major rationale of this study is to evaluate the efficacy of the selective AT_1 receptor antagonist losartan, against the deleterious consequences of KA-induced SE and the associated long-term behavioral abnormalities, diurnal disturbances, biochemical changes and neuronal damage in Wistar rats.

2. Methods

The procedures used in this study are in agreement with the European Communities Council Directives of 24 November 1986 (86/609/EEC). The experimental design was approved by the Institutional Ethics Committee at the Institute of Neurobiology and the Ethics Committees for research at the Sofia Medical University under the contract No. 30/2011 for the application grant DTK 02/56 2009–2012.

2.1. Subjects

The experiments were performed on sixty-day-old male Wistar rats obtained from the breeding facility of the Institute of Neurobiology, Bulgarian Academy of Sciences. Following arrival in the laboratory, the animals were housed under standardized conditions (20 ± 3 °C, 40–50% humidity; 12/12-h light/dark cycle with lights on at 08:00 h) and habituated for 10 days. Food and water were available ad libitum throughout the study except during test procedures. All experiments were carried out in the autumn–winter season.

2.2. Experimental design and losartan treatment

The study design with treatment groups and procedures are described in Fig. 1. Animals were randomly divided into four groups: C-veh (controls treated with vehicle, n = 10); C-los (rats treated with losartan, n = 10); KA-veh (rats treated with KA and vehicle, n = 13); KA-los (rats treated with KA and losartan, n = 12). Two groups with implanted electrodes for video- and EEG analyses were added as follows: EEG-KA-veh (n = 9) and EEG-KA-los (n = 8).

Behavioral tests started 9 weeks after SE, when the rats had already increased seizure susceptibility. Time interval between tests was at least two days. The order of behavioral tests was as follows: sucrose preference test (SPT), open field (OF), elevated plus maze (EPM) and forced swim test (FST).

Losartan treatment (kindly gifted by MERCK&CO., INC, New Jersey, USA) started 2 h after the beginning of status epilepticus (SE) at a dose of 10 mg/kg, previously shown to alleviate seizure activity in acute PTZ test (Pechlivanova et al., 2011). During the first three days, when the animals were unable to drink via bottles because of the kainate treatment, losartan/saline was injected subcutaneously (s.c.). The drug was dissolved in a lactated Ringer's and administered at a volume of 20 ml/kg. Matched animals not treated with KA were also injected with losartan or vehicle, respectively. Later, after recovering from kainite treatment, losartan was administered via drinking water for a period of four weeks. Matched animals not treated with KA were administered with losartan via drinking water or were given tap water. Daily preparation of drinking water containing losartan was adjusted to a concentration based on the individual volume of liquid consumed the previous day and body weight, and administered every day at a dose of 10 mg/kg in the morning.

2.3. Surgery

For electrode implantation rats were anesthetized with an intraperitoneal (i.p.) injection of a ketamine (40 mg/kg) and xylazine (20 mg/kg) mixture. The rats were fixated in a stereotactic frame (Narishige Sci. Inst. Labs, Japan), the skull was exposed and eight holes were drilled to place the electrodes and screws. Four custom-made epidural electrodes and two reference and ground electrodes (silver ball wire, 200 μm, Biomed Instr., Germany) were placed on both sides of the skull over the frontal (A = +1, L = ±2) and parietal (A = −4.2, L = ±3.0) cortical areas, respectively. The reference and ground electrodes were placed over the occipital bone. In the remaining two burr holes, anchor screws were placed to fix the electrodes on the skull by acrylic cement. All electrodes were linked to a connector which was attached via a swivel commutator mounted on the box's ceiling. The

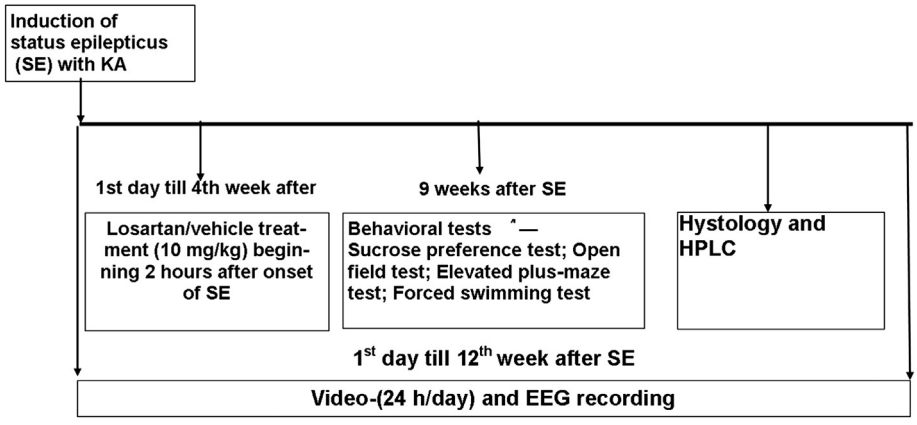

Fig. 1. Schematic illustration of the experimental protocol.

output of the connector was fed to a Nihon Kohden electroencephalograph (Japan) and EEG was recorded by means of the MP150 System (BIOPAC Systems, Inc., USA) connected to the amplifiers of the EEG machine.

2.4. Induction of status epilepticus with kainic acid

The protocol of KA-induced SE was executed according to Hellier et al. (1998) with a minor modification aiming to decrease the mortality of rats. KA was diluted in sterile saline (0.9% NaCl) at 2.5 mg/ml. SE was induced by repetitive injections of KA (Abcam, UK) starting with a dose of 5 mg/kg, i.p. (1 ml/kg) at the first hour of observation. Thereafter, KA was delivered in half of the above-mentioned dose (2.5 mg/kg) every half hour. Seizure intensity was evaluated by a modified Racine's (1972) scale. Class I and class II (immobility, facial automatism, head nodding and wet-dog shakes) were grouped as partial seizures and were not scored. Class III (forelimb clonus with lordotic posture), class IV (rearing and continued forelimb clonus), and class V (forelimb clonus and loss of posture) were grouped as secondary generalized seizures. The number of seizures of class III, IV or V was registered and used as a criterion for an additional KA injection. The onset of SE was between the third and the fourth injections of KA in a majority of rats. The severity and duration of SE during the KA treatment protocol were evaluated by means of EEG and video-monitoring (video recorder DVR-4 with AVTECH camera, Taiwan, no. AVC307R). Only the rats that developed SE (recurrent seizures with bilateral forelimb clonus for at least 3 h) and survived thereafter were included in the subsequent analyses. Matched controls were treated with an equivalent volume and number of saline injections.

