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FACILITATION OF SPIKE-WAVE DISCHARGE ACTIVITY BY LIPOPOLYSACCHARIDES IN WISTAR ALBINO GLAXO/RIJSWIJK RATS

Z. KOVÁCS,[a] K. A. KÉKESI,[b] N. SZILÁGYI,[c] I. ÁBRAHÁM,[c] D. SZÉKÁCS,[c] N. KIRÁLY,[c] E. PAPP,[a] I. CSÁSZÁR,[a] É. SZEGŐ,[c] K. BARABÁS,[c] H. PÉTERFY,[d] A. ERDEI,[d] T. BÁRTFAI[e] AND G. JUHÁSZ[c]*

[a]*Department of Zoology, Berzsenyi Dániel College, Károlyi Gáspár tér 4, Szombathely, 9700 Hungary*

[b]*Department of Physiology and Neurobiology, Eötvös Loránd University, Pázmány Péter Sétány 1C, Budapest, 1117 Hungary*

[c]*Research Group of Neurobiology, Hungarian Academy of Sciences–Eötvös Loránd University, Pázmány Péter sétány 1C, Budapest, 1117 Hungary*

[d]*Department of Immunology, Eötvös Loránd University, Pázmány Péter Sétány 1C, Budapest, 1117 Hungary*

[e]*Harold Dorris Neurobiological Center, Department of Neuropharmacology, Scripps Research Institute, North Torrey Pines Road 10550, La Jolla, CA 92037, USA*

Abstract—In normal rats the proinflammatory cytokines like interleukin-1β, interleukin-6, which are induced by bacterial lipopolysaccharides, are able to control thalamo-cortical excitability by exerting strong effects on physiological synchronization such as sleep and on pathological synchronization like that in epileptic discharges. To investigate whether proinflammatory cytokines or lipopolysaccharides could modulate absence seizures resulting from a very different generator mechanism than the already investigated bicuculline-, kindling- and kainate-induced seizures, we used a genetically epileptic Wistar Albino Glaxo/Rijswijk rat strain, which is spontaneously generating high voltage spike-wave discharges. Wistar Albino Glaxo/Rijswijk rats responded with an increase of the number of spike-wave discharges to lipopolysaccharide injection (from 10 μg/kg to 350 μg/kg). Repetitive administration of 350 μg/kg lipopolysaccharides daily for 5 days increased the number of spike-wave discharges on the first, second and third days but the number of spike-wave discharges returned to the control value on day 5, at the 5th injection of lipopolysaccharides, showing a tolerance to lipopolysaccharides. The lipopolysaccharide-induced increase in spike-wave discharges was not directly correlated with the elevation of the core body temperature, as it is in febrile seizures, although lipopolysaccharide induced prostaglandin and is clearly pyrogenic at the doses used. Indomethacin, the prostaglandin synthesis inhibitor, efficiently blocked lipopolysaccharide-induced enhancement of spike-wave discharge genesis suggesting that the spike-wave discharge facilitating effect of lipopolysaccharides involves induction of cyclooxygenase 2 and subsequent synthesis and actions of prostaglandin E2. Low dose (40 mg/kg, i.p.) of competitive *N*-methyl-D-aspartate receptor antagonist 2-amino-5-phosphonopentanoic acid, and low dose of lipopolysaccharide (20 μg/kg) showed a synergistic interaction to increase the number of spike-wave discharges, whereas at supramaximal doses of lipopolysaccharide and the *N*-methyl-D-aspartate antagonist no synergy was present. The data reveal a functional connection between absence epileptic activity and lipopolysaccharide induction of prostaglandin synthesis and prostaglandin action and suggest some common cellular targets in epilepsy and lipopolysaccharide-induced inflammation. © 2006 Published by Elsevier Ltd on behalf of IBRO.

Key words: epileptic activity, inflammation, lipopolysaccharide, indomethacin.

Thalamo-cortical synchronization is strongly affected by proinflammatory cytokines (Miller et al., 1991; Schiffelholz and Lancel, 2001; Vezzani et al., 2002). The role of endogenously released cytokines in control of synchronization is complex and contradictory in several aspects. Cytokines such as interleukin-1 β (IL-1β), endogenous antagonist of interleukin-1 receptor (IL-1Ra), interleukin-6 (IL-6) and their receptor mRNA levels were induced by kainic-acid-induced seizures in general and in amygdala, and other brain areas following kindling (Billiau et al., 2005; Jankowsky and Patterson, 2001; Lehtimaki et al., 2003; Plata-Salaman et al., 2000; Vezzani et al., 2002). IL-1β is strongly proconvulsive while the endogenous antagonist of IL-1β, IL-1Ra has anticonvulsive effect in bicuculline and kainate-induced epileptic activity (Vezzani et al., 2000, 2002), suggesting that IL-1β and its endogenous antagonist are involved in control of seizure genesis. Epileptic activity however is a symptom, which can be generated by very different cellular mechanisms. Bicuculline induces seizures through inhibition of GABAergic signaling while another type of seizure like the absence form of epilepsy is generated when there is a gentle and sustained hyperpolarization present in the thalamo-cortical system. In apparent contrast to its pro-convulsive roles and enhancement in excitation by IL-1β (Vezzani et al., 2002), it has also been demonstrated that IL-1β promotes sleep in rats (Bauer et al., 1995; Krueger et al., 1998; Schiffelholz and Lancel, 2001; Toth and Opp, 2001). Proinflammatory cytokines enhance EEG synchronization, increase slow-wave-sleep and promote sleep onset (Miller et al., 1991; Schiffelholz and Lancel, 2001). Thus proinflammatory cytokines could enhance inhibitory processes in rats because sustained inhibition of thalamo-cortical cells is a prerequisite of synchronized EEG activity in sleep (Steriade et al., 1993; Steriade, 2001, 2005).

