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Experimental chronic sleep fragmentation alters seizure susceptibility and brain levels of interleukins 1β and 6

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Brain hyperexcitability in sleep apnea is believed to be provoked by hypoxemia, but sleep fragmentation can also play a significant role. Sleep fragmentation can trigger inflammatory mechanisms. The aim of this research was to investigate the effects of chronic sleep fragmentation on seizure susceptibility and brain cytokine profile. Chronic sleep fragmentation in male rats with implanted EEG electrodes was achieved by the treadmill method. Rats were randomized to: treadmill control (TC); activity control (AC) and sleep fragmentation (SF) group. Convulsive behavior was assessed 14 days later by seizure incidence, latency time and seizure severity during 30 min following lindane administration. The number and duration of EEG ictal periods were determined. Levels of IL-1β and IL-6 were measured in the animals' serum and brain structures (hippocampus, thalamus and cerebral cortex), in separate rat cohort that underwent the same fragmentation protocol except lindane administration. Incidence and severity of seizures were significantly increased, while latency was significantly decreased in SF+L compared with TC+L group. Seizure latency was also significantly decreased in SF+L compared to AC+L group. The number and duration of ictal periods were increased in the SF+L compared to the AC+L group. IL-1β was significantly increased in the thalamus, cortex and hippocampus in the SF compared to the AC and TC groups. IL-6 was statistically higher only in the cortex of SF animals, while in the thalamic or hippocampal tissue, no difference was observed between the groups. It could be concluded that fourteen-day sleep fragmentation increases seizure susceptibility in rats and modulates brain production of IL-1β and IL-6.

Key words: chronic sleep fragmentation, brain hyperexcitability, cytokines, neuroinflammation, sleep apnea

INTRODUCTION

Sleep fragmentation is defined as a series of brief arousals repeated consecutively throughout the night (Bonnet and Arand, 2003). This type of fractured sleep causes excessive daytime sleepiness, impaired cognition and numerous neurobehavioral deficits (Bonnet and Arand, 2003; Vijayan, 2012). Clinical and translational studies suggested certain lifestyle factors may be important modulators of epilepsy, elucidating those related to sleep as particularly important. Shift work and social jetlag caused by modern lifestyles are accompanied by quantitative and qualitative sleep alterations. An altered sleep pattern has been already recognized as a cause of behavioral changes, but recent evidences suggest a bidirectional relationship between the psychiatric illnesses, disrupted circadian rhythm and disrupted sleep (Wulff et al., 2010). Since total sleeping time in fragmented sleep is insignificantly changed, most scientists tend to attribute these consequences to periodic interruptions and stress (Devinsky et al., 1994).

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Patients with wide variety of diseases, particularly those with obstructive sleep apnea (OSA) often suffer from fragmented sleep (Day et al., 1999). Treating sleep apnea can be crucial to epilepsy prevention and treatment (Malow et al., 2000; Chihorek et al., 2007). It is believed that the main cause of hyperexcitability in patients with OSA is hypoxemia (Manni and Tartara, 2000; Seyal et al., 2010), but many researchers in the field are of the opinion that sleep fragmentation, as an accompanying phenomenon, should also be considered as a powerful contributing factor (Ferrillo et al., 2000; Parrino et al., 2000; Manni et al., 2005; Seyal et al., 2010; Foldvary-Schaefer et al., 2012).

Numerous uncertainties exist on mechanisms of increased neuronal excitability upon sleep alterations. It is known that sleep fragmentation can trigger low-grade chronic inflammatory mechanisms (Mullington et al., 2009; Tang et al., 2009). Moreover, systemic or central nervous system inflammation exerts the modulation of synaptic plasticity *via* neuroinflammatory mediators, thus influencing brain neuronal networks functioning (Di Filippo et al., 2013; Hrncic et al., 2018; Šutulović et al., 2019). An increased production of different cytokines, especially IL-1β, TNF-α and IL-6 in activated astrocytes and microglia during epileptogenesis, has been already proven in several studies (Ravizza et al., 2008; Vezzani and Friedman, 2011). Indeed, these cytokines play various roles through multiple pathways and mediate complex connections between the immune system and epilepsy (Li et al., 2011).

Since epilepsy nowadays presents a major society burden, with the prevalence of 0.5–1.0% (Hauser et al., 1991) and 30% of affected individuals having continuous seizures despite antiepileptic drugs (AEDs) (Sander, 1993). The reduction of factors which may promote seizures, such as sleep fragmentation (Rajna and Veres, 1993; Frucht et al., 2000), could lead to new and more effective treatment strategies. In this study we used the lindane model of experimentally induced generalized seizures, as described earlier (Vučević et al., 2008; Hrnčić et al., 2016), because lindane induced seizures are refractory to numerous classical antiepileptic drugs and therefore believed to be suitable for exploration of refractory epilepsy (Vučević et al., 2008).

The objective of this study was to investigate the effects of chronic sleep fragmentation on seizure susceptibility using an experimental model of lindane-induced seizures and a high frequency sleep disruption model of sleep fragmentation characteristic for obstructive sleep apnea. We also wanted to examine the effect of sleep fragmentation on the IL-1beta and IL-6 levels in the hippocampus, thalamus and cortex of rat's brain tissue, and see if they could bridge the missing link between these disorders.

METHODS

Animals and housing

Two months old male Wistar albino rats were used. The animals were obtained from the Military Medical Academy breeding laboratory (Belgrade, Serbia). Rats were housed in separate transparent plastic cages (55 cm × 35 cm × 15 cm), they were given 25-30 g of food (Purina rat chow) per day and had free access to water (*ad libitum* access) continuously. Ambient conditions were set up to an optimal breeding conditions, i.e., 22-24°C, 50±5% relative humidity, 12:12 h light/dark cycle with light switched on from 8:00 h to 20:00 h. One week was the period of adaptation to these environmental conditions upon rat arrival in the laboratory. All animals were not used more than once in the experimental procedures.

All experimental procedures were approved by the Ethical Committee of the Faculty of Medicine, University of Belgrade and were in full compliance with the European Council Directive (2010/63/EU).

Implantation of intracranial electrodes for electroencephalographic (EEG) recordings: EEG registration and analysis

Three gold-plated screws were implanted into the skull of the animals for EEG recordings. Prior to the procedure rats were anaesthetized with intraperitoneal injection (i.p.) of sodium pentobarbital (50 mg/kg), and positioned in a stereotaxic apparatus afterwards. Recording electrodes were implanted over frontal (2 mm rostrally to bregma and 2 mm from the median line), parietal (2 mm rostrally to lambda and 2 mm laterally to median line) and occipital (2 mm caudally to lambda) cortices.

Upon 7-day recovery period, rats had 24 h habituation to the EEG recording settings with a full cable set-up attached.