2.5. Video and EEG monitoring of spontaneous recurrent seizures

Video-monitoring (24 h/day for 12 weeks starting 24 h after SE) was accomplished using an infrared-sensitive colored camera (S-2016, AVTECH, Taiwan, no. AVC307R) connected to a computer. The recordings were visually analyzed by independent observers to detect spontaneous motor seizures (SMS) of class IV or V. Partial seizures of classes I and II were not detected by video monitoring because without simultaneous EEG recording they could easily be omitted. EEG recordings were performed always in the morning to minimize potential circadian variations. After habituation for half an hour, each animal was recorded for at least 60 min at selected time points after SE i.e. during losartan treatment (25–30 days post-*status*) and after discontinuation of the treatment (50–60 days and 70–90 days post-*status*, respectively). Visual detection and analysis of ictal events were performed off-line by examining and analyzing EEG (Acknowledge software ACK100W, BIOPAC Inc., USA). The criterion for epileptic seizures was the abrupt onset of epileptiform activity with high frequency (≥5 Hz) and amplitude ≥ three time baseline EEG. Recordings were performed during two different periods of epileptogenesis. The first monitoring period was during losartan treatment (15–30 days after SE) and the second monitoring period after discontinuation of losartan treatment (70–90 days after SE), respectively. Recordings were performed for at least 60 min per day. Paroxysmal events were defined as seizures when they lasted more than 20 s and were separated by intervals of at least 5 s, or "spike-trains" when they were less than 20 s (Arabadzisz et al., 2005).

2.6. Sucrose preference test

The rats were tested for sucrose preference over a three-day period using a two-bottle choice test. On the first day, rats were singly housed and habituated to drinking from two identical graduated bottles, each containing 200 ml of water. On the following day, rats were trained on the sucrose choice procedure, in which regular water in one of the bottles was replaced with 1% sucrose diluted in tap water. On the 3rd (test) day rats were allowed to drink freely from both bottles. The test was performed starting from 08:00 a.m. and ran for 24 h. The bottles were weighed after 12 h or replaced by a second pair of pre-weighed bottles. The position of the bottles was switched to avoid position preferences. The volume of the sucrose solution consumed as a percentage of the total volume of fluid consumed was calculated during a 12 h-period (light phase — 08:00–20:00 h and dark phase — 20:00–08:00 h).

2.7. Behavioral tests

The open field test (OF), elevated plus-maze test (EPM) and forced swim test (FST) were performed at two time points at 15:00 p.m. and 03:00 a.m., respectively, under artificial diffused light during the light phase and in red dim light during the dark phase. The behavioral experiments were conducted in a soundproof room where the animals were moved at least 30 min before each test. The rats that exhibited SRS 1 h before starting the test were excluded from the experimental procedure. The behavior in the OF and EPM tests was recorded using an infrared sensitive CCD camera.

2.7.1. Open field test

The apparatus was made of a gray polystyrene box (100 × 100 cm × 60 cm) divided into two zones: outer square (periphery) and inner square (center). The animals were placed individually in the center of the open field. Behavior was observed for 5 min. Before each trial, the field was cleaned thoroughly with 0.1% acetic acid solution to prevent any odor traces. The open field was divided into two zones: central and peripheral areas. The center was considered an aversive place for the rats. For each rat, the total distance moved and the time spent in the central zone (sec) vs total time in % were measured by a computerized tracking system (SMART PanLab software, Harvard Apparatus, USA). An anxiety index was calculated using the following equation: anxiety index = 1 − [(Center time / Total time + (Center distance / Total distance) / 2]. Anxiety index values range from 0 to 1, with a higher value indicating increased anxiety (Mazor et al., 2009).

2.7.2. Elevated-plus maze test

The apparatus consisted of two open arms (50 × 10 cm), two enclosed arms (50 × 10 × 50 cm), and a central platform (10 ×10 cm) elevated 50 cm from the floor. Before each trial, the field was cleaned thoroughly with 0.1% acetic acid solution. Each rat was first placed in the central zone of the EPM with its head facing an open arm. The rat was allowed to freely explore the maze for 5 min. The calculated standard measures were: 1) total distance moved; 2) time (sec) spent in open arms vs total time in % and 3) anxiety index, which unifies all EPM parameters into one ration. Anxiety index = 1 − [(open arm time / Total time + (Distance open arms / Total distance) / 2].

2.7.3. Forced swim test

The forced swim test was undertaken according to the methods described by Porsolt et al. (1979). In the present study, the animals were tested in a transparent Plexiglas tank (diameter 25 cm, heath 60 cm) filled to a depth of 30 cm with tap water (24 ± 1 °C). On the first experimental day of habituation, rats were placed in the water for 15 min. They were placed in the cylinders 24 h later, and the 5 min FST was conducted. Immobility (in s) was recorded by two independent observers who were blind to the animal groups. Floating was defined as the minimum movement necessary to keep the rat's head above the water. After the test, the rats were dried and returned to their cages. The water in the apparatus was changed from rat to rat.

2.8. Histology

After a deep anesthesia with Nembutal (50 mg/kg, i.p., Abbott) (n = 5 per group) rats were transcardially perfused initially with 0.05 M phosphate buffered saline, at pH 7.3 followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB), at pH 7.3. After decapitation by

guillotine, the brains were dissected out and post-fixed overnight at 4 °C in the same fixative solution. After post-fixation, the brains were sliced in the coronal plane; the tissue blocks were washed in PB, embedded in paraffin and cut into 5 μm thick sections. The samples were then deparaffinized with xylene and ethanol, and routinely stained with hematoxylin and eosin to better identify pyknotic nuclei of damaged neurons. The sections were investigated on a Nikon Eclipse 80i light microscope (Japan), and photographed with a digital camera (Nikon DMX 1200). Sections were analyzed for major cell loss with special attention to the dorsal and ventral hippocampi as previously described (Tchekalarova et al., 2013). The staining intensity and density of nerve cell bodies were estimated using Nikon's NIS Elements Digital Imaging software. The relative neuronal densities of the selected brain areas were quantified by determining the percentage of the measurement grid occupied by stained cells. The resulting values provide a relative index of the number of stained neurons in the selected brain areas.

2.9. High performance liquid chromatography

An additional subgroup of rats (n = 8 per every group) was used for HPLC data. The rats were decapitated by guillotine under light anesthesia (inhalation with CO_2), brains were quickly dissected on ice and hippocampi were bilaterally removed. The tissue samples were frozen in liquid nitrogen, lyophilized and stored at −70 °C before analysis of 5-HT levels as was previously shown (Tchekalarova et al., 2011, 2013).

2.9.1. Statistical analysis

Parametric (for normally-distributed data) or non-parametric tests (for data not normally distributed) were used for the statistical analysis (SigmaStat® 11.0). Data which were not normally distributed were presented by data box plots. The significance of differences in behavioral, histological and biochemical data among groups was calculated by analysis of variance (ANOVA), followed by post hoc testing conducted by Bonferroni or Holm Sidak t-test for individual differences. If data were not normally distributed, ANOVA for nonparametric data (Kruskal–Wallis on ranks) followed by the Mann–Whitney U test were used. The level for statistical significance differences was set at $p < 0.05$.

