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The role of TNF-α in amygdala kindled rats

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Abstract

In the present study, the interaction between epileptogenesis and the immune system were studied in a kindling model. First, the effects of a single administration of TNF-α (5.0 μg/kg, i.p.) on seizure and EEG activity were investigated in amygdala-kindled rats. TNF-α treated rats showed more prolonged epileptiformic discharges than control rats. TNF-α also induced a decrease in the power of δ band and an increase in θ and α activity. In addition, a marked increase in the power of β and γ band was observed. The EEG changes were most numerous in the frontal cortex and amygdala. All effects were registered 24 h after TNF-α administration. Finally, electrical stimulation enhanced the level of TNF-α in blood serum from 1.9 ± 1.5 to 12.7 ± 3.8 pg/ml and in brain tissue 56.8 ± 6.0 to 109.2 ± 6.0 pg/mg, as was determined via the ELISA method. It can be concluded that there is a mutual facilitative interaction of both epileptogenic and cytokine-derived mechanisms on this type of seizure. The changes in the power spectrum of the EEG after TNF-α might contribute to intensify thalamic-derived facilitation of epileptic discharge in cortical structures.

Keywords: Amygdalar electrostimulative kindling; Seizures; Cytokines; TNF-α; EEG; Spectral analysis

1. Introduction

It is well established that the immune system is affected by kindling (Plata-Salaman et al., 2000). Kindled rats exhibited a significant up-regulation of IL-1β, IL-1R1, TNF-α and TGF-β1 mRNAs in the parietal, prefrontal and piriform cortices, amygdala and hippocampus, as measured 2 h from the moment of last seizures with a subsequent normalization of those indices in a postponed (3 weeks) period. Such an elevation of cytokines might be regarded as a mechanism of heightening of seizure susceptibility as far as some data seem to suggest that cytokines may affect epileptic activity in humans as well as in rats. High doses of TNF-α and IL-1β during immunotherapy have been found to induce seizures in humans (Mittelman et al., 1992; Redman et al., 1994). One nanogram of human recombinant IL-1β intrahippocampally injected 10 min before kainate, enhanced by 226% the time spent in seizures in rats (Vezzani et al., 1999), while blockers or antagonists of IL-1β are regarded as a new class of highly effective antiepileptic drugs (Vezzani et al., 2000).

Taking into consideration the mutual facilitator effects of IL-1β and TNF-α (Plata-Salaman and Ilyin, 1997), a role for TNF-α in the observed effects of IL-1β upon epileptogenesis might be supposed.

However, the action of TNF-α upon seizures induced by electrical kindling of the amygdala is still unclear. Hence, the aim of this work was to investigate the effects of a single dose of human recombinant TNF-α administration in rats kindled with amygdalar ES.

Taking into consideration that long-term exposure to TNF-α may lead to disruption of the blood–brain barrier (BBB) (Deli et al., 1995; Male, 1995) and that

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might be true for the kindling process, which is characterized by prolonged elevation of endogenous level of TNF-α (Van Luijtelaar et al., 2000), it was decided to use a relatively small dosage (5.0 μg/kg) of TNF-α.

The reason for usage of a TNF-α dosage of 5.0 μg/kg was explained also by the fact that it was far from being effective as inductive for fever (Fleshner et al., 1998). The intraperitoneal route of TNF-α administration was chosen on the basis that cytokines can reach the CNS directly by crossing at leaky areas in the BBB through the circumventricular organs (Watkins et al., 1995), even in healthy, basal conditions. There is convincing evidence also for active, saturable and specific transport of certain cytokines across the BBB (Banks and Kastin, 1997; Banks et al., 2001; Szelernyi, 2001).

Next, also the spectral content of the EEG after TNF-α was investigated since preliminary observations tended to suggest that EEG changes may occur and in accordance with Kubota et al. (2001), TNF-α in intact rats (50.0–200.0 μg/kg, i.p.) increased EEG δ-power and decreased EEG α- and β-power during the initial 3 h after injection. Finally, the characteristics of ES-induced epileptic manifestations and the level of TNF-α in brain tissue and plasma in the post-kindled period were investigated in order to investigate the role of ES of the amygdala on the immune system.

