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ORIGINAL PAPER

Electroencephalographic Characterization of Pentylenetetrazole Kindling in Rats and Modulation of Epileptiform Discharges by Nitric Oxide

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Abstract Epileptogenesis is a progressive process which culminates with spontaneous, recurrent and unpredictable epileptic seizures due to enhanced neuronal excitability. Well-characterized animal models of this process are needed to clarify its underlying molecular mechanisms, in which the role of nitric oxide has been a controversial component. We have used kindling with a sub-convulsive dose of pentylenetetrazole to objectively characterize early electroencephalographic changes during epileptogenesis. We used electroencephalographic recordings both during pentylenetetrazole (20 mg/kg) kindling for 20 days and then, 24 days later to quantify the number, duration and spectral power of epileptic discharges. The levels of nitric oxide were modulated locally in the cerebral cortex by pharmacological agents. The number of epileptiform discharges increased during the kindling protocol as well as 24 days later, revealing the induction of a self-sustaining epileptogenic process. Epileptic discharges were characterized by theta frequencies (4–10 Hz) that were associated with absence-like seizures. However, during kindling, the spectral power of the theta band progressively decreased, while the power of higher frequencies, in the beta band, increased. Nitric oxide in the cerebral cortex inhibited the number and amplitude of epileptic discharges. The electroencephalographic characterization of this kindling protocol provides a valuable tool to detect consequences of therapeutic interventions undertaken at initial phases of epileptogenesis, especially those targeted towards stopping this process. Increases of nitric oxide in the cerebral cortex could be a useful intervention to negatively modulate neuronal excitability, epileptic discharges and the progression of epileptogenesis.

Keywords Pentylenetetrazole · Epileptic discharges · Fourier transform · Nitric oxide

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Introduction

In adults, epilepsy develops following a brain insult by a process termed epileptogenesis, which compromises most frequently susceptible temporal lobe structures [1, 2]. Although a large number of experimental approaches exist to model seizures and epilepsy in animals, their usefulness in understanding the diagnosis, pathophysiology and development of therapeutic interventions for clinical epilepsies remains controversial [3, 4]. The outcome of these experimental strategies is most frequently detected at the behavioral level. However, behaviors vary depending on the convulsant agent or epilepsy model in use. Thus, although Racine's scale is commonly used across the literature to assess seizure intensity [5], this behavioral categorization is not applicable to kindling induced by pentylenetetrazole (PTZ), an agent that blocks type A α-aminobutyric acid ($GABA_A$) receptors [6–8]. To generate a more precise characterization of this widely used model, electroencephalographic (EEG) analysis of epileptic discharges, i.e. the consequence of synchronous neuronal firing, is a powerful tool. This approach might be especially relevant in cases where EEG abnormalities precede behavioral manifestations of epilepsy, as observed in the present work.

Epileptogenesis is initiated through over-stimulated glutamatergic networks, by increasing intracellular Ca^{2+} and the activation of Ca^{2+}-dependent enzymes such as neuronal nitric oxide synthase (nNOS), thereby leading to excitotoxicity [9, 10]. While nitric oxide (NO) in the hippocampus shows pro-convulsant properties [11, 12], it has also been reported to have the opposite effect, i.e., anti-convulsant properties [13–15]. In many of these cases, the strategy used to modulate NO levels has been systemic injection of pharmacological agents. We have recently found that NO protects against excitotoxicity in cortical neurons but is neurotoxic in hippocampal neurons, indicating that opposing effects on excitability are cell-type specific [16]. In the present studies, we characterized kindling electroencephalographically using a dose of 20 mg/kg PTZ and tested the in vivo modulation of excitability by NO in the non-limbic cerebral cortex. Interictal epileptic discharges (EDs) were observed and their time course, duration and frequency components were analyzed. We found that EDs, even in the absence of any behavioral signs, were present after the first PTZ injection. The number of EDs increased over the days following the last PTZ administration, reflecting the progressive nature of epileptogenesis even at this low PTZ dose. Furthermore, NO reduced the number and duration of EDs, revealing a protective role for this messenger in the cerebral cortex. Our results reveal that NO in the cerebral cortex might have profound effects on the initiation and propagation of EDs.

Materials and Methods

Animals and Surgical Procedures

Adult male Sprague–Dawley rats (250–300 g) were obtained from the Animal Facilities at Pontificia Universidad Catolica de Chile. The present procedures and protocols were approved by the Bioethical Committee of Universidad de los Andes. Experiments were conducted in accordance with Conicyt (Chile) Bioethical Guidelines (approved under law # 20,380 (2009) of the Ministry of Health). In total, 32 adult male rats were used, 29 of them were subjected to kindling with PTZ and $n = 3$ were injected with saline. Of the PTZ-injected rats, $n = 9$ were used for the EEG characterization of the kindling process including day 20, the first non-injection day after completion of the kindling protocol. Because in most animals the electrodes came loose due to continuous recordings in the presence of seizures, a different group of animals ($n = 5$), not recorded during kindling, were used for recordings 24 days after completion of the kindling process. Different rats were used for behavioral analysis ($n = 8$), and for modulation of EDs by NO ($n = 7$). The saline-injected rats (n3) were used primarily to demonstrate that the sole injection procedure did not evoke seizures. The rats were maintained in isolated cages under a stable 12:12 light dark cycle. The light intensity was set at 300 lux at floor level during light hours, ambient temperature was maintained at 21–23 °C, and food and water were available ad libitum.

Electrode and Cannula Implants

The rats were anesthetized with ketamine/xylazine (50/5 mg/kg) and implanted with three cranial epidural electrode screws (Fine Science Tools Inc., North Vancouver, BC, Canada) for the EEG recording and with two stainless steel neck muscle electrodes (Medwire Corp., Mt. Vernon, NY) for the electromyogram (EMG), as described by Ocampo-Garcés [17] (Fig. 1d). For the EEG, two electrodes were placed at 2.5 mm posterior to bregma and 4.0 mm lateral to the midline both on the right and the left hemispheres, while the reference electrode for bi-polar recordings was placed at the midline at 2.0 mm posterior to lambda. The guide cannulas (Plastic One, 26G and 1 mm of length were implanted close to the recording electrodes with an anterioposterior angle of 11° at the level of bregma and 4 mm lateral to the midline (Fig. 1d). Injection cannulas (Plastic One, 33G and 1.3 mm of length), used to inject substances with a syringe, were introduced through the guide cannula. Cranial anchoring screws and dental acrylic was used to attach electrodes and cannulas to the skull. At the end of the surgery, analgesic (ketoprofen, 5 mg/kg) and antibiotic (enrofloxacin 0.2 mg/kg) were administered and maintained for 3 days. During recording sessions, the animals were placed in cages (30 × 40 × 30 cm) in sound attenuated isolation chambers (65 × 60 × 60 cm).

Histological Analysis

After completion of the experiments, the rats were perfused with 4 % paraformaldehyde under deep ketamine (50 mg/kg)/xylazine (5 mg/kg) anesthesia to reduce maximally animal suffering, after which their brains were removed, dehydrated, cut into coronal slices and Nissl stained to confirm the cannula location in the somatosensory cortex.