A classical 8-channel EEG apparatus (RIZ, Zagreb, Croatia), with a modified output degree enabling the transfer of output signals to the input circuit of 12-byte AD card PCL-711B (Advantech Co. Ltd.) and sampling frequency of 512 Hz/channel installed into a computer, was used. The signals were digitized using a SCB-68 data acquisition card (National Instruments Co, Austin, Texas, USA). Data acquisition and signal processing were performed with the Lab VIEW platform software developed in the NeuroSciLaBG Laboratory (Stanojlović et al., 2009; Hrnčić et al., 2011). Following parameters of signal processing were applied: 50 Hz notch filter for ambient noise removal, and 0.3 Hz and 100 Hz the cut-

off frequencies for the high-pass and low-pass filters, respectively. All EEG recordings sessions lasted 30 min and were visually monitored. Criteria for EEG ictal period identification were: spontaneous and generalized spiking activity; lasting more than 1s., and amplitude of at least twice the background EEG activity.

We analyzed the number and duration of ictal periods using left frontal-right parietal cortex lead, since recorded electrographic seizures were generalized with simultaneous and comparable ictal activity in all leads. All ictal periods were detected visually.

Chronic sleep fragmentation

A forced awakening-protocol was used in order to experimentally fragment sleep over the period of 14 consecutive days. Sleep fragmentation was achieved by an established methodology as previously described (McKenna et al., 2007; Hrnčić et al., 2016; Grubac et al., 2019) in details using the first 6 h of light phase of the light/dark cycle as a fragmentation period. This method utilizes the protocol based on treadmill apparatus for small animals, which was applied to the control group, additional control locomotor activity group and the study group subjected to sleep fragmentation. Briefly, treadmill activity was defined by ON mode (working mode, belt moving) at the speed of 0.02 m/s and OFF mode (stop mode) at the speed of 0.00 m/s. The treadmill was programmed to work alternately for 30 s ON and 90 s OFF every 2 min during the entire period of 6 h in order to achieve sleep fragmentation (sleep fragmentation protocol SF, 180 consecutive cycles in total; n=18) similar to one in patients with OSA. The corresponding activity control protocol was also included in the study to avoid any confounding effects of the movement itself, i.e., the total movement of the rats was equal to the SF group, but without interruption of their sleep during longer periods of time (10 min ON and 30 min OFF, activity control protocol, AC; n=18). The treadmill control consisted of rats staying in the treadmill apparatus at moving speed of 0 m/s and conditions equivalent to those in the cages (treadmill control protocol: TC, consecutive cycles in total over 6 h, n=18). After each treadmill session, the rats from the SF, AC and TC protocols were transferred to home cages and the procedure was repeated in the same manner the next day for fourteen consecutive days.

On the 15th day, one third of the animals from each protocol received intraperitoneal injection of subconvulsive dose of lindane (L, 4 mg/kg) according to our previous study (Hrnčić et al., 2016), comprising the SF+L, AC+L and TC+L group (n=6 per group). Lindane was dissolved in dimethylsulfoxide (DMSO) immediately prior to use. The other third of the animals from each protocol received only DMSO, comprising the SFc, ACc and TCc groups (n=6 per group), which served as control groups to chemoconvulsant.

The remaining third of the animals from the SF, AC and TC protocol (SF, AC, TC, n=6 per group) were immediately sacrificed to obtain serum and brain structure isolation for determination of cytokines.

The study design time-flow chart is presented in Fig. 1.

Convulsive behavior

Convulsive behavior parameters were observed 30 min upon lindane injection and were defined as follow: incidence – number of convulsing rats in group, expressed as percentage; latency period – time between lindane administration and first convulsion and seizure severity – modified descriptive scale with grades from 0 to 4. The grades were defined as in our previous studies (Stanojlović et al., 2009): 0 – no convulsive behavior; grade 1 – head nodding, lower jaw twitching; grade 2 – myoclonic body jerks (hot plate reaction), bilateral forelimb clonus with full rearing (Kangaroo position); 3 – progression to generalized clonic convulsions followed by tonic extension of fore and hind limbs and tail and grade 4 prolonged severe tonic-clonic convulsions lasting over 5 min (status epilepticus).

Determination of interleukin levels

Blood samples were collected from the tail vein, and centrifuged at 1575 g for 10 min and frozen at -20°C until assayed for cytokine levels. Duplicate serum aliquots for all analyses were used. Brain levels of IL-1 beta and IL-6 were determined in rats from the SF, AC and TC groups (n=6). These groups have undergone the same protocol of sleep fragmentation like SF+L, AC+L and TC+L and SFc, ACc and TCc, but were sacrified on day 15th, at 8th a.m. by decapitation (so it wouldn't interfere with the IL levels), instead of being treated with lindane or DMSO, respectively. The isolation of brain and dissection of the hippocampus, thalamus and fronto-parietal cerebral cortex was performed in the previously described method (Paxinos and Watson, 2007). All tissue samples were homogenized on ice in the commercial RIPA buffer (Sigma-Aldrich, USA) supplemented with protease inhibitory cocktail (#P8340, Sigma-Aldrich, USA) and stored at -80°C until further usage. The IL-1β and IL-6 concentrations were assayed in the above-mentioned brain structures using the commercial ELISA kit (Rat IL-1β ELISA Kit, Sigma-Aldrich, USA and Rat IL-6 ELISA Kit, Sigma-Aldrich, USA) according