3. Results

3.1. Seizure activity in rats treated with KA

During the baseline electrographic recordings no seizures were detected in both groups (Fig. 2A, B). The average dose of KA needed to induce SE was as follows: median ± SD: 15 ± 5.2 mg/kg, range 7.5–29 mg/kg. Rats with implanted electrodes exhibited EEG spiking activity accompanied by wet-dog shakes and repetitive electrographic seizures in the cortical leads, which afterwards culminated in SE (Fig. 2A, B). The mortality rate as a consequence of SE was about 10%. The latent phase was characterized by normal patterns of EEG alternating occasionally with abnormal non-convulsive EEG activity with freezing of the animal (Fig. 2A, B). The first detected non-convulsive seizure in the KA-veh group usually preceded the first convulsive seizure by about a week, which is in line with previous reports (Bertram and Cornett, 1993). Losartan treatment (10 mg/kg) significantly increased the latent seizure-free period (median ± SD: 28.5 ± 23 days; range 7 −86 days) compared to vehicle treatment (median ± SD: 17.5 ± 14.2 days; range 6–67 days) (Mann–Whitney Rank Sum Test: T = 393; p = 0.017).

Spontaneous EEG epileptic seizures were accompanied by behavioral motor seizures during the chronic phase (Fig. 2Ad). The number and total duration of paroxysmal events and seizures detected during the first and the second period of EEG recording are presented in Table 1A and B. As a result of high variability in EEG data a tendency toward a decrease in paroxysmal events and number of spike-trains was observed only in the KA-los group unlike the KA-veh group (Table 1B). A significant difference in the frequency of the SMS was detected between the KA-los group and the KA-veh group at the time of losartan treatment (the 1st month) and after its discontinuation (the 2nd and the 3rd months, respectively) ($^{*}p < 0.05$) (Fig. 3). A significant increase in seizure frequency as a function of time was also detected in both the KA-veh group and the KA-los group, respectively ($^{o}p < 0.05$ the 3rd vs the 1st month). The daily seizure frequency for the KA-veh group during the 3rd month was: median ± SD 1.4 ± 2.9 seizures (range: 0.6–10.3); for the KA-los group: 0.27 ± 0.8 seizures (range: 0.03–2.8), respectively.

3.2. Sucrose preference test

Except the controls, all groups exhibited diurnal variations in SPT ($^{\#}p < 0.05$) (Fig. 4). Lower preference to sucrose consumption was detected both in the C-los and in the KA-veh groups during the light phase ($^{*}p < 0.05$). A month after discontinuation of losartan treatment, both groups: C-veh and KA-los showed similar preference for sucrose solution.

3.3. Open field test

Vehicle-treated rats displayed typical thigmotaxic behavior, with locomotor activity confined to the peripheral zone and a comparatively short time spent in the central zone. The C-los group showed a significant decrease in motor activity during the light phase ($^{*}p < 0.05$) (Fig. 5). The KA-veh rats were hyperactive ($^{*}p < 0.05$ vs C-veh group) and demonstrated disturbed diurnal variations of motor activity. The long-term losartan treatment (10 mg/kg) alleviated KA-induced hyperactivity during the light phase ($^{o}p < 0.05$ vs KA-veh group) and restored diurnal variations of motor activity ($^{\#}p < 0.05$).

The KA-veh group demonstrated a lower anxiety level compared to the C-veh group with increased time spent in the aversive central area of the field and decreased anxiety index during both the light and dark phases, respectively ($^{*}p < 0.05$) (Fig. 6A, B). The long-term losartan treatment (10 mg/kg) prevented the KA-induced change in anxiety level only during the dark phase ($^{o}p < 0.05$ vs KA-veh group). Diurnal variations in anxiety level were demonstrated by both the C-veh and KA-los groups.

3.4. Elevated plus-maze test

Like in the OF test, the KA-veh group was characterized by low anxiety level without diurnal fluctuations ($^{*}p < 0.05$ vs C-veh group) (Fig. 7A, B). Both the C-veh and KA-los groups had similar anxiety level during the dark phase.

3.5. Forced swim test

The KA-veh group spent a significantly longer time immobile during the light phase ($^{*}p < 0.05$ vs C-veh group) (Fig. 8). The long-term losartan treatment (10 mg/kg) significantly reduced immobility time compared with that of the KA-veh group ($^{o}p < 0.05$). Diurnal fluctuations with less immobility time during the dark phase was detected only in the KA-veh group ($^{\#}p < 0.05$).

3.6. Histopathological changes and 5-HT levels in the hippocampus

The histological examination of E&H-stained sections from the dorsal hippocampal formation in C-veh group showed densely arranged and intensely stained neurons in all layers of the hippocampus and dentate gyrus (Fig. 9A). A severe neuronal loss in the CA1 (septo-temporal and temporal), CA3a (septo-temporal and temporal) and CA3c (septo-temporal and temporal) areas of the hippocampus, as well as in the septo-temporal hilus of the dentate gyrus was detected in the KA-veh group ($^{*}p < 0.05$ vs C-veh) (Figs. 9B, 10, 11). In contrast, the long-term losartan treatment (10 mg/kg) during epileptogenesis caused