Drugs and Experimental Protocols

PTZ (Sigma, St. Louis, MO, USA, Catalogue Number P6500) was dissolved in saline solution (40 mg/mL) for intra-peritoneal (i.p.) administration at a dose of 20 mg/kg. PTZ was administered 2 h after turning on lights, and immediately thereafter the 2-h lasting recording session began. The time course of the experimental protocol is shown in Fig. 1. After surgery, the rats recovered for 7 days. Baseline recordings were performed for 2 h daily

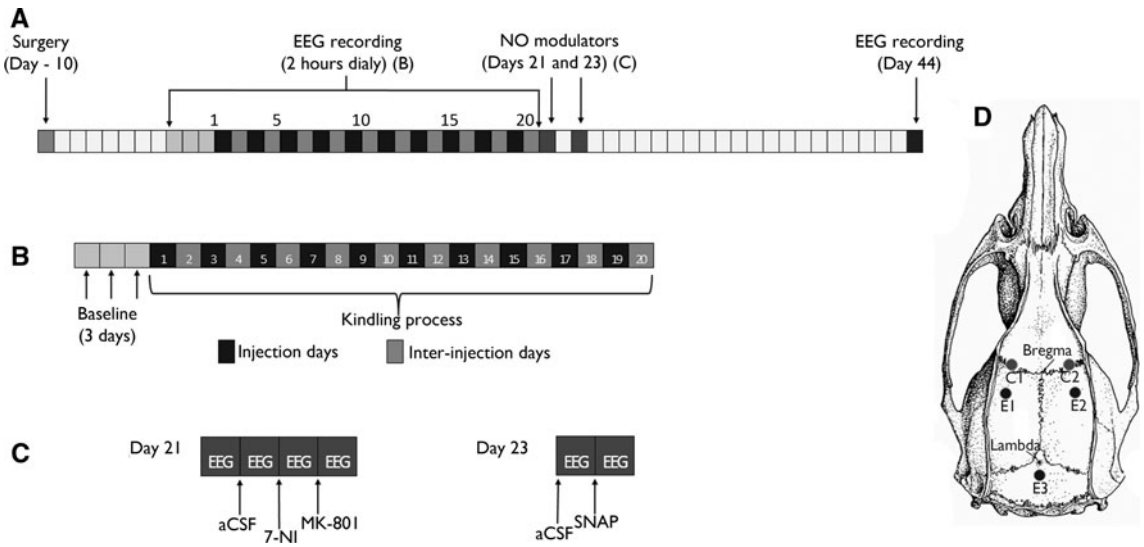


Fig. 1 The scheme of the electrode placement and the experimental design is shown. **a** To perform EEG recordings, dural recording electrodes were implanted 10 days before beginning with PTZ injections (*arrow*, surgery). After 7 days of recovery, the baseline EEG was obtained for 2 h on 3 consecutive days (*light gray*). The kindling process was carried out for 20 days to perform: EEG recordings during the kindling protocol including day 20, the first non-injection day after completion of the protocol (n = 9) and EEG recordings 24 days after completion of the kindling protocol (n = 5). In different groups, kindling was characterized behaviorally (n = 8) or cannulas were implanted to test NO modulators on days 21 and 23 (n = 10). **b** The kindling protocol consisted of 10 PTZ injections (odd days, *black*) spaced by inter-injection days (even days, *grey*). Days during which baseline recordings were performed are indicated by *arrows*. **c** On days 21 and 23, the NO levels were modulated. After completion of the kindling process, the EEG was recorded on day 21 and the following substances were sequentially delivered through implanted cannulae: artificial cerebrospinal fluid (aCSF) as a control, the preferential nNOS inhibitor 7-NI, or the NMDA receptor antagonist MK-801. The intervals between applications were 90 min, and the EEG was continuously recorded. On day 23, 0.5 % DMSO in aCSF and the NO donor SNAP (dissolved in 0.5 % DMSO) were applied. **d** Electrode location on the skull surface (adapted from Paxinos and Watson 1998) is indicated by E1 and E2 while cannula placement is indicated by C1 and C2. E3 was the reference electrode

during the 3 days preceding the first PTZ injections. Thereafter, 10 doses of PTZ were administered every other day. Recordings were performed for 2 h after PTZ injection, considering the rapid effect of the drug (within minutes) and a half-life of about 2 h [18, 19]. In a different group, the EEG was recorded 24 days after completion of the kindling protocol, i.e., on day 44, when, based on the pharmacokinetic properties of PTZ, the drug had been eliminated from the organism. Seizure intensity was categorized by a blind observer according to the scale of Luttjohann, that includes absence-like seizures [8].

After completion of kindling, i.e. on days 21 and 23, 0.5 μL of NO modulators were manually applied with a syringe during 1 min (corresponding to ~8 nl/s) through the implanted cannulas. In this case, the control recording was not the baseline EEG but the EEG obtained after 10 injections of i.p. saline instead of PTZ. In such a way, it could be demonstrated that the stress of the sole injection procedure did not induce EDs. To modulate NO levels, stock solutions of 200 μM *S*-nitroso-*N*-acetyl-D, L-penicillamine (SNAP, Cayman Chemical # 82250) and 300 μM 7-nitroindazole (7-NI, Cayman Chemical # 81340) were dissolved in 0.5 % dimethyl sulfoxide (DMSO) in artificial cerebrospinal fluid (aCSF) with the following composition: 124 NaCl, 26 $NaHCO_3$, 3 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 1.25 KH_2PO_4, and 10 glucose (in mM) at pH 7.3–7.4. 5 μg/μL (+)-MK-801 hydrogen maleate (MK801, Sigma # M107) was prepared in aCSF. The corresponding vehicle and volume controls (0.5 μL) for each test agent was performed using 0.5 % DMSO or aCSF.

Electroencephalographic and Electromyographic Recordings

To allow recovery, recordings started one week after surgery. The rats were connected to the recording system by means of a counterbalanced cable attached to a slip-ring (Airflyte Electronics, Bayonne, NJ). A computer-based data acquisition system sampled, displayed and stored the EEG and EMG signals. Signals were sampled at 250 Hz and acquired by means of an amplifier (Grass Model 15LT, Astromed, Inc., West Wartwick, RI) after analogue filtering and conditioning of signals (notch filter at 50 Hz). The EEG channel was set at band-pass 0.5–30 Hz and amplified by a factor of 2,500–5,000 for a 1 V input. The band-pass for EMG was set at 30–100 Hz and amplified by a factor of 5,000.

State Scoring

Successive 5-s epochs were visually assigned to wakefulness, NREM sleep or REM sleep by off-line analysis. Each epoch was assigned to the state that occupied more than 50 % of that epoch. Wakefulness was identified by the presence of desynchronized EEG and phasic EMG activity. NREM sleep presented high-amplitude slow-wave (1–4 Hz) EEG activity accompanied by sleep spindles (10–15 Hz) and low neck muscle tonus. REM sleep was identified by the presence of desynchronized EEG and sustained theta (4–10 Hz) activity coupled with low EMG tone relative to NREM sleep. EEG epochs with movement artifacts were excluded from analysis.