2. Materials and methods

2.1. Animals

Two-month-old male Wistar rats, with a starting bodyweight of 150–180 g, were used as experimental subjects. They were kept under standard laboratory conditions, i.e. constant temperature of 23 °C, 60% relative humidity, 12 h dark/light cycles, standard diet and tap water present ad libitum.

Procedures involving animals and their care were conducted according to University guidelines that comply with international laws and policies (European Community Council Directive 86/609, OJ L 358, I, December 12, 1987; National Institute of Health Guide for Care and Use of Laboratory Animals, US National Research Council, 1996).

2.2. General surgery

Animals were anesthetized with Nembutal ('Ceva', France; 35 mg/kg, i.p.) and implanted stereotaxically with bipolar electrodes (nichrome wires insulated except for the tips, wire diameter 0.12 mm, interelectrode distance 0.25 mm) in the left basolateral amygdala (AP = 2.2; L = 4.7; H = 8.5), according to the rat brain atlas of Paxinos and Watson (1998).

Reference monopolar electrodes were implanted in the right ventral hippocampus (AP = −4.3; L = 4.5; H = 8.0), left frontal cortex (AP = 1.7; L = 2.0; H = 1.0) and left occipital cortex (AP = −6.3; L = 3.0; H = 1.0). Indifferent electrodes were fixed in the nasal bones. Electrodes were fixed to the skull with dental cement. Starting 1 week after surgery, the rats were handled daily and adapted to the experimental setup.

2.3. Kindling procedure and behavioral investigations

Kindling was started 10–14 days after surgery. Electrical stimulation of the amygdala was performed using an ESU-2 universal electrostimulator (former Soviet Union). Electrical stimuli (60 Hz, duration 1 ms) were applied for a total of 1 s. The intensity of electrical current used for kindling was 80–140 μA, depending on its ability to induce after-discharge (Racine, 1972).

Generalized clonic-tonic seizures were seen following daily stimulation for 16–25 days. The severity of convulsions was evaluated according to the scale described by Racine (1972).

General response to TNF-α was investigated via determination of sleep–wakefulness behaviour with the identification of next stages: active-, passive-wakefulness, slow wave sleep and paradoxical sleep (Shandra et al., 1999). Besides, a 2-min 'open field' test was performed and a number of crossed squares were calculated.

2.4. EEG acquisition and analysis

The EEG signals were sampled at 256 samples per second using a data acquisition board (National Instruments, USA) and stored for off-line analysis. The signals were band pass filtered, only frequencies between 0.5 and 40 Hz were allowed to pass. The polygraph records were inspected visually and epochs containing artifacts discarded. Fast Fourier transform analysis was performed on 16-s samples ('Labview-5.0' software modified for EEG). To normalise the data for differences in the EEG baselines for each rat, the pre-TNF-α power of different bandwidths of EEG (μV^2) was compared with the correspondent indices in post-TNF-α period (24 h from the moment of TNF-α administration). The frequencies were grouped into five bands of 0.5–4, 4–8, 8–12, 12–25 and 25–40 Hz.

All registrations were made from 10:00 to 12:00 h, only epochs with a clear waking EEG were analysed.

2.5. TNF-α (h)

TNF-α was administered i.p. 24 h after the last kindled ES. Human recombinant TNF-α (Sigma, USA, Lot N096H6656) was dissolved in phosphate buffered saline, pH 7.4 and administered at a dosage of 5.0

μg/kg in a volume of 0.3 ml/100 g. Kindled rats administered i.p. with an analogous volume of saline were used as controls when the effects of TNF-α on seizures and epileptiformic activity in brain structures were investigated.

2.6. ELISA

Twenty four hours after kindled ES, the experimental group of rats ($n = 8$) and their controls (implanted with electrodes, $n = 8$) were killed by decapitation and their brains were rapidly removed at 4 °C and frozen on dry ice. Brain tissue was weighted and homogenized in ice-cold PBS (5 g/ml) using a Potter homogenizer (1000 rpm, ten strokes). The homogenates were centrifuged for 10 min (5000 rpm, 4 °C). One hundred microliters of the supernatant were taken in duplicate to measure TNF-α.