*Corresponding author. Tel: +361-209-0555x8110; fax: +361-319-6723.
E-mail address: gjuhasz@dec001.geobio.elte.hu (G. Juhász).

Abbreviations: ACSF, artificial cerebrospinal fluid; AP5, 2-amino-5-phosphonopentanoic acid; IL-1R, interleukin-1 receptor; IL-1Ra, endogenous antagonist of interleukin-1 receptor; IL-1β, interleukin-1 β; IL-6, interleukin-6; LPS, lipopolysaccharide; LTP, long-term potentiation; NMDA, *N*-methyl-D-aspartate; PGE2, prostaglandin E2; SWD, spike-wave discharges; TLR4 receptor, toll-like receptor 4; TNF-α, tumor necrosis factor-α; WAG/Rij, Wistar Albino Glaxo/Rijswijk.

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It is likely that cytokines could alter sustained inhibition of thalamo-cortical neurons and enhance not only sleep but also absence seizures. Recently, it has been published that IL-1β contributes to fever induced hyperexcitability underlying febrile seizures (Dubé et al., 2005; Heida and Pittman, 2005). Fever-induced febrile seizures and absence epileptic activity are linked to the same polymorphisms in the GABA receptor γ2 subunit gene in humans (Marini et al., 2003), suggesting common molecular mechanisms behind these two types of seizure activity. With this background, we set out to examine whether proinflammatory cytokines or the bacterial lipopolysaccharide (LPS) that induces proinflammatory cytokines in the brain and cause fever could also modulate absence epilepsy in a genetically epileptic rat strain. There is a genetically determined form of epileptic spike-wave discharges (SWD) in Wistar Albino Glaxo/Rijswijk (WAG/Rij) rats, which might be a promising model for studying LPS/cytokine mechanisms in synchronization of SWDs of thalamic origin (Coenen et al., 1992; Drinkenburg et al., 1993; Weir, 1965). SWD genesis correlates with the transitional states of sleep cycle (Coenen et al., 1991; Danober et al., 1998; Drinkenburg et al., 1991, 1995) and it is based on sustained synchronous activity of thalamic relay cells and/or cortical neurons performing calcium spikes due to activation of T-type calcium channels at 8–10 Hz frequency (Avanzini et al., 2000; Manning et al., 2004; Meeren et al., 2002; Pinault et al., 2001; Russo et al., 2004; Sitnikova and van Luijtelaar, 2004; Steriade et al., 1993; Steriade, 2001; Ure and Perassolo, 2000). T-type calcium channels open when the membrane potential is gently hyperpolarized (Steriade et al., 1993). Most studies on the role of proinflammatory cytokines in fever and sleep are performed either by inducing their rapid synthesis *in vivo* by the systemic injection of bacterial LPS, that through the toll-like receptor 4 (TLR4) receptor induces the synthesis and release of numerous proinflammatory mediators such as IL-β, tumor necrosis factor-α (TNF-α) and IL-6, thus instead of using one of the proinflammatory cytokines alone we decided to use LPS. Using freely moving WAG/Rij rats, we report here first on dose dependent effects of LPS on SWD number, duration and SWD power spectra which are independent from LPS induced changes in core body temperature and independent of LPS effects on sleep. We also show that the LPS effect on SWDs is not restricted to the genetically inbred strain WAG/Rij but it was observed in old Wistar rats performing spontaneous SWDs. The LPS effect on SWD numbers is synergistic with the blockade of *N*-methyl-D-aspartate (NMDA) receptors, as shown by combined application of LPS and a reversible NMDA receptor blocker 2-amino-5-phosphonopentanoic acid (AP5). An important link between SWD generation and LPS-induced inflammation may be represented by prostaglandins, as it was shown that both of these LPS induced responses could be inhibited with indomethacin that blocks the synthesis of prostaglandins.

EXPERIMENTAL PROCEDURES

Animals

All animal treatments and surgery procedures were carried out in correspondence with the local ethical rules which are in conformity with the guidelines of the European Communities Council Directive 24 November 1986 (86/609/EEC) to use and treat animals in experimental laboratories. All efforts were made to eliminate animal suffering and to reduce the number of animals used.