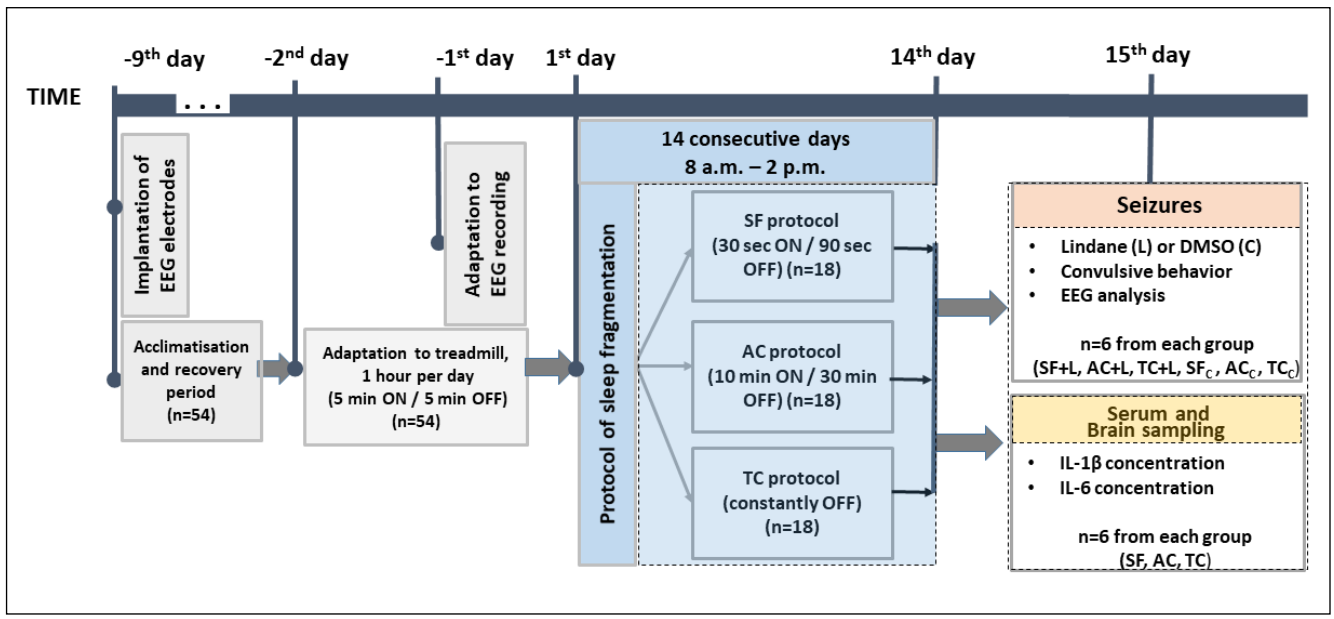

Fig. 1. Schematic illustration of experimental design time flow. Upon animal arrival, acclimatization to laboratory conditions was allowed for 7 days. Sleep fragmentation was achieved by procedure using treadmill for small laboratory animals. Prior to fragmentation all animals were adapted to the moving of the treadmill track for 2 days in sessions of 1 h (5 min ON and 5 min OFF working regime, treadmill belt speed ON=0.02 m/s, OFF=0 m/s). For the sleep fragmentation procedure (its beginning is denoted as day 1 at time line), animals (n=54) were subjected to one of the three protocols (n=18 in each) in the treadmill during 6 h of the light period (between 8 a.m. – 2 p.m.) for 14 consecutive days: sleep fragmentation protocol (SF: 30 s OFF: 90 s ON regime, 30 sleep interruptions per h), activity control protocol (AC: 10 min ON: 30 min OFF regime, 1.5 sleep interruptions per h), treadmill control protocol (TC: constantly OFF regime, 0 sleep interruptions per h). On the 15th day, one third of the animals from each protocol received lindane (L) comprising the SF+L, AC+L and TC+L groups (n=6 per group). The other third of the animals from each protocol received only vehicle, comprising the SFc, ACc and TCc groups (n=6 per group), which served as control groups to chemoconvulsant. The remaining third of the animals (n=6 per group) form each protocol (SF, AC and TC group) were immediately sacrificed to obtain serum and brain structure isolation for determination of cytokines.

to manufacturer's instructions which did not require any specific protein isolation procedure apart from the aforementioned homogenization. Briefly, sandwich assay procedure included addition of samples/standards into antibody pre-coated wells with an overnight at 4°C incubation after which biotin-labelled detection antibody was added. Concentrations of IL-1β and IL-6 were calculated based on standard curve plot.

Data analysis

The statistically significant difference in the incidence was determined by using Fisher's exact probability test. The non-parametric Kruskal-Wallis ANOVA and Mann-Whitney U tests were applied in further data analyses for the assessment of statistically significant difference between group since the normal data distribution of the data regarding latency period, convulsive episodes' severity, as well as number and duration of ictal periods in EEG, has not been determined by the Kolmogorov-Smirnov test (*p<0.05 and **p<0.01). On the other hand, the normal distribution of the data on interleukin levels has been proven by the Kolmogorov-Smirnov test and one-way ANOVA, with LSD *post hoc* testing used to evaluate differences in interleukin levels between groups. For parametric variables, data were presented with means ± standard error, while non-parametric variables were presented as medians with 25th and 75th percentiles. Significance was set as p<0.05.

RESULTS

Convulsions analysis

There were no seizures in the SFc, ACc and TCc groups, while seizures have been noted in every lindane-treated group. The incidence of seizures was highest in the SF+L group, where they were uniformly present (incidence=100%). The incidence in the SF+L group was significantly higher compared to the AC+L group, with the incidence of 33.33% (Fisher's exact *P*=0.0303, Fig. 2), and also compared to the TC+L group, which demonstrated the incidence of 16.67% (Fisher's

exact P=0.0076, Fig. 2). There was no significant difference in incidence between the TC+L and AC+L group (33.33% *vs.* 16.67%, Fisher's exact P=0.5000, Fig. 2).

The next parameter of convulsive behavior response that we analyzed was latency time to first seizure sign upon lindane administration. Kruskal-Wallis ANOVA showed significant (H_2=12.15, P<0.05) difference in latency time among TC+L, AC+L and SF+L group. Latency time was significantly shorter in the SF+L group than in the TC+L (U=0.50, z=2.85, P<0.01) and AC+L groups (U=13.00, z=2.75, P<0.01, Fig. 3A). Although the median latency to seizure was somewhat shorter in the AC+L group compared to the TC+L, the difference was not statistically significant (U=13.00, z=0.85, P>0.05, Fig. 3A).

Grading the severity of seizures using the behavioral scale previously described, median value in SF+L group was 2, with some animals reaching even grade 4 (Fig. 3B). Severity in other groups were similar, with both the AC+L and TC+L groups having the median grade 0, albeit somewhat higher range was observed in the AC+L group (Fig. 3B). Kruskal-Wallis ANOVA showed significant (H_2=9.13, P<0.05) difference in severity of seizures among TC+L, AC+L and SF+L group. Statistically, the severity in the SF+L group was statistically higher compared to the TC+L group (U=13.00, z=-2.77, P<0.01). There were no statistically significant differences AC+L *vs.* SF+L (U=6.00, z=-1.91, P>0.05) and TC+L *vs.* AC+L (U=14.00, z=-0.74, P>0.05).

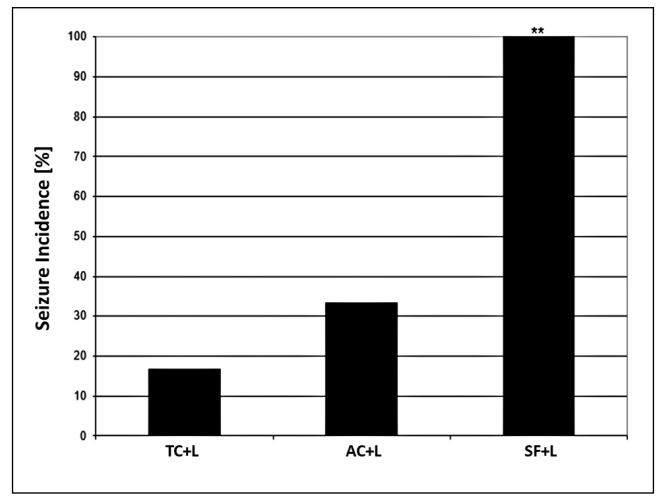

Fig. 2. Incidence of seizures in sleep-fragmented lindane-treated animals. After being adapted to the environment and on the 14th day upon completion of sleep fragmentation protocol, Lindane (L), was administered. Rats were distributed into following groups: treadmill control (TC+L, treadmill protocol OFF only), activity control (AC+L, alternating treatmill protocol 10 min ON and 30 min OFF), sleep fragmentation (SF+L, treadmill protocol 30 s ON and 90 s OFF). Seizure incidence was expressed as convulsive rats percentage (%) of total. Fisher's exact probability test was performed for statistical assessment (**p<0.01).