Frequency-Domain Analysis of EEG

Portions of EEG recordings (5 s epochs or EDs of variable length) were analyzed in the frequency domain with Fourier analysis. A Hanning operation was applied to avoid spectral artifacts. State-specific and event-related spectrograms of the EEG were obtained ranging from 1 to 30 Hz and resampled to a resolution of 0.5 Hz. The total power (spectrogram area) was normalized to 1. To evaluate the contribution of specific spectral bands, the power spectra were further collapsed into discrete bands [20]: delta (1.5–4 Hz), theta (4–10 Hz), low beta (10–15 Hz) and high beta (15–30 Hz) [20]. Thus, the calculated area under the curve in each of these discrete bands corresponds to a fraction of the total spectral power.

Identification of Epileptiform Discharges (EDs)

After each PTZ injection, EDs emerged as conspicuous graphoelements in the EEG record. The typical ED displayed a fusiform high-amplitude spike-wave pattern. The time span of each event was estimated visually by defining a starting and ending time (Fig. 2b, c). Events lasting less than one second were discarded (i.e. less than 3 cycles) while two consecutive EDs separated by less than a second were considered to be part of a single event.

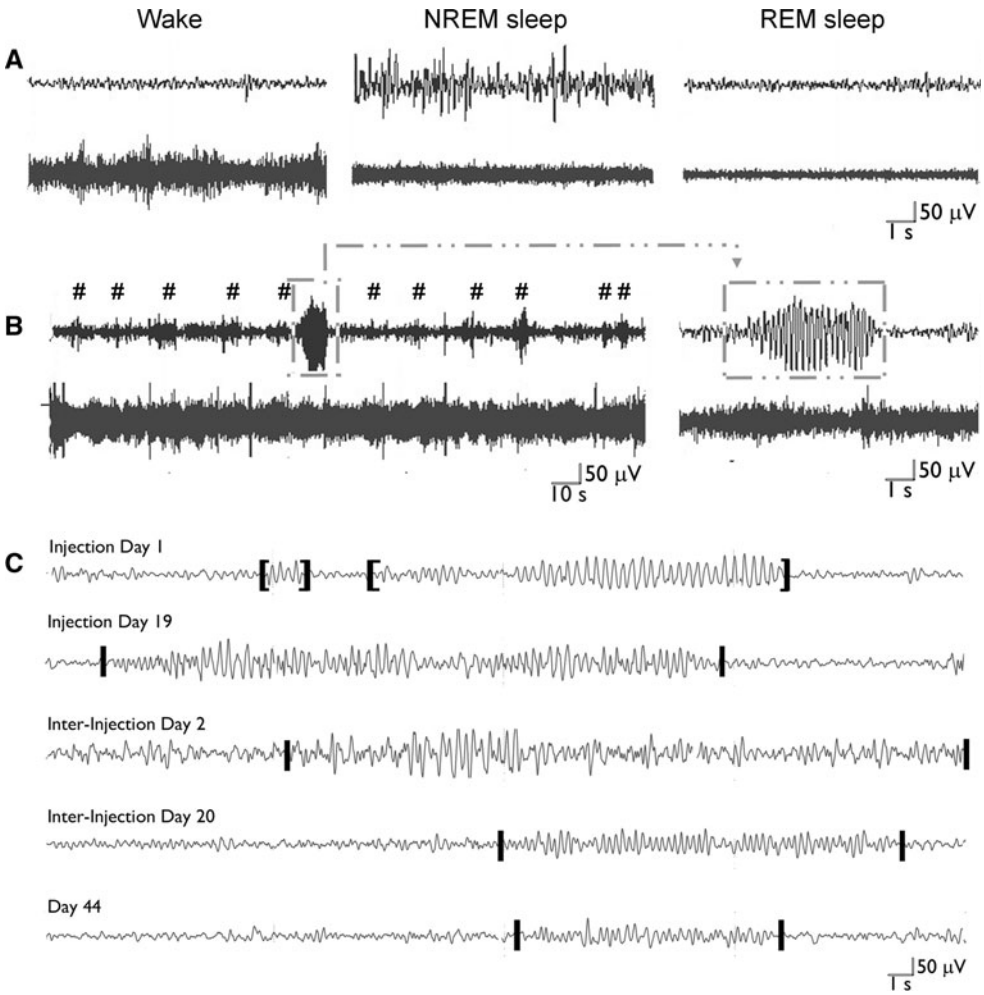


Fig. 2 Epileptiform discharges (EDs) occur during wakefulness. **a** EEG and EMG recordings of the sleep-wake states were performed in rats during baseline days prior to the PTZ injection protocol. Representative traces of each of the sleep-wake states: wakefulness, non-REM and REM, are shown in 2-epoch traces (10 s). **b** After PTZ injection, EDs appeared in the EEG exclusively during wakefulness. A representative recording at different time scales is shown. The time scale in the *left panel* is the same as in **a**. # marks EDs; the ED shown in the *box* is presented in an extended timescale on the *right*. **c** Representative examples of EDs are shown from different days of the protocol. High amplitude rhythmic discharges separated by less than 1 s were considered part of a single ED. In the first trace, 2 consecutive EDs were separated by more than 1 s. In this case, each ED is shown *between brackets*. In the following traces, *two vertical lines* indicate the beginning and end of each ED

Data Analysis and Statistics

The analyzed variables were the number, mean duration (in seconds) and spectral power of the EDs. The number of EDs was expressed as the EDs per minute of wakefulness. The statistics for the power spectra were performed on normalized data, i.e., the area under each spectral power curve was set at 1.

To evaluate the progression of the epileptogenic process in terms of number of EDs, a mixed model Poisson regression analysis for repeated measures was used, while the duration of EDs was analyzed by a mixed model analysis of variance (ANOVA) for repeated measures. In turn, the spectral power of bands across time was subjected to mixed model trend analysis for repeated measures (nested in rats). The power of each frequency was analyzed by a paired Student's *t* test.

EDs (number and duration and spectral power) on days 20 and 44 were compared with two-tailed unpaired Student's *t* tests while after modulation of NO levels, EDs were compared by two-tailed paired Student's *t* tests.

Results

Epileptiform Discharges (EDs) in EEG Recordings

To assess the quality of the recordings of each rat and the characteristics of the normal EEG prior to kindling, recordings were acquired for 2 h during 3 days before the initiation of the kindling protocol (baseline days, indicated by arrows in Fig. 1b). As expected, the three stages of the sleep-wake cycle: wakefulness, non-REM sleep and REM sleep, could be identified (Fig. 2a). The kindling protocol started with a PTZ dose of 20 mg/kg, which is subthreshold for seizure induction. Following the first PTZ injection, EDs were observed in the electroencephalographic recording during wakefulness, but never during sleep, without any accompanying behavioral abnormalities. Total sleep during the 2 h recording period, diagnosed on a base of 5 s epochs, was longer on inter-injection days (mean percentage ± SEM, non-REM: 26.6 ± 3.3 %; REM 2.1 ± 1.1 %) than on injection days (non-REM: 4.6 ± 1.4 %; REM 0.1 ± 0.2 %) ($p = 0.0002$ and $p = 0.0004$, respectively). EDs were identified by their distinctive high amplitude and slow frequency, which could be ascribed to the theta range (Fig. 2b, c). These EEG events were distinguishable from REM sleep because in REM sleep, muscle tone, recorded by the electromyogram (EMG), was absent. In addition, when behavioral signs appeared, based on the classification of Luttjohann [8], i.e. absence seizures and facial and neck jerks, after the 5th PTZ injection, and tonic–clonic seizures after the 8th injection, all of them occurred exclusively during wakefulness. The length of EDs was determined visually, as depicted in Fig. 2c, and single events could be interrupted with a low voltage and high frequency EEG of less than 1 s. If the intervals were longer than 1 s, the two consecutive EDs were counted as independent events. The mean amplitude of such events was 6.6 ± 0.05 times larger than the amplitude of the preceding or successive EEG recording epochs on injection days and 5.4 ± 0.04 larger on inter-injection days (n = 5, $p < 0.001$ in both cases). The Fourier analysis of EDs in the frequency domain revealed a large power increase in the theta band (4–10 Hz). Therefore, the presence of muscle tone and twitches and a direct transition from wakefulness, but never from non-REM sleep, were additional criteria used to identify EDs. Note that EDs occurred from the very beginning of the protocol, when behavioral manifestations were still absent (Fig. 3). In Fig. 3, the mean of the highest seizure score per rat was plotted along the PTZ injection days, revealing a remarkable small variability of the development of kindling-like behaviors along the protocol.