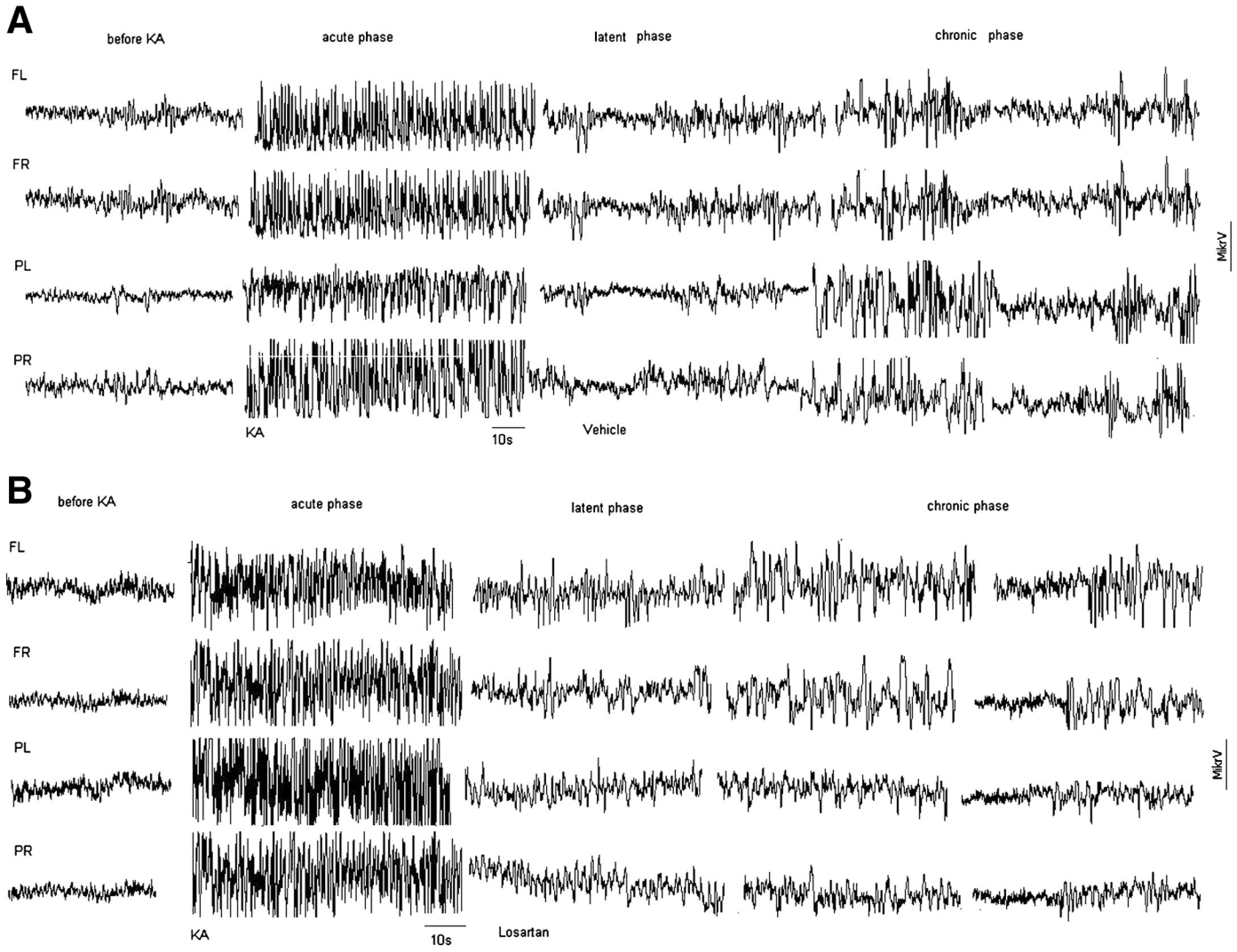

Fig. 2. Electrocorticographic activity from the left and right frontal (FrL and FrR) and parietal cortices (PL and PR) in two representative rats, a rat from kainate the (KA)-veh group (A); a rat from the KA-los group (B). The first panels are representative baseline EEG recordings before KA-induced status epilepticus (SE), the second panels from the latent phase, and the third panels from the chronic phase after the first spontaneous motor seizure. The theta EEG activity was seen in cortical leads during the latent phase. Spontaneous electrographic seizures during the chronic phase were characterized with large amplitude sharp waves accompanied by a train of spikes and terminating with an isolated spike after a 5-s delay. In the KA-los rat paroxysmal events appeared later and more rarely than in the KA-veh rat. Calibration: 10 s, 100 μV.

neuroprotection in KA-treated rats selectively in the CA1 area of the hippocampus ($^{o}p < 0.05$ vs KA-veh), while the neuronal loss in the CA3 area and septo-temporal hilus of the dentate gyrus was not as severe as in the KA-veh group (Figs. 9C, 10, 11).

A significant decrease in the hippocampal 5-HT levels was detected in the KA-veh group ($^{*}p < 0.05$ vs C-veh) (Fig. 12). The long-term losartan treatment (10 mg/kg) decreased the 5-HT levels in the hippocampus both in the naive and KA-treated rats ($^{*}p < 0.05$ vs C-veh).

4. Discussion

In the present study, daily administration of 10 mg/kg of losartan for 30 days, starting after the onset of the KA-induced SE, alleviated seizure activity through extending seizure-free latent period and decreased the frequency of spontaneous motor seizures. Our findings are in agreement with several other studies, including our recent reports which considered the putative role of AT_1 receptors in seizure phenomena and epilepsy (Lukawski et al., 2010, 2014; Pechlivanova et al., 2011; Pereira et al., 2010). These results correlate well with findings that AT_1 receptors and their mRNA expression are up-regulated in the hippocampus of the Wistar audiogenic rat, a model of TLE (Pereira et al., 2010), as well as in patients diagnosed with TLE (Argañaraz et al., 2008). Our results support the hypothesis that AT_1 receptor subtype should exert a putative role in limbic epilepsy. It is interesting to note that although AT_1 receptor density is moderate or low in other areas involved in seizure propagation, such as the hippocampus, amygdala, and the piriform cortex, both the inhibitory $GABA_A$ receptor complexes and the AT_1 receptors are co-expressed in these structures (Gehlert et al., 1991; Richards et al., 1987; reviewed in Wright et al., 2008). Literature data supports the presumption that blockade of AT_1 receptors may modulate inhibitory responses to GABA and vice versa. Thus, GABA system exerts an inhibitory control on specific neuronal pathways integrated in various central effects of angiotensin II, such as blood pressure responses, drinking and release of vasopressin (Chen and Toney, 2003; Unger et al., 1983). Johren and Saavedra (1996) suggested that angiotensin II regulates GABAergic interneurons in the brain areas associated with excitability changes, which might lead to epileptogenesis.

The present study demonstrated that KA-treated rats were characterized by hyperactivity, low anxiety level, depressive-like behavior and abolished diurnal behavioral rhythms two months after SE, which is in agreement with our previous works (Tchekalarova et al., 2010, 2011, 2013) and those of others (Brandt et al., 2006; Jones et al., 2008; Mazarati et al., 2008; Polascheck et al., 2010). Abnormally increased total activity demonstrated in the OF and EPM tests, which is considered

Table 1
Characterization of cortical paroxysmal activity for each rat in vehicle- or losartan-treated Wistar groups after KA-induced SE.

First monitoring period								
Rat	KA-veh				KA-los			
	No. of paroxysmal events per h	No. of seizures per h	No. of spike-trains per h	Median duration of paroxysmal events (sec)	No. of paroxysmal events per h	No. of seizures per h	No. of spike-trains per h	Median duration of paroxysmal events (sec)
1	11	8	3	1014	5	2	3	148
2	14	8	6	340	15	14	1	933
3	9	8	1	1188	18	11	7	621
4	18	15	3	1639	5	2	3	215
5	32	21	12	1410	20	17	3	1135
6	14	11	3	776	23	14	9	1092
7	20	8	12	618	18	9	9	388
Mean ± SD	17 ± 8	11 ± 5	6 ± 5	455 ± 172	15 ± 7	10 ± 6	5 ± 3	647 ± 413
Second monitoring period								
Rat	Wistar				SHR			
	No. of paroxysmal events per h	No. of seizures per h	No. of spike-trains per h	Total duration of paroxysmal events (sec)	No. of paroxysmal events per h	No. of seizures per h	No. of spike-trains per h	Total duration of paroxysmal events (sec)
1	87	42	35	3334	18	15	3	148
2	69	28	41	2620	52	25	27	933
3	61	25	36	1099	13	12	1	621
4	18	7	11	388	7	2	5	215
5	16	12	14	756	10	9	3	1135
6	n.d.	n.d.	n.d.	n.d.	14	10	9	1092
7	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	9	388
Mean ± SD	50 ± 32	23 ± 14	27 ± 14	1639 ± 1272	15 ± 7	10 ± 6	5 ± 3	647 ± 413