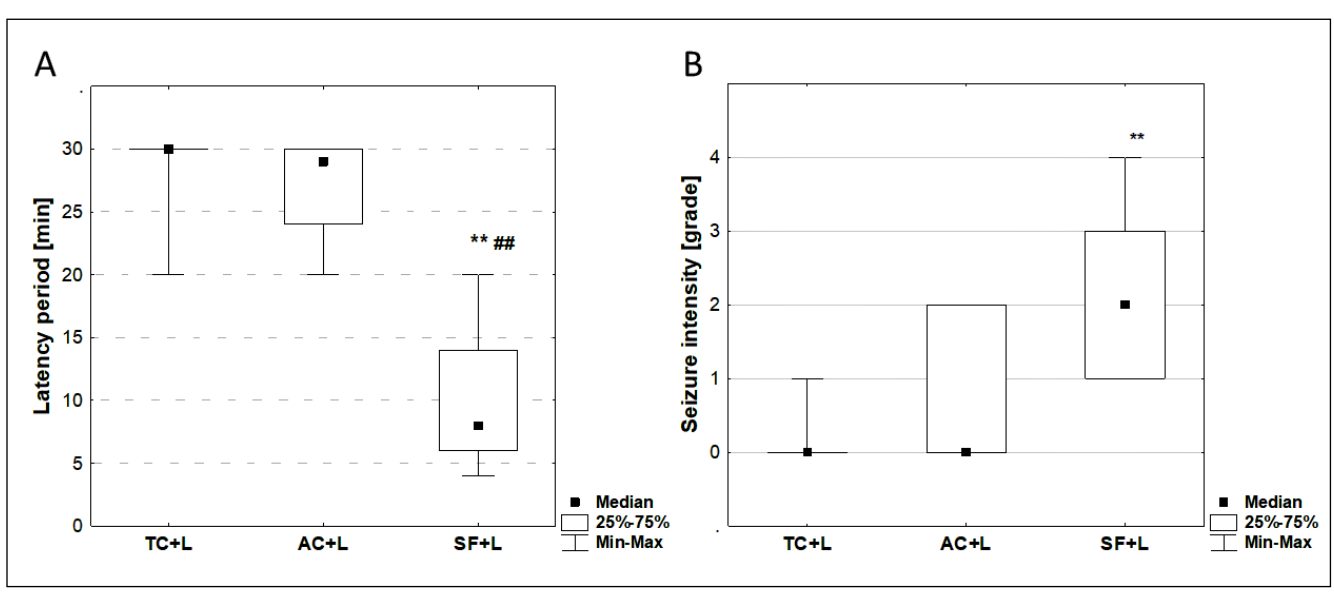

Fig. 3. Latency to convulsions and intensity of seizures (seizure grade). Latency to seizure is expressed as minutes elapsed following lindane administration to the occurrence of first convulsive signs. Statistical significance was examined using the Kruskal-Wallis ANOVA followed by Mann-Whitney U-test. (**p<0.01 *vs.* TC+L, ##p<0.01 *vs.* AC+L). (B) Lindane-provoked seizure intensity grade. Seizures were graded based on behavioral observations in convulsive rats based on a descriptive scale: 0: no seizure; 1: head nodding, lower jaw twitching; 2: "hot plate reaction" (myoclonic body jerks), "Kangaroo position" (bilateral forelimb clonus with full rearing); 3: generalised tonic-clonic seizures with extension of fore and hind limbs and tail; 4: status epilepticus (tonic-clonic seizures lasting over 20 s) or frequently repeated episodes of clonic convulsions for an extended period of time (over 5 min). Statistical significance was examined using the Kruskal-Wallis ANOVA followed by Mann-Whitney U-test. (**p<0.01 *vs.* TC+L as well as *vs.* AC+L).

EEG analysis

The EEG activity in rats from the control SFc, ACc and TCc groups showed no signs of ictal activity (Fig. 4A, C, E). Rats from groups receiving lindane developed ictal activity in EEG consisting of sporadic, smaller groups or series of high-amplitude spikes (EEG ictal periods, Fig. 4B, D, F). We analyzed the number and duration of these ictal periods.