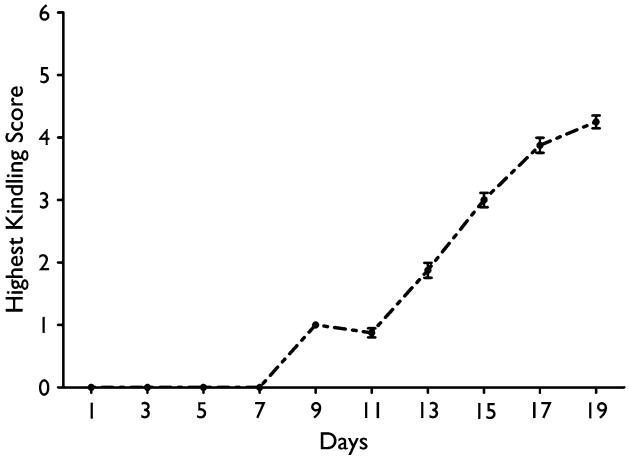
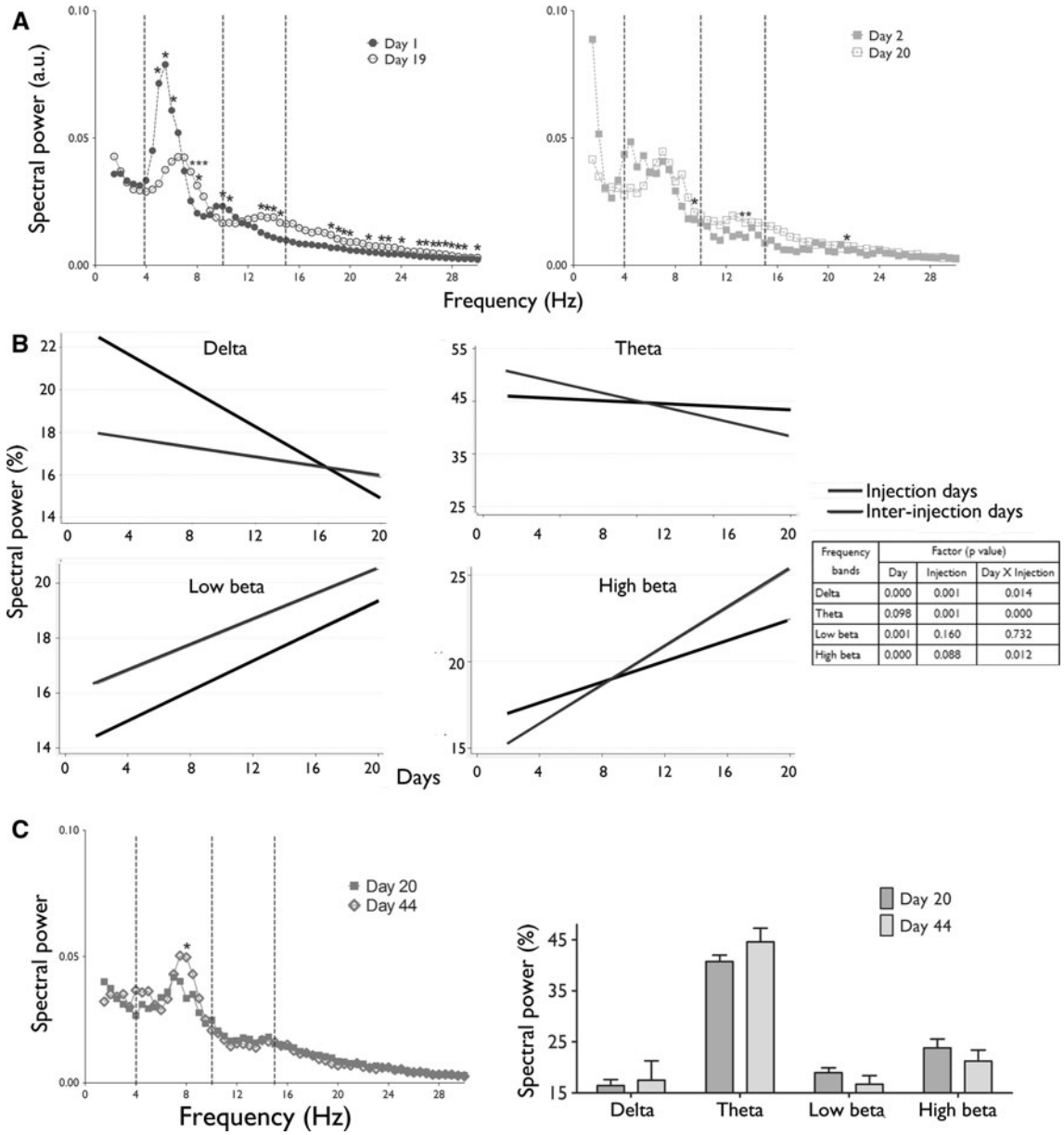


Fig. 3 Seizures along the kindling protocol were categorized following the scale of Luttjohann. The maximum seizure score reached for each rat on consecutive days was used to calculate its mean value for n = 8 rats. After the 5th PTZ injection, i.e. on day 9 of the protocol, the first absence-like seizures were observed. Maximum seizure intensity increased over the protocol reaching stage 5 in most rats on day 19

Fourier Analysis of EDs During Kindling

To obtain further insight into the stability of the spectral power of EDs, Fourier analysis was performed during the 20 consecutive days of the kindling protocol (Fig. 4). Note in Fig. 4a (left panel) that the high power theta frequencies (4–10 Hz), conspicuously observed during the first injection day, became depressed compared to the last injection day (i.e. 5, 5.5, 6, 7.5, 8 and 10 Hz). Similarly, the power at 11.5 Hz decreased on inter-injection days (right panel)



Frequency bands	Factor (p value)		
	Day	Injection	Day X Injection
Delta	0.000	0.001	0.014
Theta	0.098	0.001	0.000
Low beta	0.001	0.160	0.732
High beta	0.000	0.088	0.012