TNF-α was determined using selective antibody ('Biotrak' system from Amersham Pharmacia Biotech, USA). Absorbency was read at 450 nm. The detection limit was 4.0 pg/ml.

2.7. Histology

At the end of the experiments, the rats were anesthetized with pentobarbital sodium and perfused with paraformaldehyde. Frozen sections (32 μm) of the brain were then prepared and every alternate section mounted on gelatin-coated slides, stained with neutral red, covered with a cover-slip and examined by light microscopy. In all rats used in the analysis of the data, the electrodes were inserted at the appropriate location.

2.8. Data analysis

The bandwidth power data were analyzed by a one-way ANOVA, followed by Newman–Keuls test. The number of rats that showed seizures was analyzed using the Fisher test. Duration of sleep–wakefulness cycle stages and the number of crossed squares in 'open field' test were analyzed using the Wilcoxon–Whitney *U*-test.

3. Results

3.1. Effects of on behaviour of rats

The comparison of the rats behaviour 60 min before kindled ES (23 h from the moment of TNF-α administration) revealed the prevalence of active and passive wakefulness, which possessed from 45 to 72%, pertained to the whole period of observation. This index was no different from an analogous index determined in saline-treated control animals (38–57%) ($P > 0.05$, Student's *t*-test). Rat investigation in 2-min 'open field' test showed that TNF-α treated animals crossed from ten to 26 squares, while saline-treated control rats from 12 to 31 squares ($P > 0.05$, Wilcoxon–Whitney *U*-test).

3.2. Effects of TNF-α on ES-induced seizures and epileptiform discharges

When amygdalar ES was carried out in the 24 h following the administration of TNF-α, repeated generalized clonic seizures of body muscles were seen in all six rats. In the control group of kindled rats, six animals demonstrated generalized clonic-tonic fits, the animals falling and showing post-seizure depression, while rearings and clonic seizures of forelimbs were seen in two rats. Hence, the total number of rats with repeated generalized clonic-tonic fits was significantly greater in TNF-α treated animals ($P < 0.025$, Fisher's test). The duration of the ES-induced epileptiformic activity was 47.3 ± 5.3 s in the control rats and 92.5 ± 10.5 s in the group treated with TNF-α ($P < 0.05$) (Student's *t*-test). Row-EEG revealed clear prevalence of duration of afterdischarge in experimental (TNF-α-treated) rats in control (i.p. saline) (Fig. 1).

3.3. EEG of kindled rats before TNF-α

As shown in Table 1, the largest power seen in all brain areas in kindled rats before the administration of TNF-α was seen in the δ band. θ and β activities were in second and third position, respectively, in the majority of structures, while the power of α and γ frequency bands were less pronounced.

3.4. EEG of kindled rats after TNF-α

TNF-α was followed by changes in the power of δ activity, which was reduced by 22.2% in the hippocampus in comparison with the pre-TNF-α level ($P < 0.05$) (Table 1) (Fig. 2).

Theta activity in the hippocampus did not change significantly after TNF-α ($P > 0.05$), whereas, in all other structures, a marked increase in θ activity was clearly seen, most markedly in the frontal cortex (2.3 times increased) ($P < 0.05$).

Marked increasing in the power of α activity was seen in the post-TNF-α period, with a 2.2 time increase in the frontal cortex ($P < 0.05$) and a 28.1% increase in the hippocampus ($P > 0.05$).

Beta activity was increased by 54.8% ($P < 0.05$) in the frontal cortex after TNF-α. γ Activity was increased by 64.8% in the zone of left amygdala and by 46.8% in the hippocampus ($P < 0.05$).

3.5. Level of TNF-α

The level of TNF-α in blood serum was 6.3 times greater (in control—from 0 to 12.0 pg/ml, average: 1.9 ± 1.5 pg/ml; in kindled—from 0 to 27.0 pg/ml, average: 12.7 ± 3.8 pg/ml) ($P < 0.05$) and in brain tissue, 1.9 times greater (in control—from 31.0 to 79.0 pg/mg, average: 56.8 ± 6.0 pg/mg; in kindled—from 75.0 to 126.0 pg/mg, average: 109.2 ± 6.0 pg/mg) ($P < 0.05$) in kindled than in control rats.