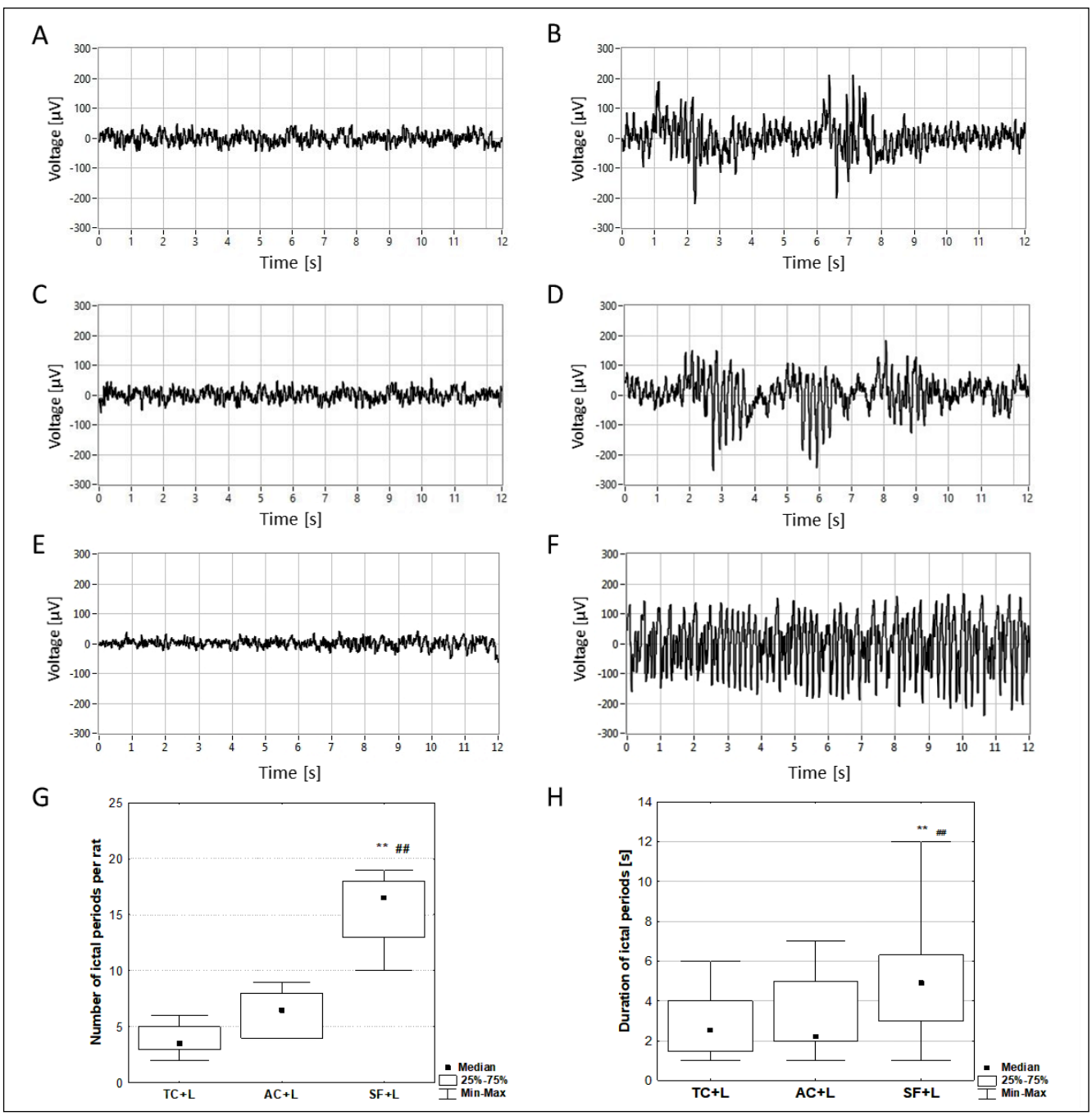

Fig. 4. Representative EEG tracings recorded in TCc, ACc and SFc groups (A, C, E) and TC+L, AC+L and SF+L groups (B, D, F). The number (G) and duration (H) of EEG ictal periods upon lindane administration. Representative EEG tracings recorded in control groups (TCc, ACc and SFc, A, C, E) showing baseline activity without signs of epileptiform discharges. Ictal activity has been recorded upon lindane administration in TC+L, AC+L and SF+L group (B, D, F) in form of spike bursts of different duration and number. Lead: left frontal–right parietal cortex. The number (G) and duration (H) of ictal periods was measured during 30 min EEG recordings upon lindane administration. The significance of the differences between the groups was estimated by Kruskal-Wallis ANOVA and Mann-Whitney U test (**p<0.01 *vs.* TC+L, $^{\#\#}$p< 0.01 *vs.* AC+L).

According to Kruskal-Wallis ANOVA, number of ictal periods was significantly different (H_2=13.28, P<0.05) among the groups receiving lindane (TC+L, AC+L and SF+L). The SF+L group exhibited significantly higher number of ictal periods per rat in comparison to the TC+L (U=0.00, z=-2.81, P<0.01), as well as in comparison to the AC+L group (U=0.00, z=-2.80, P<0.01, Fig. 4G). At the same time, there were no statistically significant differences between the AC+L and TC+L groups regarding the number of ictal periods per rat (U=5.50, z=-1.94, P>0.05), Fig. 4G), On the other hand, the same holds true for duration of ictal period among the groups receiving lindane (TC+L, AC+L and SF+L, H_2=15.67, P<0.01, Fig. 4H).

Interleukin analysis

According to one-way ANOVA analysis, there were significant differences ($F_{2,15}$=7.22, P<0.01) among TC, AC and SF group in IL-1β hippocampal levels. Post hoc analysis showed that hippocampal levels of IL-1β showed significant increase in the SF group contrasted with both the TC (P<0.01) and AC groups (P<0.05, Fig. 5C). IL-1β levels were noted to be higher in the AC group compared to TC, but the difference was not statistically significant (P>0.05, Fig. 5C). Similar distribution has been observed in the thalamus ($F_{2,15}$=63.28, P<0.01), with the SF group having the highest IL-1β level among the three groups (P<0.01 *vs.* both AC and TC, Fig. 5E). Again, AC group levels were higher than in the TC group, but not statistically significant (P>0.05, Fig. 5E). The same holds true for IL-1β in the cortex ($F_{2,15}$=5.31, P<0.05): the SF group expressed significantly higher level of IL-1β compared to both the TC (P<0.05) and AC groups (P<0.05, Fig. 5A), while no significant differences between TC and AC group were observed (P>0.05, Fig. 5A).

In contrast to hippocampal IL-1β, the hippocampal levels of IL-6 post-hoc analysis revealed that the SF group had the lowest values among the three groups, the TC group levels were somewhat higher, and the AC group had the highest IL-6 level values; however, no statistical significance was noted in the observation ($F_{2,15}$=0.88, P>0.05, Fig. 5D). Thalamic IL-6 levels were distributed in the following manner: SF<TC<AC, but no statistical significance was obtained ($F_{2,15}$=0.08, P>0.05, Fig. 5F). On the other hand, according to one-way ANOVA analysis, there were significant differences ($F_{2,15}$=4.67, P<0.05) among TC, AC and SF group in IL-6 levels in the cortex, IL-6 was higher in the cortex of animals from the SF group *vs.* the animals from the TC and AC group (P <0.05, Fig. 5B).

Serum levels of IL-1β and IL-6 showed no statistical difference between groups ($F_{2,15}$=0.71, P>0.05; Fig. 6A and $F_{2,15}$=0.88, P>0.05, Fig. 6B, respectively).

DISCUSSION

The effects of altered sleep pattern are known to have profound effects on seizure susceptibility, and the role of total or partial sleep deprivation in patients with epilepsy has been already explained (Scalise et al., 2006; Lucey et al., 2015). However, the relationship between sleep fragmentation and epilepsy has not been studied extensively. Sleep fragmentation is common in patients with sleep apnea (Day et al., 1999), which consists of abnormal breathing as a main symptom and cyclic appearance of sleep interruptions with frequency of 30 or more times per h in severe forms (Devinsky et al., 1994). In our previous study, we have proven that acute sleep fragmentation of 6 h in the light phase of the light/dark cycle potentiated epileptic activity in rats by increasing the number of seizures and their severity, while shortening the seizure latency at the same time (Hrnčić et al., 2016). However, one of the major limitations of that study was the length of the induced fragmentation, i.e., chronic effects have not been evaluated in that study. It should be emphasized that the effects of acute and chronic sleep interruption are quite different in terms of stress response, somatic pain, reduced quality of life, emotional distress, mood disorders, and cognitive, memory, and performance deficits (Medic et al., 2017). In both, our previous and this research we used the model of sleep fragmentation which mimic the sleep disturbance in patients with proven OSA (Grubac et al., 2019). We should emphasize that, using this specific pattern of fragmentation, shift in the circadian rhythm or REM rebound effect is possible or even likely to happen, however that matter is irrelevant in our case since it also occurs in everyday situations with OSA.

There are several assumptions regarding the underlying cause of the difference between acute and chronic effects, ranging from alternations in HPA axis (hypothalamic-pituitary-adrenal) (Bonnet and Arand, 2003) to REM rebound effect in chronic sleep loss (Shrivastava et al., 2014) and modified sleep architecture (Bonnet and Arand, 2003). However, a definite answer to this question has not been given yet. In the present study we have decided to evaluate the effects of long-term sleep fragmentation which lasted for 14 consecutive days in accordance with Baud et al. (2015), with the difference in duration of the fragmented period since Baud et al. used 24 h fragmentation, and on the other hand we used only 6 h/24 h sleep fragmentation which is, in our opinion, better for understanding its effects due to the fact that the period of 6 h is average sleeping time. The effects of long-term sleep fragmentation were more severe than the effects of the short-term in our previous research, increasing not only seizure susceptibility