Fig. 4 The spectral power of the theta band decreased during the kindling protocol, but the spectral power of the beta bands increased. **a** *Left* spectral profiles on injection days 1 and 19 of the protocol were plotted; *Right* spectral profiles on non-injection days 2 and 20 are shown. At each frequency, the power among curves was compared by a 2-tailed paired *t* test. *Vertical lines* indicate separations between the bands: delta (0–4 Hz), theta (4–10 Hz), low beta (10–15 Hz) and high beta (15–30 Hz). **b** The spectral power data during PTZ administration was subjected to a mixed model trend analysis for repeated measures (nested by subject). The *inset* shows the *p* values of the factors: day, injection and the interaction of both, n = 5 rats. **c** *Left* the spectral profiles on days 20 and 44 are shown. At each frequency, the power among *curves* was compared by a 2-tailed unpaired *t* test. *Right* the mean spectral power of each EEG band was normalized to the total area under the *curve* (100 %). $*p < 0.05$, $***p < 0.001$

while in both cases, an increase of the powers at beta frequencies was observed .6. The trends of the mean spectral power bands of the EDs obtained on injection and non-injection days are shown in Fig. 4b, indicating significant decreases of the delta and theta bands but increases in the beta bands. The inset indicates the p-values of the factors day, injection, and the interaction of both factors. Therefore, the tendencies of the beta band did not significantly differ between injection and inter-injection days. However, the day of the protocol (i.e., the number of the injection) affected the spectral power of each of the defined bands. As epileptogenesis progressed through day 44, the power at 8.5 Hz increased (Fig. 4c, left panel) while no changes in the bands were visible (Fig. 4c, right panel).

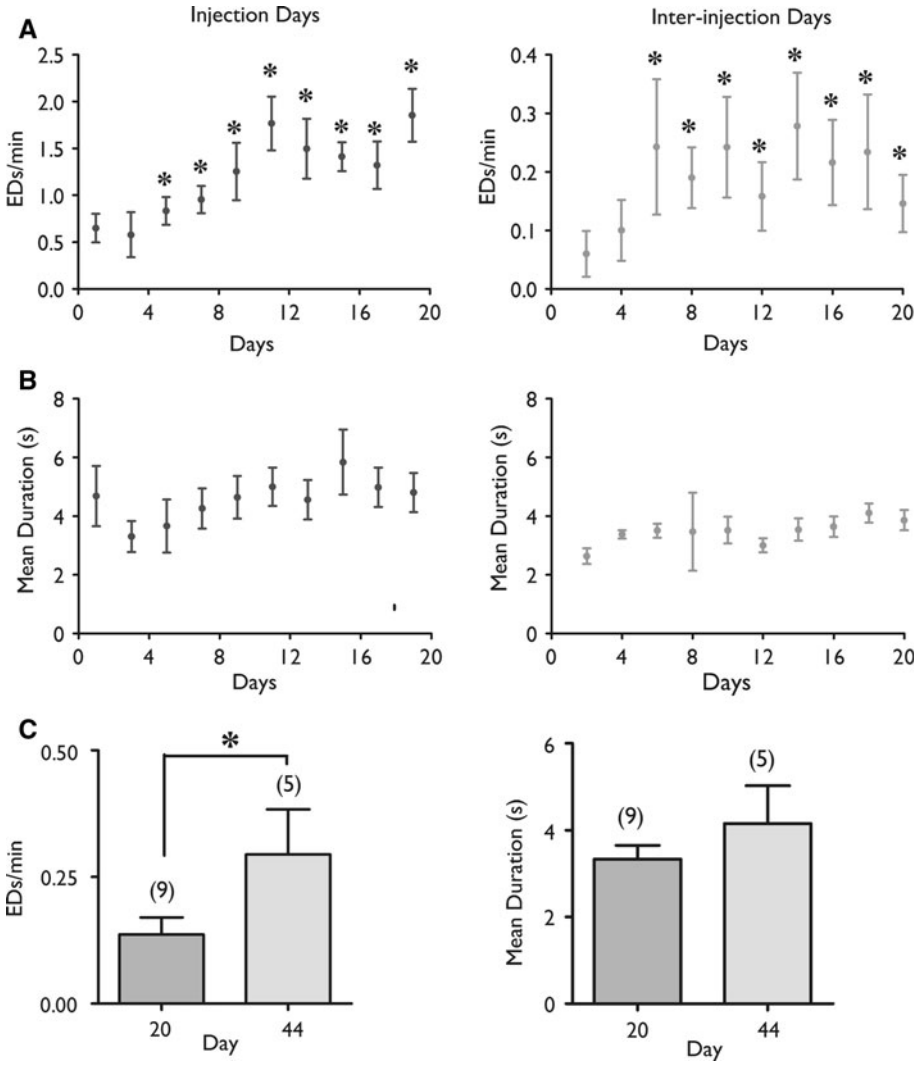


Fig. 5 The number and duration of EDs increased during the kindling process. **a** The numbers of EDs on injection (*left*) and inter-injection (*right*) days are shown. Note the different scales on the *Y* axis (n = 5 rats). **b** The durations of the EDs on injection and inter-injection days are shown (n = 5 rats). **c** The numbers and durations of EDs on day 20 (non-injection day) and day 44 are shown (n = 9 and 5, respectively). $^{*}p < 0.05$

Taken together, this analysis reveals a continuous re-organization in the frequency domain.

The number (Fig. 5a) and duration (Fig. 5b) of EDs was quantified during the 2-h daily recordings. The number of EDs was expressed as EDs per minutes of wakefulness because EDs occurred exclusively in this state. On injection days (left panels), the probability of an ED increased at a rate of 10.9 ± 0.5 %, while on inter-injection days (right panels), the probability increased by 5.4 ± 1.7 % (Fig. 5a). The same analysis shows that both factors, the day of the protocol and injection versus non-injection day, significantly affected the results. However, the mean duration of EDs on injection and non-injection days, of 4.6 ± 0.7 s and 3.5 ± 0.4 s, respectively, was not different on injection or inter-injection days. Interestingly, although the last injection was on day 19, the number of EDs on day 44 increased when compared to day 20, in which residual PTZ might have been built up, thus confirming that a gradual pathological process had been initiated and continued independently of further PTZ injections (Fig. 5c).

Effect of NO Modulators on EDs

Finally, the effect of unilateral application of NO modulators on EDs was tested in saline- and PTZ-injected animals (Fig. 6). First, the histologic analysis revealed that 40 % of the cannulas were positioned bilaterally on the meninges and did not cause cell destruction, 40 % caused bilateral cell destruction reaching layer II/III and 20 % caused unilateral cell destruction reaching layers II/III. Using 7-NI, a preferential nNOS inhibitor, an increased number of EDs in both saline and PTZ-treated animals was induced (Fig. 6a, left panel). The duration of EDs increased in saline-treated animals (compared to 0) but was not affected in PTZ-treated rats (Fig. 6a, right panel) while it was larger in PTZ-treated rats than in saline-treated rats.