4. Discussion

Three major results were obtained: an increase in behavioral seizures was seen following a single injection with TNF-α after electrical stimulation of the amygdala, the level of TNF-α in brain and plasma was elevated after electrical stimulation and quite large effects of TNF-α were found on the background EEG. The first outcome, an increase in severity of the seizures, supports the idea that TNF-α initially affects central mechanisms of generation of epileptogenic excitation. That might also be the main mechanism of action of increased level of TNF-α in blood plasma. Mechanisms of mutual intensification of production of different cytokines should be additionally considered (Plata-Salaman and Ilyin, 1997). This is especially true for IL-1β and TNF-α; since it was shown in our laboratory in the same rats that IL-1β was also enhanced by 6.5 and 4.0, respectively in blood plasma and brain tissue (Van Luijtelaar et al., 2000). The combination of the latter and the outcomes of the present study suggest that IL-1β might be of inductive significance for TNF-α in kindled rats. It should also be noted that TNF-α was not changed in WAG/Rij rats of different age (2–6 months), which suffered from a quite mild type of non-convulsive epilepsy (unpublished data). This suggests some form of specific role for this cytokine in convulsive types of epilepsy.

Development of pronounced effects after i.p. TNF-α administration in relatively low dosage clearly points to good permeability of BBB to this cytokine in kindled animals. That might be the indication on the deteriorated state of BBB induced in the course of kindling, presumably with the participation of endogenously overexpressed TNF-α. Meanwhile, a used dosage of TNF-α is at least ten times less than that one effectively