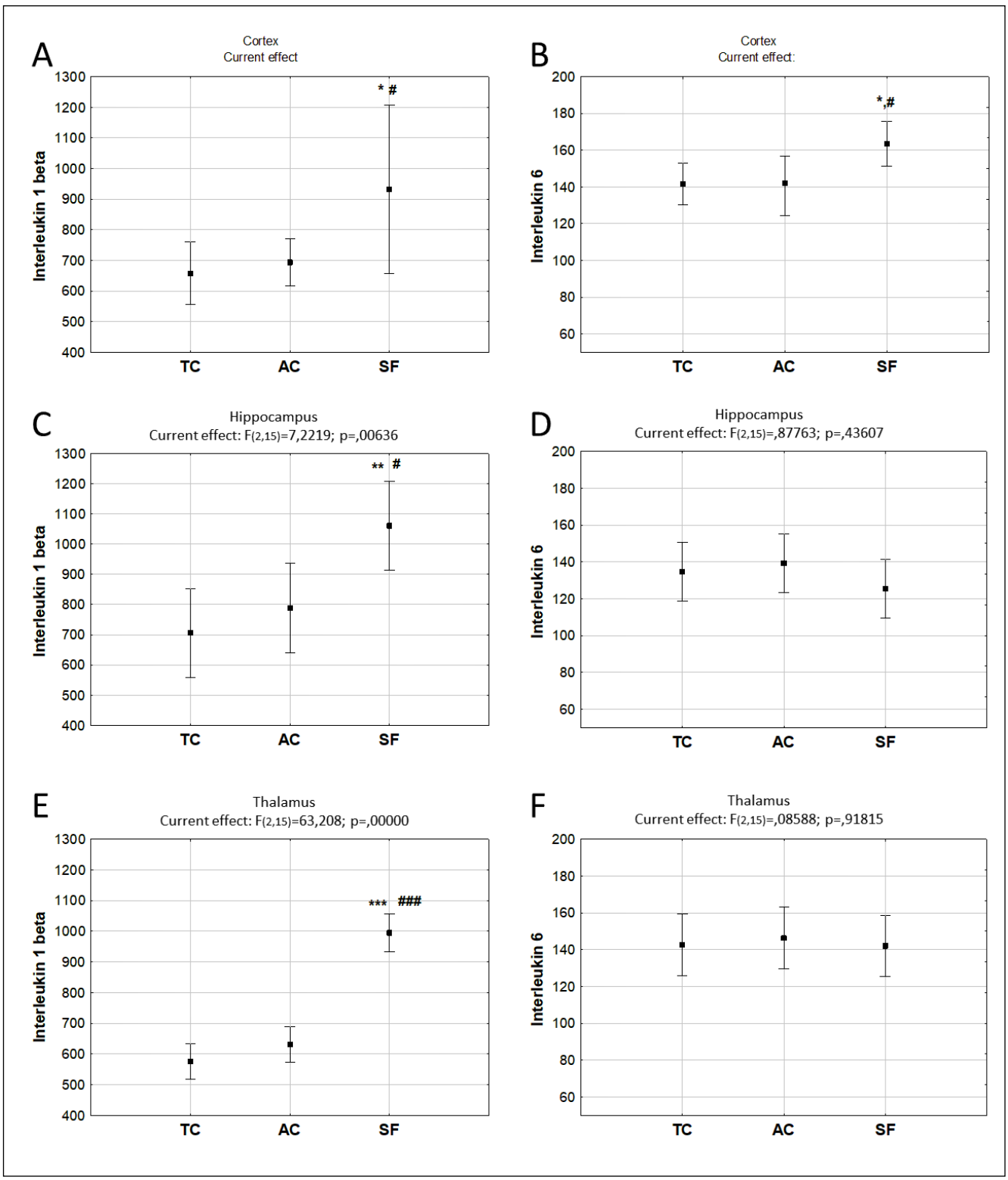

Fig. 5. Interleukin levels in rat brain structures – cortex, thalamus and hippocampus. (C) demonstrated significantly higher values in SF group compared to others (**p<0.01 *vs.* TC, $^{\#}$p<0.05 *vs.* AC). The results were similar in both: thalamic IL-1β levels (E), with SF group having the highest value (***p=0.001 *vs.* TC, $^{\#\#\#}$p=0.001 *vs.* AC) and cortex IL- β levels (A) (SF *vs.* TC, *p<0.05; SF *vs.* AC, $^{\#}$p<0.05; AC *vs.* TC p>0.05). IL-6 levels were also analyzed in rat hippocampi (D), thalami (F) and cortex (B). Difference in IL-6 levels among the three groups was not statistically significant in the hippocampus and thalamus (p>0.05 *vs.* both TC and AC) while significant difference was observed in cortex (*$^{\#}$p<0.05 *vs.* both TC and AC). Statistical significance was tested using Student's T-test.

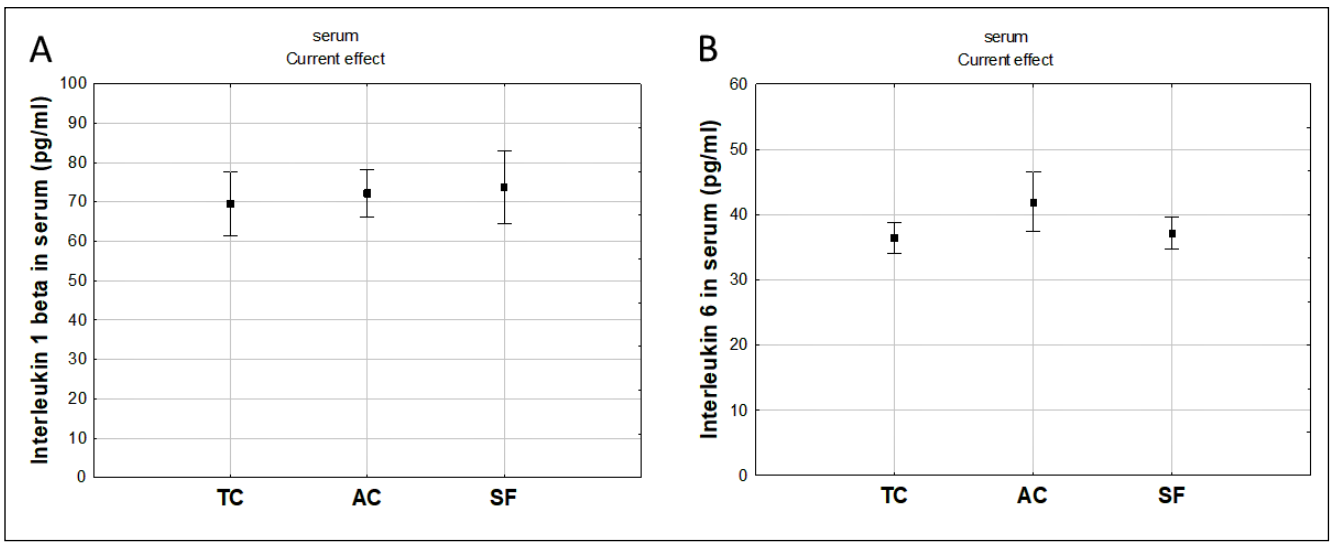

Fig. 6. Serum levels of IL-1β and IL-6. Levels IL-1β and IL-6 in animals' serum were measured using commercial ELISA kit according to the instructions of the manufacturer. Difference in both, IL-1β (A) and IL-6 (B) levels among the three groups was not statistically significant in serum ($p>0.05$ *vs.* both TC and AC).

but inducing both behavioral and EEG epileptic activity after administration of lindane in subconvulsive dose. Our present data also showed significant increase of IL-1β levels in the thalamus, cerebral cortex and hippocampal tissue in rats undergoing protocol of chronic sleep fragmentation compared to the corresponding controls. In addition, the levels of IL-6 were significantly increased in the cortex of sleep fragmented animals, but no statistical difference was found in the thalamus or hippocampus.