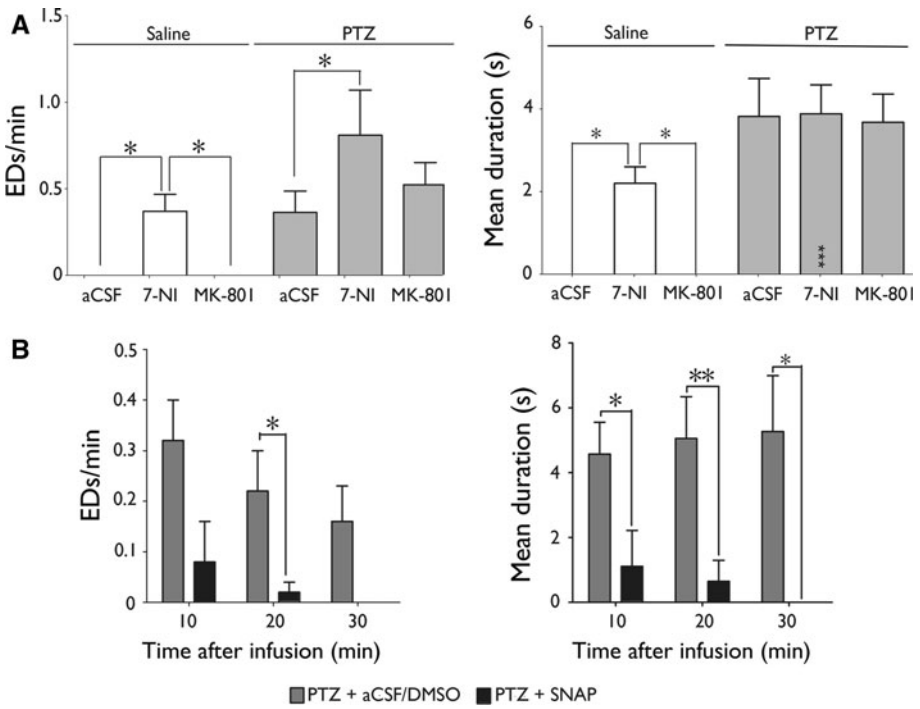


Fig. 6 Local application of pharmacological agents that modulate NO levels revealed a protective effect of NO on the number and duration of EDs. **a** Application of 300 μM 7-nitroindazole (7-NI, a preferential nNOS inhibitor) and 5 μg/μL MK-801 (NMDA receptor antagonist). *Left panel* number of EDs; *right panel* mean ED duration in both saline (n = 3) and PTZ-treated (n = 7) rats. Statistical significance indicated within the column indicates comparison between saline- and PTZ-treated animals after 7-NI applicaton. **b** The effect of local application of the NO donor SNAP, dissolved in 0.5 % DMSO in aCSF on the number of EDs (*left panel*) and mean ED duration (*right panel*) over 3 time intervals: 0–10, 10–20 and 20–30 min after application through the cannula (n = 5). The corresponding control was 0.5 % DMSO in aCSF. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$

Interestingly, ED induction was ipsilateral to the applied drug in saline-treated rats. Similarly, a unilateral effect was observed in PTZ-treated rats regarding the amplitude of EDs that increased by 20 ± 5 % ($p < 0.01$) at the ipsilateral derivation compared to the contralateral derivation (not shown).

As expected, in saline-treated animals, EDs appeared only after 7-NI had been applied and were not present after an innocuous stimulus such as aCSF, revealing that the potential damage caused by cannula implantation per se, or the injection procedure, did not induce EDs. This means that inhibition of NO production in the cerebral cortex led spontaneously to increased excitability irrespective of kindling. The occurrence of EDs was inhibited after application of the NMDA receptor blocker MK801 in saline-treated animals, while they returned to normal levels in the PTZ-treated animals, revealing that NMDA receptor-mediated neurotransmission contributed to epileptiform manifestations. In turn, when SNAP, a NO donor of short half-life, was applied through the cannula to PTZ-treated rats, both the frequency and the duration of EDs decreased (Fig. 6b), indicating that NO decreased excitability. Taken together, bi-directional modulation of NO reveals an inhibitory effect on cortical excitability.

Discussion

Although many animal models recapitulate the central symptoms of epilepsy, well defined models of the process of epileptogenesis, i.e., the development of epilepsy in a slow gradual manner-taking often years in humans-, are less prevalent and are definitely not available for the very frequently used PTZ kindling model. This model presents several unique opportunities to study the epileptogenesis process: it is easy to use, can therefore be reliably reproduced in different laboratories, presents small inter-individual differences compared to other pharmacological models such as kainate-induced *status epilepticus*, and is therefore of importance for the testing of various therapeutic interventions aimed at stopping epileptogenesis even in early stages. This study revealed that PTZ injections at sub-convulsive doses lead to immediate abnormalities in the EEG during a behaviorally "latent" period, of apparently normal behavior. The number of EDs increased progressively through the protocol both on injection and non-injection days and after completion of the protocol, reflecting an epileptogenesis process. In turn, EDs could be modulated by pharmacological agents without overt effects on behavior. These results demonstrate the advantages of

more accurate measurements of assessing modulators of excitability and epileptic activity. In the present study, NO had location selective effects in the cerebral cortex and its effect could be interpreted as neuroprotective by reducing the number and duration of EDs irrespective on the pharmacological agent used.

PTZ Kindling as a Model of Epileptogenesis

PTZ at low doses induces behavioral arrest and/or motionless staring reminiscent of absence-like seizures [8, 21] as well as neuronal loss in the hippocampal CA1 subfield and mossy fiber sprouting in the dentate gyrus, recapitulating key features of acquired human epilepsy [22]. In our study, the first behavioral manifestations were evident on day 9 and then reached level 5 (i.e., convulsions and/or tonic–clonic seizures) on day 17, revealing a latent period regarding the onset of behavioral abnormalities. This was accompanied by a progressive shift in the spectral power of EDs from theta towards higher frequency bands (i.e., low and high beta bands). While the number of EDs was increased on day 44, i.e. 24 days after completion of the kindling process, the spectral power of the frequency bands did not change. Thus, it is evident that shifts in the frequency domain occur consistently during early epileptogenesis. The fact that on day 44 the proportion of absence-like seizures increased compared to day 20 suggests that lower frequencies in the EDs are associated to behavioral arrest. In such a way, we agree with reports in which it has been shown that frequencies of 4–6 Hz correlate with absence-like seizures in PTZ-treated rats [23], while a shift towards higher discharge frequencies correlates with the presence of clonic and tonic–clonic seizures.

The transition of lower kindling scores, such as absence-like seizures, to higher scores following repeated injections of PTZ is accompanied by the activation of multiple brain structures in seizure activity [7, 24, 25]. The hippocampal subfields are compromised only after tonic, clonic or tonic–clonic seizures. Thus, absence-like seizures mainly induce the activation of the cingulate and piriform cortices and limbic structures, such as the amygdala and thalamic nuclei, consistent with a thalamo-cortical activation. In turn, our observation that EDs, even without obvious behavioral manifestations, occurred exclusively during wakefulness agree with the high sensitivity of the anterior and midline thalamic nuclei to PTZ, which induce arousal and play a role in seizure prevention [26]. Indeed, reductions in REM sleep after repeated PTZ injections have been reported [27].