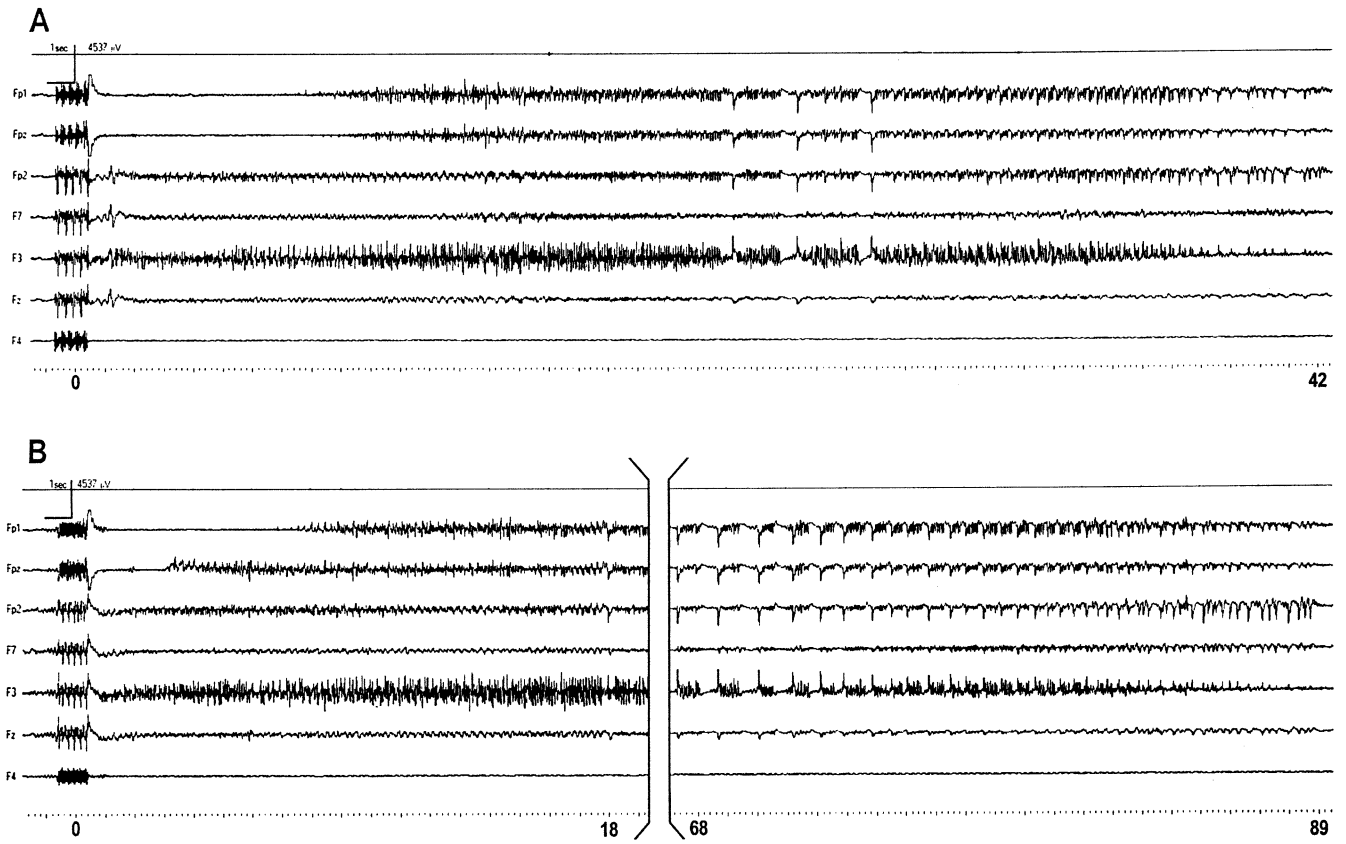

Fig. 1. Row EEG data in kindled rat after testing ES of amygdala (A: control) and ES of amygdala after treatment with TNF-α (5.0 mcg, i.p.) (B). Fp1, occipital left; Fpc, frontal left; Fp2, amygdala hippocampus (right); Fz, bipolar lead-amygdala-hippocampus; F4, bipolar lead occipital-frontal cortex. At (B) the 50-s period of continous epileptic activity was cut; mean power of EEG was equal to the one established in the 3 s before.

Table 1
EEG effects of TNF-α in kindled rats (mean ± S.E.M.)

Groups of animals	Amygdalar (left)	Frontal cortex (left)	Occipital cortex (left)	Hippocampus (ventral right)
δ Rhythm				
Control ($n=7$)	62.3 ± 2.6	58.2 ± 4.1	61.0 ± 4.2	57.3 ± 3.1
After TNF-α ($n=8$)	65.2 ± 3.7	64.5 ± 5.4	65.3 ± 6.9	44.6 ± 2.2 #
θ Rhythm				
Before ($n=7$)	31.4 ± 2.4	21.5 ± 1.8	34.0 ± 4.4	27.5 ± 1.8
After TNF-α ($n=8$)	43.7 ± 3.3 #	48.7 ± 5.8 #	55.3 ± 6.4 #	31.4 ± 3.5
α Rhythm				
Before ($n=7$)	14.7 ± 1.2	11.3 ± 0.8	16.4 ± 1.6	13.9 ± 1.2
After TNF-α ($n=8$)	21.5 ± 2.1 #	24.6 ± 2.5 #	25.6 ± 3.1 #	17.8 ± 2.2
β Rhythm				
Before ($n=7$)	24.6 ± 2.7	21.7 ± 2.3	28.5 ± 3.2	24.1 ± 3.2
After TNF-α ($n=8$)	31.9 ± 4.7	33.6 ± 4.2 #	34.1 ± 3.6	32.7 ± 3.9
γ Rhythm				
Before ($n=7$)	8.8 ± 0.7	9.3 ± 0.8	9.9 ± 0.6	10.9 ± 0.8
After TNF-α ($n=8$)	14.5 ± 1.4 #	10.7 ± 1.0	11.2 ± 1.1	16.0 ± 1.3 #

# $P<0.05$ in comparison with corresponding data in the control group.

induced not-REM sleep in intact rats (Kubota et al., 2001). The same is true for fever induction (Fleshner et al., 1998). Hence, presumably we observed direct effect of TNF-α on EEG, which were not mediated via affecting of non-REM somnogenic mechanisms. In this respect it should also be stressed that rats continued to keep the state of active or passive wakefulness after TNF-α administration and their behaviour was no different from that of saline-treated control rats.