Recordings were performed during two different periods A—the period of losartan treatment (15–30 days after SE) and B—after discontinuation of the treatment (70–90 days after SE), respectively. Paroxysmal activity lasting longer than 5 s was defined as a paroxysmal event. They were subdivided further into seizures, when they were longer than 20 s, and "spike-trains" when they were shorter than 20 s. The median duration and total of paroxysmal events (in sec), the proportion of time spent in paroxysmal activity were determined from at least of 1 h artifact-free recording.

to be an index of impulsivity and increased emotionality (Werboff et al., 1961), reflects disinhibited hyperactive behavior associated with pathogenesis of epilepsy (Brandt et al., 2006; Stafstrom et al., 1993). Selective lesion of afferents from the neocortex to hippocampus impairs exploration in the OF test (Myhrer, 1988). The increased motor activity (total distance in the OF and EPM tests) in epileptic rats suggests a common mechanism underlying SE-induced hippocampal damage and/or damage to other extra-limbic structures contributing to lack of adaptive response and impulsivity. The long-term losartan treatment after SE decreased motor activity both in the naive rats and in the KA-treated rats. A previous report showed that an acute injection of losartan is able to alleviate hyperactivity in hypertensive rats (Srinivasan et al., 2003). The long-term losartan treatment after SE counteracted most of the deleterious behavioral alterations induced during epileptogenesis in a phase-dependent manner. Moreover, losartan restored the disturbed diurnal behavioral fluctuations of motor activity in epileptic rats. However, it

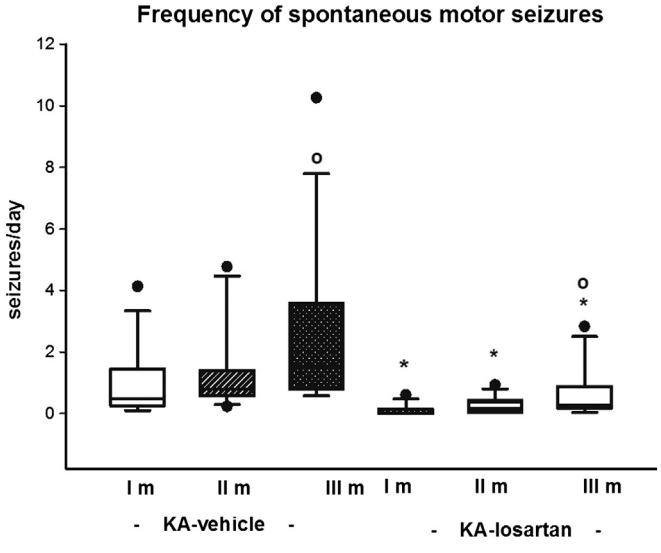

Fig. 3. Frequency of spontaneous motor seizures detected by 24-h video-recording in rats during the 1st, the 2nd and the 3rd months after KA-induced SE. The data represent seizure frequency (number of seizures per day as a function of time of the KA-veh group (n = 10) and the KA-los group (n = 12)). Vehicle and losartan were administered for up to 4 weeks after SE. *p < 0.05 between groups (vs. the KA-veh group); °p < 0.05 within a group (Kruskal–Wallis test).

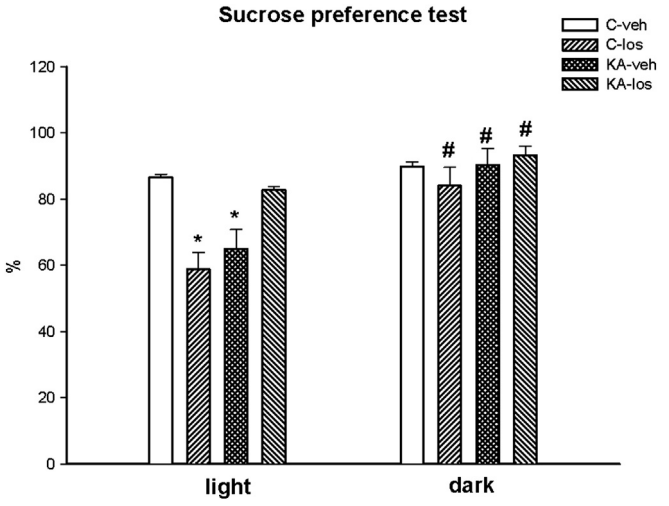

Fig. 4. Sucrose preference test during a 12 h-period (light phase — 08:00–20:00 h and dark phase — 20:00–08:00 h) performed 9 weeks after KA-induced SE. Data are mean ± SEM (n = 10–15). Analysis of data by three-way ANOVA indicated a main Phase effect [$F_{1,90}$ = 44.603, p < 0.001], as well as Condition × Drug × Phase interaction [$F_{1,90}$ = 11.971, p < 0.001]. *p < 0.05 vs controls, #p < 0.05 within a group (15:00 h vs 03:00 h).

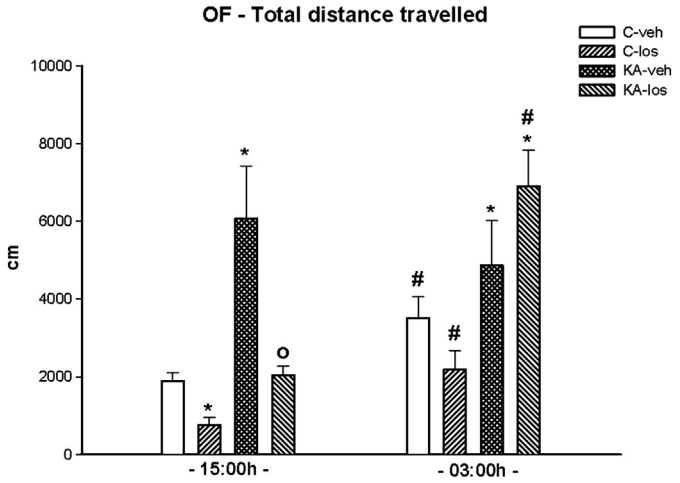

Fig. 5. Diurnal variations of locomotor activity in the open field (OF) test indicated by total distance traveled (cm). Data are mean ± SEM (n = 10–15). Analysis of data by three-way ANOVA indicated: a main Condition effect [$F_{1.88} = 18.631$, $p < 0.001$], Phase effect [$F_{1.88} = 7.191$, $p < 0.009$], as well as Drug × Phase interaction [$F_{1.88} = 3.743$, $p < 0.05$]. *$p < 0.05$ vs controls, °$p < 0.05$ vs KA-treated rats, #$p < 0.05$ within a group (15:00 h vs 03:00 h).