Epileptogenesis has been studied at the behavioral or electroencephalographic level after electrical kindling, after kainate-induced *status epilepticus* and after perinatal hypoxia–ischemia [15, 20, 28]. However, PTZ kindling, although a very frequently used model, has mostly been assessed at the behavioral level, at doses ranging from 25 to 50 mg/kg while single higher doses (up to 70 mg/kg) induce immediate tonic–clonic convulsions [29–33]. One of the advantages of PTZ-induced kindling is the small inter-individual differences in the response: this can even be detected at the behavioral level, as revealed in Fig. 3 in which the highest kindling score reached by each rat was plotted along injection days. In turn, the frequency composition of EDs was remarkably stable among animals on the different recording days and correlated with behavioral intensity stages. Therefore, based on the spectral analysis, the present model could be characterized with daily 2-h recordings, although with this method circadian variations of ED incidence could not be detected. However, even at doses of 50 mg/kg, behavioral manifestations were limited and were observed in the following 30–45 min after PTZ injection while full behavioral recovery was observed at 2 h [33, 34] thus showing that 2-h recordings are adequate to capture EDs. As PTZ does not originate active drug metabolites, the EDs at non-injection days reflect spontaneous discharges in virtual absence of the drug considering a half-life of about 2 h [22] while at 24 days (i.e. 3–4 weeks) after stopping PTZ injections, a clear self-sustained process independent of PTZ presence is developing. Therefore, this model will be useful to test anti-epileptogenic therapy that should act early on EDs at time points and doses that cannot be detected at the behavioral level. It is very conceivable that early epileptogenesis mechanisms differ qualitatively, not only quantitatively, from later stages at which behavioral manifestations are evident. The biological nature of these processes is not clear, but could be addressed with models such as the present one.

Nitric Oxide: Region-Specific Effects?

NO has been reported to either increase or decrease excitability depending on the pharmacological agent used and the route of administration. For example, *i.p.* administration of 7-NI or *N*(*G*)-nitro-L-arginine methyl ester (L-NAME, a non-selective NOS inhibitor) has anti-convulsant properties on PTZ-induced seizures [35, 36]. However, additional selective nNOS inhibitors administered either *i.p.* or subcutaneously, acted dose-dependently as either pro- or anti-convulsants [14]. The NOS isoform involved in the here reported effect is not clear: 7-NI has been reported to inhibit nNOS and eNOS with similar potencies, although its selectivity in biological systems remains under debate [37, 38]. In any case, NO by itself, as revealed by SNAP application, had a negative impact on excitability and this was also shown with 7-NI, irrespective of the NOS isoform implied in NO synthesis. It was suggested that the negative

effects of NO depend on its relative concentration reached in a specific area or micro-environment [39]. However, nNOS−/− mice revealed increased seizure susceptibility to PTZ [14]. Therefore, a complex picture emerges suggesting that the modulation of excitability by NO might depend on NOS-associated cell-specific signaling complexes [16]. Indeed, it has been reported that the opposing effects of NO depend on the dose of PTZ used to induce convulsions [13]. Because different PTZ doses affect different brain areas, this suggests that NO acts on excitability in a region-specific manner. Accordingly, in our experiments, carried out in the somatosensory cortex, NO had the same effect on cortical excitability regardless of whether animals had been treated with PTZ. In addition, NO had opposing effects on viability in neuronal cultures of hippocampal compared to cortical origin [16]. Taken together, these results strongly suggest that NO inhibits neuronal depolarization in cortical areas but has the opposite effect in the hippocampus [12].

Targets of NO

At least two different signal transduction pathways are activated by NO: soluble guanylyl cyclase and the *S*-nitrosylation of proteins [40, 41]. *S*-nitrosylated proteins are either activated or inhibited by this post-translational modification. Depending on the biological function of the protein, the consequences are protective, such as inhibition of *N*-methyl-D-aspartate (NMDA) receptors [42], or toxic, such as activation of cyclooxygenase-2 [43]. It is unknown how NO targets a specific repertoire of proteins. One explanation is that physical proximity of the NO source is one of the determining factors and limits the NO levels that are sensed by the target. Thus, NO itself can be either protective—in the case of epilepsy, it can inhibit excitability—or it can be pro-epileptic by enhancing excitability and apoptosis [39]. It is also possible that NOS isoforms are differentially expressed and induced by activity among neuronal or glial subtypes and/or targeted to different cell compartments, or that various cell types express NO targets to a different degree. This latter possibility would explain why NO decreases excitability in the cerebral cortex, but not in the hippocampus.

Conclusions

Epileptic discharges and their spectral components constitute a sensitive measure of ongoing epileptogenesis in PTZ kindling. NO inhibits excitability in brain structures and neuronal types such as the sensory neocortex, highlighting the neuroprotective effects of this molecule. More studies should be conducted in the next future to identify NO targets, e.g. by mass spectrometry of *S*-nitrosylated proteins, to develop interventions aimed at blocking epileptogenesis and the propagation of neuronal discharges underlying epilepsy.

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References

1. Duncan JS, Sander JW, Sisodiya SM, Walker MC (2006) Adult epilepsy. Lancet 367:1087–1100
2. Engel J, Williamson P, Wieser HG (2008) Mesial temporal lobe epilepsy with hippocampal sclerosis. In: Engel J, Pedley TA (eds) Epilepsy: a comprehensive textbook, 2nd edn. Lippincott Williams & Wilkins, Philadelphia, pp 2479–2486
3. Jefferys JG (2003) Models and mechanisms of experimental epilepsies. Epilepsia 44(Suppl 12):44–50
4. Bertram E (2007) The relevance of kindling for human epilepsy. Epilepsia 48(Suppl 2):65–74
5. Racine RJ (1972) Modification of seizure activity by electrical stimulation. II. Motor seizure. Electroencephalogr Clin Neurophysiol 32:281–294
6. Huang RQ, Bell-Horner CL, Dibas MI, Covey DF, Drewe JA, Dillon GH (2001) Pentylenetetrazole-induced inhibition of recombinant gamma-aminobutyric acid type A (GABA(A)) receptors: mechanism and site of action. J Pharmacol Exp Ther 298:986–995
7. Szyndler J, Maciejak P, Turzynska D, Sobolewska A, Taracha E, Skorzewska A, Lehner M, Bidzinski A, Hamed A, Wislowska-Stanek A, Krzascik P, Plaznik A (2009) Mapping of c-Fos expression in the rat brain during the evolution of pentylenetetrazol-kindled seizures. Epilepsy Behav 16:216–224
8. Luttjohann A, Fabene PF, van Luijtelaar G (2009) A revised Racine's scale for PTZ-induced seizures in rats. Physiol Behav 98:579–586
9. McNamara JO, Huang YZ, Leonard AS (2006) Molecular signaling mechanisms underlying epileptogenesis. Science's STKE: signal transduction knowledge environment 2006:re12
10. Waxman EA, Lynch DR (2005) N-methyl-D-aspartate receptor subtypes: multiple roles in excitotoxicity and neurological disease. Neuroscientist 11:37–49
11. Chuang YC, Chen SD, Lin TK, Liou CW, Chang WN, Chan SH, Chang AY (2007) Upregulation of nitric oxide synthase II contributes to apoptotic cell death in the hippocampal CA3 subfield via a cytochrome c/caspase-3 signaling cascade following induction of experimental temporal lobe status epilepticus in the rat. Neuropharmacology 52:1263–1273
12. Kovacs R, Rabanus A, Otahal J, Patzak A, Kardos J, Albus K, Heinemann U, Kann O (2009) Endogenous nitric oxide is a key promoting factor for initiation of seizure-like events in hippocampal and entorhinal cortex slices. J Neurosci 29:8565–8577
13. Jelenkovic A, Jovanovic M, Ninkovic M, Maksimovic M, Bokonjic D, Boskovic B (2002) Nitric oxide (NO) and convulsions induced by pentylenetetrazol. Ann N Y Acad Sci 962:296–305
14. Itoh K, Watanabe M (2009) Paradoxical facilitation of pentylenetetrazole-induced convulsion susceptibility in mice lacking neuronal nitric oxide synthase. Neuroscience 159:735–743