Sleep fragmentation and intermittent hypoxia are hallmarks of OSA in which reduction in seizure threshold has been reported (Malow et al., 2003). Unanswered question in the contemporary literature is to which extent sleep fragmentation contribute to this seizure threshold reduction in apneic patient. Our study assessed this question from the viewpoint of chronic sleep fragmentation modeled in experimental conditions since intermittent hypoxia and sleep disruption are very difficult to study separately in clinical settings because they occur simultaneously in apneic patients. Our current study has proven that long-term sleep fragmentation, modeled according to severe form of OSA, aggravates seizures. This is in agreement with line that seizures are very sensitive to alterations in sleep pattern. Namely, good quality sleep is important for optimal human functioning, but it is particularly essential in patients with epilepsy who may find themselves in a cycle of worsening seizures, further sleep disruption, and intractable epilepsy (Bazil, 2017). Moreover, this study could actually give the answer to the question why continuous positive airway treatment (CPAP) is not sufficient treatment of OSA in terms of seizure control (Malow et al., 2003; Bazil, 2017). Beneficial effect of CPAP on epilepsy was shown in about 40% of treated patients with OSA (Vaughn et al., 1996; Beran et al., 1999; Sonka et al., 2000), with approximately 50% seizure reduction in treated apneic patients (Pornsriniyom et al., 2014). Indeed, hypoxia has been already established as a proconvulsive factor (Jensen et al., 1991; 1992) by facilitating glutaminergic neurotransmission (Rubaj et al., 2003; Yang et al., 2013), while CPAP can result in new onset of frontal lobe seizures if applied incorrectly (Miano et al., 2008). Based on our results, we could hypothesize that sleep fragmentation also could represent the mechanism responsible for lowering the threshold to seizure in sleep apnea conditions.

Epileptogenesis is a complex process characterized by an enduring predisposition to generate epileptic seizures and it has been found to be associated with changes in immunological profile (Vezzani et al., 2002; Lehtimäki et al., 2007; Rosa et al., 2016). In our current study, the levels of IL-1β in the SF group showed significant increase in the thalamus, cortex and hippocampus when compared to the AC and TC groups. There was no statistical difference between the AC and TC groups, but levels of IL-1β were higher in the activity control group compared to sedentary controls, so we cannot completely rule out physical activity as the confounding factor for this increase. As for the levels of IL-6, statistically higher levels were only found in the cortex of fragmented animals, and no difference between the groups was observed in the thalamic or hippocampal tissue.

Relationship between epilepsy and IL-1β levels are not completely clear in clinical studies, i.e., in some link has been proven and in some not (Tilgen et al., 2002; Virta et al., 2002; Kanemoto et al., 2003; Ozkara

et al., 2006). On the other hand, basic experimental studies give us better opportunity to establish a link between elevated levels of IL-1β and enhanced propensity for seizure activity. Vezzani group (1999) was the first to report that IL-1β enhances focal electrographic seizures induced by kainate *via* glutamatergic signaling.

Inflammatory alterations in the rats during the acute epileptic events are characterized by rapid increase of IL-1β in activated microglia and astrocytes (Fabene et al., 2008). Moreover, this elevated Il-1β level persisted even upon seizure cessation, what adds to the presumption on IL-1β involvement in the mechanisms underlying the onset of spontaneous seizures (Allan et al., 2005; Fabene et al., 2008). The activation of IL-1β signaling occurs through nontranscription-dependent enzymatic pathway which includes IL-1R1–mediated activation of the neutral sphingomyelinase (Sanchez-Alavez et al., 2006) and the subsequent production of ceramide, which in turn activates the tyrosine kinase protein, Src (Sanchez-Alavez et al., 2006). IL-1β than enhances calcium influx into hippocampal pyramidal cells exposed to *N*-methyl-d-aspartate (NMDA) by phosphorylation (*via* Src kinase) of the NR2B subunit of the NMDA receptor complex, which is responsible for regulating its calcium permeability properties (Viviani et al., 2003). When released, IL-1β binds with high affinity to the type 1 IL-1 receptor (IL-1R1) that is expressed by hippocampal neurons (Ravizza and Vezzani, 2006). In contrast, when seizures evoke rapid production of IL-1β, IL-1RA is upregulated to a far lower extent and with a several hour delay (De Simoni et al., 2000; Ravizza and Vezzani, 2006). This finding suggests that the brain is much less effective than the periphery in inducing a crucial mechanism for rapidly terminating the effects of a sustained rise in endogenous IL-1β. Seizures are often accompanied by extensive neuronal injury, which may account for IL-1 production by microglia (Sanchez-Alavez et al., 2006). At the cellular level, Toll-like receptors (TLRs), activated by seizure-induced heath shock proteins (HSPs), induce the activation of nuclear factor kappa B (NF-κB), which finally results in IL-1β production (Hanke and Kielian, 2011). TLR3 contributes to hippocampal excitability through its upregulation of pro-inflammatory cytokines such as IFN-β (Costello and Lynch, 2013). FOX3P is also expressed by microglia to downregulate inflammatory processes through modulation of NF-κB, a key inflammatory transcription factor (Chung et al., 2010). Involvement of these mechanisms in the elevation of Il-1β observed in our current study is still open question that should be addressed in one of the following studies.

This mechanism has been supported by exploring anticonvulsive effects of IL-1R in bicuculline induced seizures in rodents (Vezzani et al., 2002). In our current study, increased levels of IL-1β in the cerebral cortex, hippocampus and thalamus found in the SF group of rats are congruent with theories about proconvulsive and neurotoxic effects of IL-1β. Proconvulsive role could be achieved through several mechanisms: IL-1β acts as a direct proconvulsive agent through inhibition of $GABA_A$ receptors and K^+ efflux (Wang et al., 2000); inhibits glial uptake of excitatory neurotransmitters (Hu et al., 2000) and causes intracellular calcium ion surge, thus resulting in modification of voltage-dependent ion channels and reduction of seizure threshold (Viviani et al., 2003; Xu et al., 2013). Other mechanisms could also be involved, for example IL-1β is known to increase nitric oxide production in the brain (Meini et al., 2000), as well as to stimulate glutamatergic neurotransmission pathway and evoke neuronal hyperexcitability (Zhu et al., 2006). Contrary to our results, Sayyah et al. (2005) reported an anticonvulsive effect of intraventriculary injected IL-1β in amygdala-kindling mice, which could potentially be explained with IL-1β ability to modulate intracellular concentration of Ca^{2+} in dose-dependent manner, thus balancing excitatory and inhibitory neurotransmission (Zhu et al., 2006; Li et al., 2011). For schematic illustration of proposed mechanisms see Fig. 7.