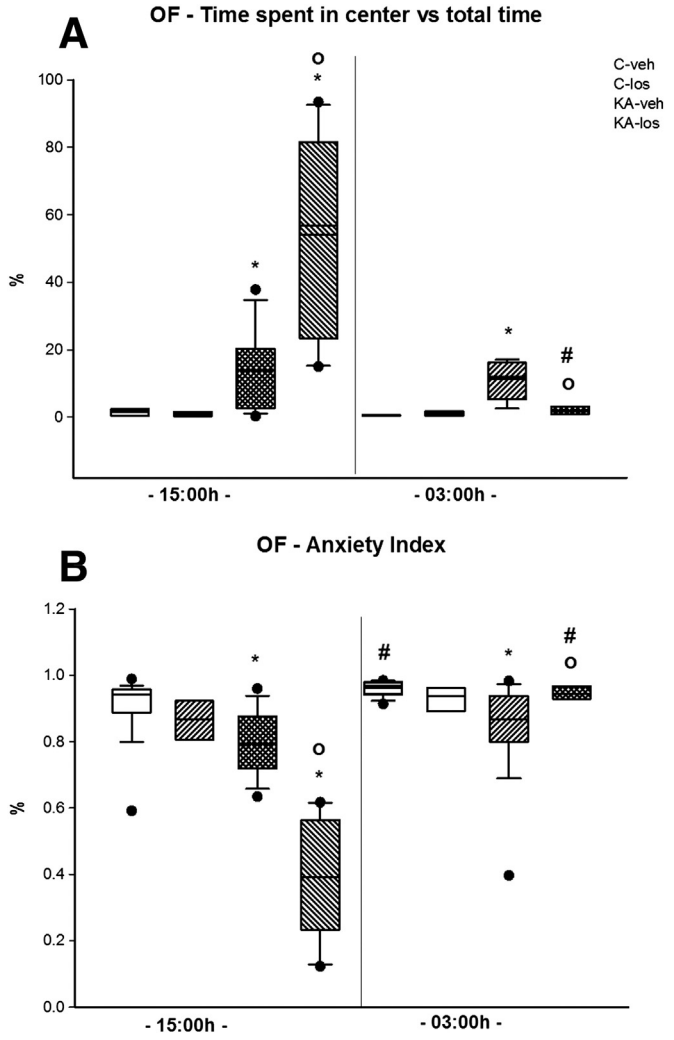

Fig. 6. Diurnal variations of anxiety level in the OF test measured by time spent in the central zone (sec) vs total time spent in the OF (%) (A) and anxiety index (%) (B). Data are mean ± SEM (n = 10–15) Analysis of data by three-way ANOVA indicated: (A) a main Condition effect [$F_{1.62} = 35{,}709$, $p < 0.001$], Drug effect [$F_{1.62} = 5.771$, $p < 0.019$], Phase effect [$F_{1.62} = 18.528$, $p < 0.001$], as well as Condition × Drug × Phase interaction [$F_{1.62} = 15.659$, $p < 0.001$]; (B) a main Condition effect [$F_{1.87} = 50.337$, $p < 0.001$], Drug treatment effect [$F_{1.87} = 21.323$, $p < 0.001$], Phase effect [$F_{1.87} = 66.165$, $p < 0.001$], as well as Condition × Drug × Phase interaction [$F_{1.87} = 27.062$, $p < 0.001$]. *$p < 0.05$ vs controls, °$p < 0.05$ vs KA-treated rats, #$p < 0.05$ within a group (15:00 h vs 03:00 h).

is unclear why these disease-modifying effects of losartan treatment on epilepsy-associated behavioral changes are different during the light and dark phases and that needs further exploration.

Anxiety, psychosis, and aggressive behavior are among the most frequent comorbid psychiatric disorders in patients with epilepsy (Andres and Kanner, 2006). However, several reports, including ours, demonstrated low anxiety level in different experimental models of TLE (Brandt et al., 2006; Detour et al., 2005; Tchekalarova et al., 2010), the behavior of which is difficult to extrapolate to high anxiety in epileptic patients. The damaged networks of the ventral hippocampus, entorhinal cortex and amygdala involved in fear expression and impulsive inadaptive behavior of rats was suggested to underlie the reduced anxiety and impulsive behavior of epileptic rats (Detour et al., 2005). In the present study, losartan restored KA-induced low anxiety level to control level during the dark phase. Again this disease-modifying effect was phase-dependent confirming the idea that AT_1 receptors might be involved in diurnal behavioral variations. There is evidence that the angiotensin system can regulate circadian rhythms in the periphery. Nonaka et al. (2001) reported that angiotensin II is able to influence the circadian gene expression through AT_1 receptor activation in cultured vascular smooth muscle cells. A review of published data on the effect of AT_1 receptor antagonist on anxiety revealed a lack of effect (Shepherd et al., 1996) or anxiolytic effect in rats (Benicky et al., 2011; Kulakowska et al., 1996; Nayak and Patil, 2008; Srinivasan et al., 2003). We found a positive correlation between the beneficial behavioral effects of losartan treatment and neuroprotection in the hippocampus of epileptic rats, which might explain its disease-modifying effect.

The regulatory role of the brain angiotensin system in depression and anxiety has been suggested earlier (Gard et al., 1999). Losartan at a single dose of 10 mg/kg and angiotensin converting enzyme inhibitors have been reported to possess antidepressant and anxiolytic activities in rodents (Nayak and Patil, 2008; Srinivasan et al., 2003). In the present study, the observed antidepressant effect during the light phase and effect on anxiety level during the dark phase of long-term losartan treatment may be directly associated with plastic changes in epileptogenesis.

There was a positive correlation between the low hippocampal levels of 5-HT and the disturbed emotional responses in KA-treated rats during the chronic epileptic phase. Compromised serotoninergic system is associated with impulsive and aggressive behavior, which on their turn is associated with post-status model of TLE and neuronal hyperexcitability in the limbic system (Kondziella et al., 2007). Although losartan pretreatment elicited antidepressant effect in epileptic rats during the light phase, receptor antagonist did not affect KA-induced low hippocampal 5-HT levels. In contrast, long-term losartan treatment caused a marked decrease of 5-HT levels in the hippocampus of control and epileptic rats. The latter finding supports a previous report by Nahmod et al. (1978) in which a biphasic effect of angiotensin II on 5-HT release and synthesis i.e. stimulating at high doses and inhibiting of the octapeptide at low doses had been demonstrated. Our results agree with previous reports where KA-treated Wistar rats were characterized with neuronal damage in the hippocampus and the piriform cortex during chronic phase (Tchekalarova et al., 2010, 2013). In the present work, long-term losartan treatment exerted a significant neuroprotection, which is in line with numerous findings showing that AT_1 receptor antagonists are able to protect cerebral blood flow during stroke, decrease brain inflammation, β-amyloid and glutamate-induced neurotoxicity, and reduce traumatic brain injury (Benicky et al., 2011; Danielyan et al., 2010; Ito et al., 2002; Saavedra et al., 2011; Timaru-Kast et al., 2012; Villapol et al., 2012; Wang et al., 2014). A possible explanation for this beneficial neuroprotective, as