Obtained data that showed the marked increase of TNF-α in kindled rats are in good agreement with the results of Plata-Salaman et al. (2000), who showed a marked increase of the TNF-α and IL-β mRNA in 2 h after the last kindled seizures in rats. It should be noted that the relative increase of the level of endogenous compounds was far greater (30 times) compared with that observed in rats after intra hippocampal administration of kainate (16 times) (Vezzani et al., 1999). Meanwhile, epileptogenesis induced via kainate injection is more severe and is manifested in the form of status epilepticus precipitation (Vezzani et al., 1999). This fact might be in favor for a special role played by interleukines in kindling pathogenesis of the amygdala which might be linked with the predisposition for the epileptic phenomena creating, but not with the membrane depolarization which is an intimate effect of kainic acid epileptogenic action (Lothman and Collins, 1981). In other words, epileptogenic effects of cytokines might be connected with the facilitation of epileptic activity spreading, but not with the lowering of the threshold of epileptic phenomena precipitation. As far as early post-kindled state is regarded as highly sensitive to antiepileptic drugs (AEDs) action (Löscher and Rundfeld, 1991; Shandra et al., 1999), it might be assumed that a high level of cytokines (TNF-α) serves for such an increase in seizure susceptibility. But, direct administration of TNF-α also caused a marked increase in seizure susceptibility and this controversial role of TNF-α might be explained by the dependence of cytokine action on their concentration (Plata-Salaman, 1996). Only high levels tend to be associated with neurotoxicity and neurodegeneration. It is also possible that the known stimulating action of cytokines on the metabolic (microsomal) activity of hepar (Breulle et al., 1993) serves for the activation of AEDs and achieving more pronounced antiseizure action. The final major finding was that TNF-α caused profound changes in the EEG of kindled rats. The most marked effect was the general increasing in the power of α activity in all brain structures. Elevated θ activity was seen in all structures, except the hippocampus. In contrast, the increase in the power of the β and γ band was less widespread, and δ activity was even decreased in the hippocampus. It is also of interest to note that the level of increased IL-s mRNA was largest in prefrontal cortical zones (Plata-Salaman et al., 2000)—that brain structure where we noted maximal deteriorations of the EEG pattern in 24 h from the moment of TNF-α administration in kindled rats. The increase in α activity might be regarded as intensification of the thalamic-derived facilitation of epileptic discharge in cortical structures (Neal and Keane, 1980). The increase in the higher frequencies of EEG activity might also contribute to the TNF-α induced EEG changes of epileptogenesis, since EEG desynchronization is determined by activation of the ascending reticular formation during the course of which seizure susceptibility is reduced (Okudzava et al., 1979; Shouse et al., 1989). The relationship between δ-type activity and epileptogenesis is not settled—there are effects of inhibition of cortex susceptibility during