Beside IL-1β, epileptogenesis is also related to alterations in IL-6, however its role is still unclear and given research results are contradictory (D'Arcangelo et al., 2000). After seizures, IL-6 mRNA is rapidly induced in the hippocampus, cortex, dentate gyrus, amygdala, and meninges, whereas up-regulation of IL-6-receptor mRNA seems to be limited to the hippocampus (Lehtimäki et al., 2003). Several clinical studies demonstrated rapid and transient post-ictal increase of IL-6, which peaked at 12 h and remained for 24 h after seizures. However, some patients with chronic epilepsy also had higher levels of IL-6 in basal conditions (Virta et al., 2002; Lehtimäki et al., 2004, 2007; Alapirtti et al., 2009). Different types of epilepsy showed different patterns in IL-6 elevation. Taking into account the lower post-ictal levels of IL-6 in some serious forms of epilepsy, it can presumably act as a neuroprotective agent (Bauer et al., 2009). In contrast to this finding, there is also a report about exogenous IL-6 attenuated seizures in rats (Fukuda et al., 2007). Studies using transgenic animals reflect the double-edged nature of IL-6 with IL-6 deficient mice more prone to seizures, and those with over expression of IL-6 showing lower threshold to spontaneous and NMDA-induced seizures, probably due to reduced GABA-mediated inhibition (De Sarro et al., 2004).

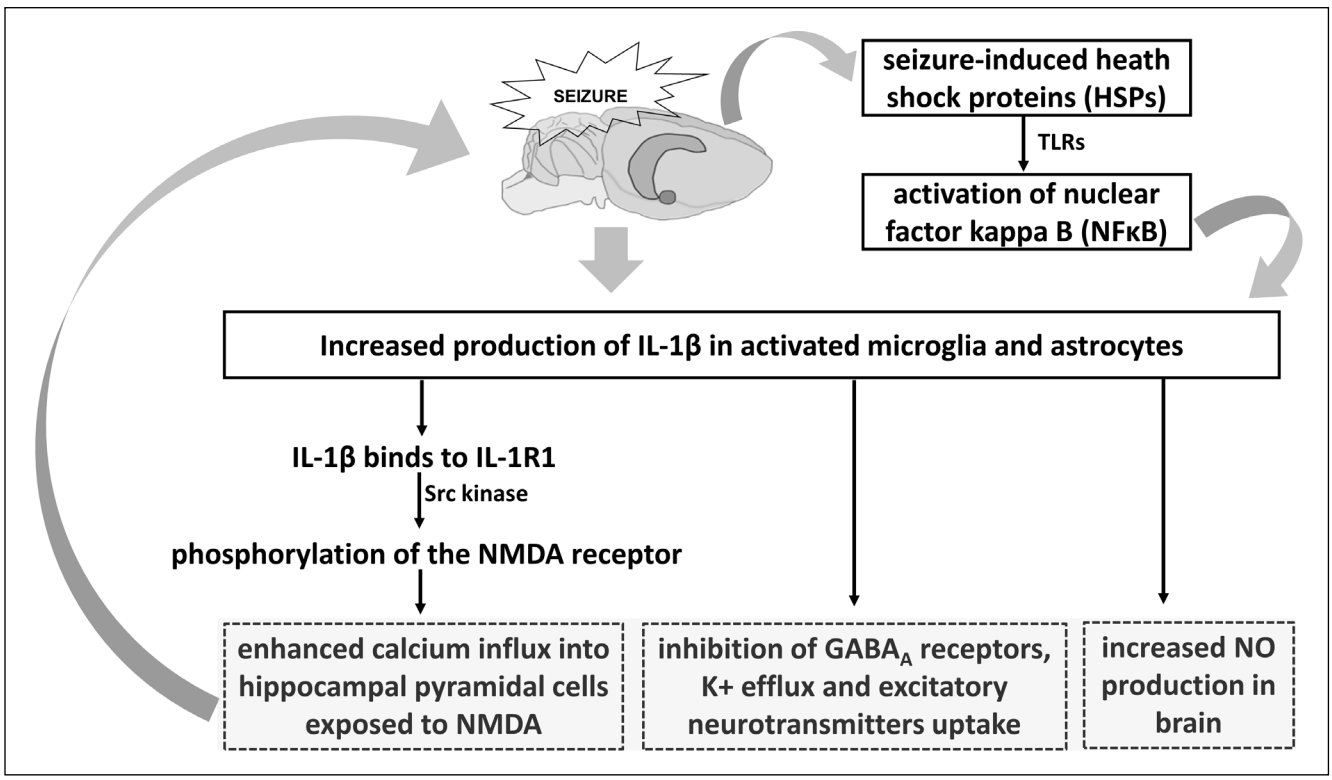

Fig. 7. Schematic representation of the proposed mechanism of IL-1β activation and subsequent production of seizure.

In our study elevated levels of IL-1β and IL-6 were found in the cortex, which is always involved in epileptic discharge (Alapirtti et al., 2018).

IL-1β has been recognized as a fully proconvulsive and proinflammatory cytokine, as it is obvious from our discussion above. On the other hand, IL-6 is a pleiotropic cytokine that has both pro- and anti-inflammatory actions with predominantly anticonvulsive nature (Erta et al., 2012). We found increased IL-1β levels in the cortex, hippocampus and thalamus, with IL-6 level was increased only in the cortex upon chronic sleep fragmentation. We could speculate that IL-1β may contribute to epileptogenesis in our study by modulating or blocking IL-6 signal transduction in the hippocampus and thalamus, since such a crosstalk between these two cytokines has been demonstrated in other organs (Deon et al., 2001). Namely, antagonism may occur at the level of signal transduction, i.e., IL-1β has been shown to suppress Janus kinase-STAT signalling by IL-6 and antagonize IL-6 induction of tissue inhibitor of metalloproteases 1 expression (Deon et al., 2001). Neuroinflammation depends on cytokines, glial and neuronal signalling, as well as on extracellular matrix, and all of the components being interconnected (Hrncic et al., 2018). Additionally, increased IL-6 level in the cortex could be compensatory response in order to reestablish dysregulated cytokine balance. Indeed, upregulated IL-6 has been revelled in conditions of higher neuronal excitability (Uludag et al., 2013), which is presumably consequence of sleep fragmentation in our study.

There are several limitations of this study. The study has been performed in male rats having in mind their stability regarding hormonal status. However, further studies on female rats should be also done, in diestrus phase of their estrus cycle due to possible influence of sex hormones (Scharfman and MacLusky, 2014). Namely, there are important sex-dependent differences that influence seizures and epilepsy. Studies in which pooling male and female are likely to obtain confusing and different results unless experimental conditions are fully controlled (Tan and Tanb, 2001). We focused herein on sleep fragmentation effects on IL-1β and IL-6 production in different brain structures, while finding the exact cell type (astrocytes, microglia, neurons) as a source of these cytokine production should be addressed in further immunohistochemical studies.

CONCLUSIONS

In summary, we have demonstrated herein that a 14-day chronic sleep fragmentation increases sei-

zure susceptibility in rats, which is objectively proven by convulsive behavioral response and EEG analysis and altered brain production of inflammatory cytokines IL-1β and IL-6, which could be potential explanation for increased seizure susceptibility. For sure, other mechanisms may also be involved in the development of brain hyperexcitability. Further studies are needed with IL-1β and IL-6 antagonists, as well as modifications of IL-1β and IL-6 genes, in order to confirm the proexcitatory role of these cytokines in sleep fragmentation and find exact cellular source of its production.

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