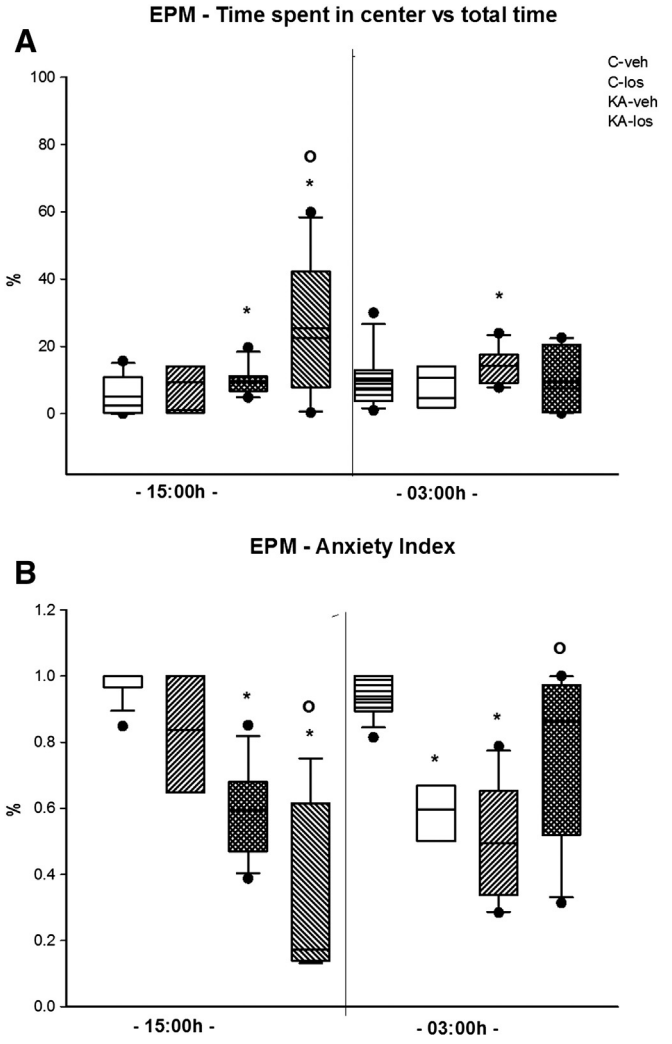

Fig. 7. Diurnal variations of anxiety level in the elevated plus maze test measured by time (sec) spent in open arms vs total time in closed and open arms (%) (A) anxiety index (%) (B). Data are mean ± SEM (n = 10–15) Analysis of data by three-way ANOVA indicated: (A) a main Condition effect [$F_{1,72} = 96.575$, $p < 0.001$], Phase effect [$F_{1,72} = 6.941$, $p < 0.001$], as well as Condition × Drug × Phase interaction [$F_{1,72} = 14.550$, $p < 0.001$]; (B) a main Condition effect [$F_{1,80} = 69.406$, $p < 0.001$], Drug effect [$F_{1,80} = 11.733$, $p < 0.001$], Condition × Phase interactions [$F_{1,80} = 22.400$, $p < 0.001$], as well as Condition × Drug × Phase interaction [$F_{1,80} = 28.627$, $p < 0.001$]. *$p < 0.05$ vs controls, °$p < 0.05$ vs KA-treated rats, #$p < 0.05$ within a group (15:00 h vs 03:00 h).

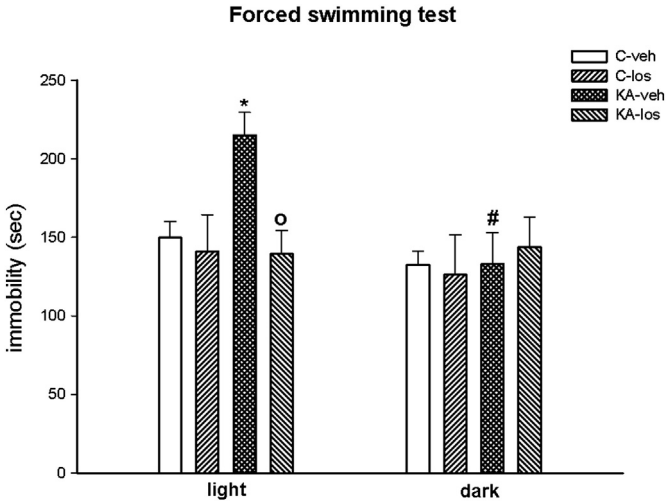

Fig. 8. Diurnal variations of immobility time (sec) in the forced swim test. Data are mean ± SEM (n = 10–15). Analysis of data by three-way ANOVA indicated: a main Condition effect [$F_{1,94} = 6.896$, $p < 0.010$], as well as Condition × Drug × Phase interactions [$F_{1,94} = 4.691$, $p < 0.033$]. *$p < 0.05$ vs controls, °$p < 0.05$ vs KA-treated rats, #$p < 0.05$ within a group (15:00 h vs 03:00 h).

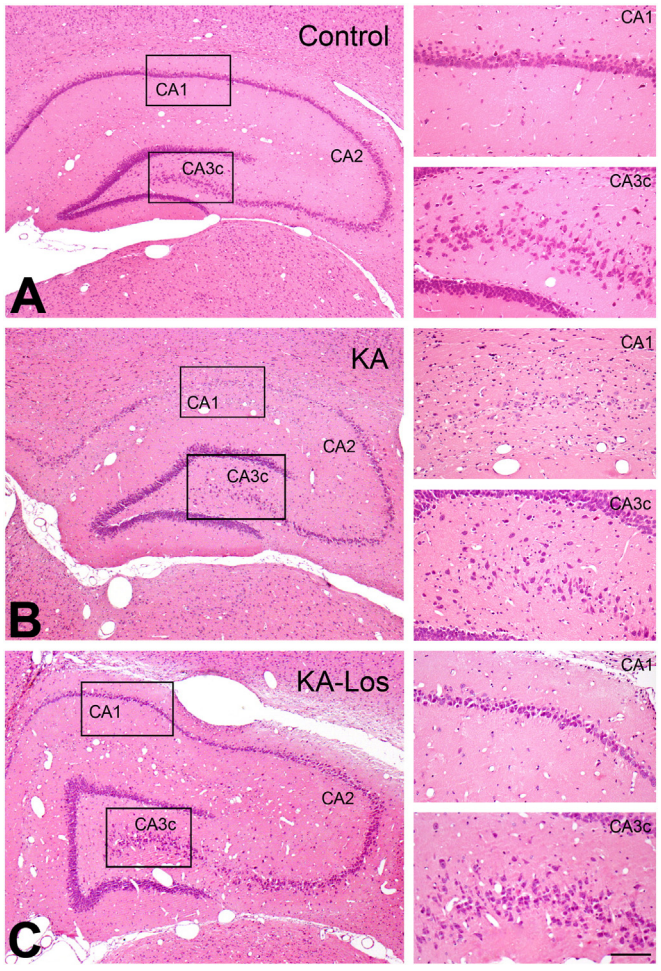

Fig. 9. Representative conventionally H&E-stained sections of the hippocampal formation of a control rat (A), a KA-veh rat (B), and KA-los rat (C). The representative images on the right panels are higher magnifications of the boxed areas in the left images from the CA1 and CA3c areas of the hippocampus, respectively. Please note that the KA-veh rats (B) showed severe neuronal loss in CA1 and CA3 pyramidal cell layers, and the hilus of the dentate girus (DG) when compared to the control rats (A). In contrast, KA-los rats (C) did not differ from controls (A). Scale bars = 200 μm (A–C, left) and 75 μm in high-resolution insets (A–C, right).

well as seizure-alleviating effect, might include the antioxidant activity of AT_1 receptor antagonists (de Cavanagh et al., 2004). Increased ROS production is reported during the acute seizure phase of KA-induced SE in a number of brain areas, particularly in the hippocampus (Atanasova et al., 2013). Recently, we have found that chronic systemic infusion with losartan via osmotic mini-pumps at a dose of 10 mg/kg prevents the KA-induced oxidative stress and neurotoxicity in both normotensive Wistar rats and spontaneously hypertensive rats (Tchekalarova et al., 2013). The observed effectiveness of this AT_1 receptor antagonist against KA-induced neurotoxicity and oxidative stress might underlie the partial restoration of behavioral responses associated with motor activity and anxiety.