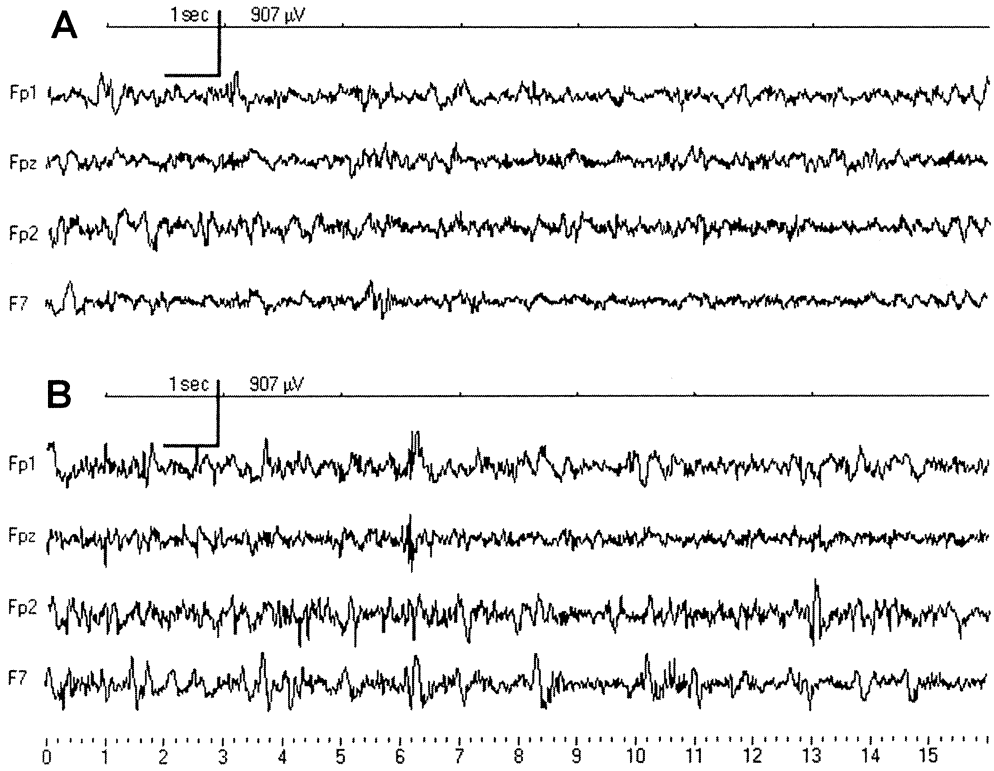

Fig. 2. Background EEG activity of kindled rat before (A) and after (B) TNF-α (5.0 mcg, i.p.) administration. Fp1, occipital left; Fpz, frontal left; Fp2, amygdala (left); F7, hippocampus (right).

slow wave precipitation (Gloor, 1979) and spontaneous generation of spikes in isolated cortex on the background of δ-activity generation (Biniaurishvili et al., 1985).

In accordance with the Stam and Pritchard (1999) data, periodical type of δ-rhythmicity is followed by low thalamic-cortical input (afferentation) and inactivation of both positive and negative feedback. The abolishment of negative backward influences is important as far as this type of regulation is essential for the generation of α-rhythmicity, which is not compatible with δ type of activity (Steriade et al., 1990; Lopes da Silva, 1991).

Hence, our observed tendency of a reduction of δ activity along with the intensification of α might be understood on the basis of their mutual antagonism, which is facilitated by a possible indirect mechanism of TNF-α affecting electrogenesis. Besides, direct effects of TNF-α upon pacemakers of different rhythms are at forehand not excluded. It should be noted that the antiepileptic drug, remacemide, caused the opposite effect of reducing the spindle-range of activity in EEG along with increasing the amplitude of δ activity in the cortex of rats that had suffered from the absence type of epilepsy (Van Luijtelaar and Coenen, 1995).

Observed effects are opposite to data obtained by (Kubota et al., 2001) with respect to spectral EEG dynamics. Hence, those authors observed an enhancement of δ activity along with decrease of α- and β-activity—a picture that reflects activation of non-REM sleep. It should be noted that Kubota et al. (2001) worked with intact animals and used dosages that were at least ten times greater than the dosage we explored. It is also important to note that the establishment of the full-kindled state is characterized by a reduction of REM sleep (Sterman and Shouse, 1981). Hence, the results obtained in the EEG of kindled animals after TNF-α might be different from those in intact (sham-operated) rat. The general character of EEG spectral changes in our experiments might be pertinent to REM sleep activation. Moreover, it should also be noted that TNF-α action in kindled rats at a dosage of 5.0 μg/kg was connected with a reduction of muscle tonus of rats (unpublished data)—a sign which is quite characteristic for REM sleep. That is why we can come to the intriguing idea that we have observed effects which are in favor of a role of TNF-α as an inductor of a pathological form of REM sleep. It might be supposed that those neuronal centers that are involved in REM sleep supporting serve as a target for TNF-α action, and they might be localized in the hypothalamic region, which is most penetrable for i.p. administered TNF-α, while structures in the pons are less likely involved (Banks et al., 2001). Hence, gained data showed that increasing TNF-α in ES amygdalar-kindled rats might serve as a self-perpetuating mechanism for seizure development.

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