15. Marangoz AH, Yildirim M, Ayyildiz M, Marangoz C (2012) The interactions of nitric oxide and acetylcholine on penicillin-induced epilepsy in rats. Neurochem Res 37:1465–1474
16. Sandoval R, Gonzalez A, Caviedes A, Pancetti F, Smalla KH, Kaehne T, Michea L, Gundelfinger ED, Wyneken U (2011) Homeostatic NMDA receptor down-regulation via brain derived neurotrophic factor and nitric oxide-dependent signalling in cortical but not in hippocampal neurons. J Neurochem 118:760–772
17. Ocampo-Garces A, Ibanez F, Perdomo G, Torrealba F (2011) Orexin-B-saporin lesions in the lateral hypothalamus enhance photic masking of rapid eye movement sleep in the albino rat. J Sleep Res 20:3–11
18. Esplin DW, Woodbury DM (1956) The fate and excretion of C14-labeled pentylenetetrazol in the rat, with comments on analytical methods for pentylenetetrazol. J Pharmacol Exp Ther 118:129–138
19. Ramzan IM, Levy G (1985) Kinetics of drug action in disease states. XV: effect of pregnancy on the convulsive activity of pentylenetetrazol in rats. J Pharm Sci 74:1233–1235
20. Buzsaki G, Draguhn A (2004) Neuronal oscillations in cortical networks. Science 304:1926–1929
21. Snead OC 3rd (1992) Pharmacological models of generalized absence seizures in rodents. J Neural Transm Suppl 35:7–19
22. Mortazavi F, Ericson M, Story D, Hulce VD, Dunbar GL (2005) Spatial learning deficits and emotional impairments in pentylenetetrazole-kindled rats. Epilepsy Behav 7:629–638
23. Erbayat-Altay E, Yamada KA, Wong M, Thio LL (2008) Increased severity of pentylenetetrazol induced seizures in leptin deficient ob/ob mice. Neurosci Lett 433:82–86
24. Andre V, Pineau N, Motte JE, Marescaux C, Nehlig A (1998) Mapping of neuronal networks underlying generalized seizures induced by increasing doses of pentylenetetrazol in the immature and adult rat: a c-Fos immunohistochemical study. Eur J Neurosci 10:2094–2106
25. Brevard ME, Kulkarni P, King JA, Ferris CF (2006) Imaging the neural substrates involved in the genesis of pentylenetetrazol-induced seizures. Epilepsia 47:745–754
26. Miller JW (1992) The role of mesencephalic and thalamic arousal systems in experimental seizures. Prog Neurobiol 39:155–178
27. Schilling M, Wetzel W, Grecksch G, Becker A (2006) Pentylenetetrazole kindling affects sleep in rats. Epilepsia 47:2075–2082
28. Hirase H, Qian L, Bartho P, Buzsaki G (2004) Calcium dynamics of cortical astrocytic networks in vivo. PLoS Biol 2:E96
29. Wilczynski GM, Konopacki FA, Wilczek E, Lasiecka Z, Gorlewicz A, Michaluk P, Wawrzyniak M, Malinowska M, Okulski P, Kolodziej LR, Konopka W, Duniec K, Mioduszewska B, Nikolaev E, Walczak A, Owczarek D, Gorecki DC, Zuschratter W, Ottersen OP, Kaczmarek L (2008) Important role of matrix metalloproteinase 9 in epileptogenesis. J Cell Biol 180:1021–1035
30. Szyndler J, Maciejak P, Wislowska-Stanek A, Lehner M, Plaznik A (2013) Changes in the Egr1 and Arc expression in brain structures of pentylenetetrazole-kindled rats. Pharmacol Rep PR65:368–378
31. Stafstrom CE, Ockuly JC, Murphree L, Valley MT, Roopra A, Sutula TP (2009) Anticonvulsant and antiepileptic actions of 2-deoxy-D-glucose in epilepsy models. Ann Neurol 65:435–447
32. Nasir SA, Sharma A, Khanam R, Vohora D (2012) Effect of medroxyprogesterone on development of pentylenetetrazole-induced kindling in mice. Neuroscience 207:283–287
33. Park JH, Cho H, Kim H, Kim K (2006) Repeated brief epileptic seizures by pentylenetetrazole cause neurodegeneration and promote neurogenesis in discrete brain regions of freely moving adult rats. Neuroscience 140:673–684
34. Kumar A, Lalitha S, Mishra J (2013) Possible nitric oxide mechanism in the protective effect of hesperidin against pentylenetetrazole (PTZ)-induced kindling and associated cognitive dysfunction in mice. Epilepsy Behav 29:103–111
35. Han D, Yamada K, Senzaki K, Xiong H, Nawa H, Nabeshima T (2000) Involvement of nitric oxide in pentylenetetrazole-induced kindling in rats. J Neurochem 74:792–798
36. Akula KK, Dhir A, Kulkarni SK (2008) Nitric oxide signaling pathway in the anti-convulsant effect of adenosine against pentylenetetrazol-induced seizure threshold in mice. Eur J Pharmacol 587:129–134
37. Beamer E, Otahal J, Sills GJ, Thippeswamy T (2012) N (w)-propyl-L-arginine (L-NPA) reduces status epilepticus and early epileptogenic events in a mouse model of epilepsy: behavioural, EEG and immunohistochemical analyses. Eur J Neurosci 36:3194–3203
38. Yin XH, Yan JZ, Hou XY, Wu SL, Zhang GY (2013) Neuroprotection of S-nitrosoglutathione against ischemic injury by down-regulating Fas S-nitrosylation and downstream signaling. Neuroscience 248C:290–298
39. Calabrese V, Mancuso C, Calvani M, Rizzarelli E, Butterfield DA, Stella AM (2007) Nitric oxide in the central nervous system: neuroprotection versus neurotoxicity. Nat Rev Neurosci 8:766–775
40. Garthwaite J (2008) Concepts of neural nitric oxide-mediated transmission. Eur J Neurosci 27:2783–2802
41. Foster MW, McMahon TJ, Stamler JS (2003) S-nitrosylation in health and disease. Trends Mol Med 9:160–168
42. Takahashi H, Shin Y, Cho SJ, Zago WM, Nakamura T, Gu Z, Ma Y, Furukawa H, Liddington R, Zhang D, Tong G, Chen HS, Lipton SA (2007) Hypoxia enhances S-nitrosylation-mediated NMDA receptor inhibition via a thiol oxygen sensor motif. Neuron 53:53–64
43. Tian J, Kim SF, Hester L, Snyder SH (2008) S-nitrosylation/activation of COX-2 mediates NMDA neurotoxicity. Proc Natl Acad Sci USA 105:10537–10540