5. Conclusion

Although losartan, the AT_1 receptor antagonist, did not prevent the development of epileptogenesis, it exerted disease-modifying effects on seizure activity, neuronal damage, behavioral abnormalities and

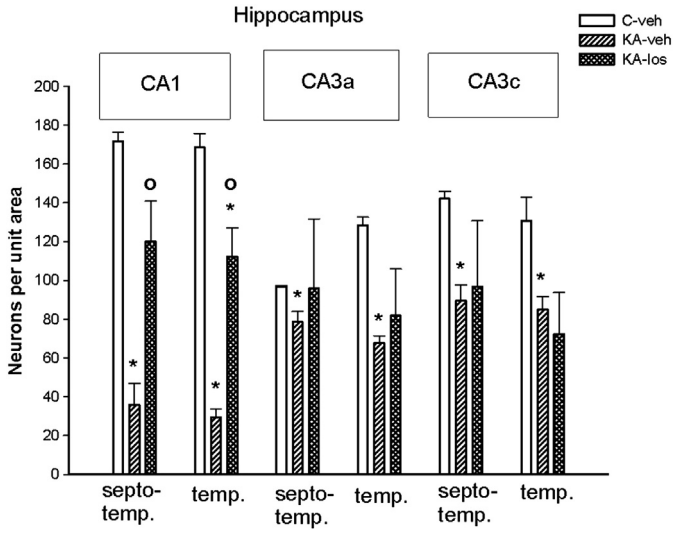

Fig. 10. Effect of long-term losartan treatment on KA-induced temporal lobe epilepsy on the histology scores. Neuronal damage in the hippocampus (septo-temporal and temporal) — CA1, CA3a and CA3c pyramidal cell layers. Analysis of data by two-way ANOVA indicated: CA1 area (septo-temporal), a main Condition effect [$F_{1.8} = 76.076$, $p < 0.001$] and Drug effect [$F_{1.8} = 22.693$, $p < 0.003$]; (temporal), a main Condition effect [$F_{1.9} = 125.803$, $p < 0.001$] and Drug effect [$F_{1.9} = 44.660$, $p < 0.001$]. CA3a area (temporal), a main Condition effect [$F_{1.9} = 12.017$, $p < 0.010$]. CA3c area (septo-temporal): a main Condition effect [$F_{1.8} = 9.039$, $p < 0.024$]; (temporal): a main Condition effect [$F_{1.8} = 5.923$, $p < 0.045$]. *$p < 0.05$ vs. C-veh group; °$p < 0.05$ vs KA-treated rats.

disturbed diurnal variability of motor activity in epileptic rats. The data suggests that the brain AT_1 receptor might play a significant role in neuronal injury after SE and unveils its role in abnormal as well as normal behavior.

Acknowledgements

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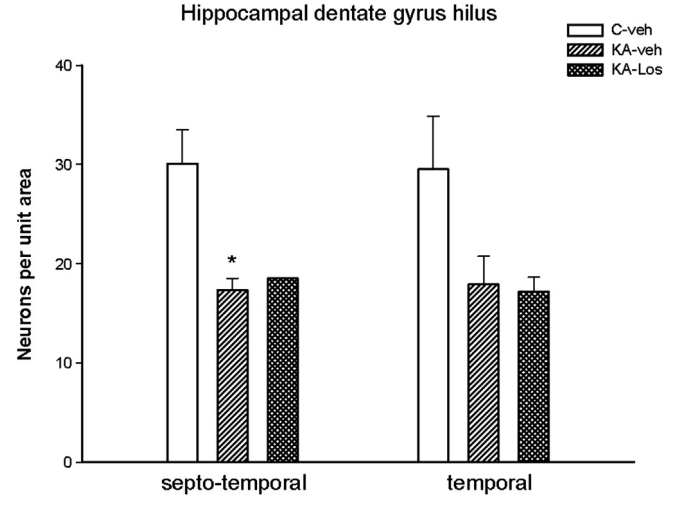

Fig. 11. Effect of long-term losartan treatment on KA-induced temporal lobe epilepsy on the histology scores. Neuronal damage in the DG (septo-temporal and temporal) (A) and in the hilus of DG (septo-temporal and temporal) (B). Analysis of data by two-way ANOVA indicated: in the hilus of DG (septo-temporal): a main Condition effect [$F_{1.8} = 19.547$, $p < 0.004$]; (temporal: a main Condition effect) [$F_{1.9} = 5.777$, $p < 0.047$]. *$p < 0.05$ vs. C-veh group; °$p < 0.05$ vs KA-treated rats.

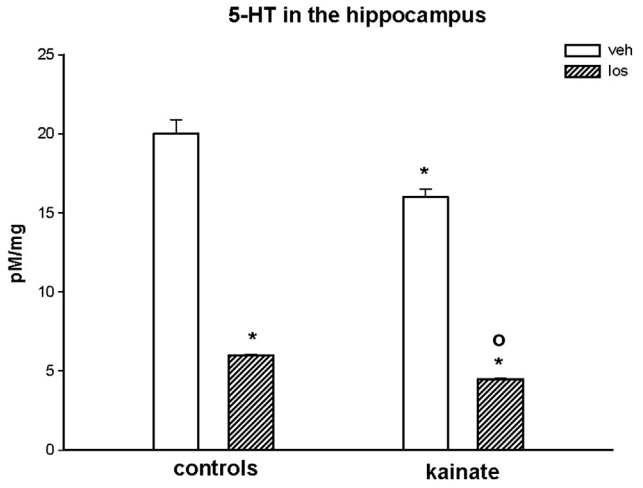

Fig. 12. Hippocampal concentration of 5-HT in pM/mg wet tissue measured by HPLC method. Data are mean ± SEM (n = 10–15). Two-way ANOVA + Bonferroni test: a main Condition effect [$F_{1,45} = 4.668$, *$p < 0.036$], Drug effect [$F_{1,45} = 13.833$, *$p < 0.001$]; Condition × Drug interaction [$F_{1,45} = 3.838$, *$p < 0.05$]. *$p < 0.05$ vs. C-veh group.

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