Adult (6.5 months old, S.E.M.$\pm$0.25) WAG/Rij and Wistar (12 months old, S.E.M.$\pm$0.5) mail rats weighing 250–330 g were used. Animals were housed in groups of three to four and they were separated after surgery under standard laboratory conditions of Charles-River Co. Technology (12-h light/dark cycle, light was on from 8:00 AM to 8:00 PM), with free access to water and food. Rats were maintained in an air-conditioned room at 22$\pm$2 °C.

Implantation of animals for EEG recording

WAG/Rij (n=45) and Wistar (n=3) rats were implanted in halothane–air mixture (1%) anesthesia with 0.8 mm stainless screw electrodes for EEG recording. Screws were placed into the bone above the frontal (A 2.0, L 2.1) and parietal (A −6.5, L 2.1) cortices. Ground electrode was placed above the cerebellar cortex. Electrodes were soldered to a 10-pin socket and glued to the skull with dentacrylate cement. Lidocaine ointment was used as post-implantation pain reliever. Rats were allowed to recover for at least 2 weeks. The recovery was controlled by recording the EEG and the full recovery was established when SWD frequency was stabilized in the transition phase from sleep to the active period in late afternoon. Animals were gently handled several times daily to reduce stress-induced changes of SWD number.

We applied thermo-resistors (Pt 100) for body temperature measurement implanting them to the surface of the skull. The thermo-resistors were covered with silicon grease making thermal connection between the bone and the aluminum holder and we applied silicone oil for enhancing the thermal conduction between the holder and the thermo-resistor. Body temperature was measured at 0.05 °C accuracy with a thermometer (Supertech Ltd., Pécs, Hungary) in each 10 min.

Implantation of animals for collecting blood samples and cytokine measurement by ELISA

Blood samples were collected for measuring cytokines from freely moving WAG/Rij rats (n=6) 4 days after implantation of a jugular vein cannula. The out-leading tube of the cannula was attached to the skull to avoid stressful handling of rats when samples were taken. Heparin–sodium citrate (0.01% heparin in 1% citrate) solution was applied to prevent blood clotting. Each day the tubes were flushed with heparin (0.01%) to prevent clogging. Animals were gently handled several times daily to prevent stress-induced increase of cytokines. Blood sample collection started with collecting three control samples (50 μl), then 20 μg/kg or 350 μg/kg LPS (Sigma-Aldrich Ltd., Budapest, Hungary; *E. coli*, serotype O111:B4) was injected respectively and 50 μl blood samples were collected in each hour for 6 h. The Quantikine kits of R&D Systems (Minneapolis, MN, USA) were applied for IL-1β, IL-6 and TNF-α measurements. Results were obtained in pg/ml concentrations using a standard calibration solution series of 0, 15.6, 31.2, 62.5, 125, 250, 500 pg/ml for IL-1β; 0, 6.25, 12.5, 25, 50, 100, 200 pg/ml for TNF-α; and 0, 15.6, 31.2, 62.5, 125, 250, 500 pg/ml for IL-6, respectively. We were not able to measure cytokine levels from blood samples during SWDs because of sampling procedure abolished SWD genesis.

Recording SWD and data scoring

Recording was carried out in the animal home cage placed into an electrically shielded and soundproof box lighted with a bulb (1000 lux). EEG of the frontoparietal leads was recorded by a differential preamplifier (Supertech Bioamp 4; Supertech) attached to a CED 1401 mkII a data capture and analysis device using SPIKE 2 software of the company (Cambridge Electronic Design Ltd., Cambridge, UK). The bandwidth of the EEG recording was 0.53 Hz to 75 Hz and it was sampled at 500 Hz sampling rate.

SWDs abundantly appear during transition from the sleeping period (daytime) to active period (nighttime) in WAG/Rij rats (Coenen et al., 1991; Drinkenburg et al., 1991, 1995). Therefore rats were recorded from 3:30 PM to 8:00 PM. In that period, rats usually are in continuous drowsy state performing sleep spindles, theta waves and only a few delta waves interrupted by short waking periods because of reduction of the sleep drive (Gottesmann et al., 1995). The automatic separation of SWD activity from the rest of the EEG is not properly solved yet so we applied the generally accepted criteria and performed the evaluation manually (Schridde and van Luijtelaar, 2004). A typical SWD was defined as a train of asymmetric spikes and slow waves starting and ending with sharp spikes (Schridde and van Luijtelaar, 2004; Sitnikova and van Luijtelaar, 2004). The power spectra showed 7–11 Hz dominant frequency and some of its upper harmonics. The average SWD amplitude was at least twice as high as the EEG delta activity and the duration of spindles was longer than 2 s. We eliminated some ultra-short SWD looking spindles from the evaluation and also we selected out some atypical spindles of only a little higher amplitude as an ordinary sleep spindle. The selected SWDs were cut off from the row data files and checked by FFT analysis.

It is known that cytokines could change the sleep–waking cycle of rats, therefore analysis of sleep stages during the experiments was carried out on the basis of EEG applying sleep staging criteria of Gottesmann et al. (1995). Briefly, we considered the rats awake when EEG showed dominantly beta (30–40 Hz) and theta (6–8 Hz) activity interrupted by high slow waves of motor artifacts. Rats were active and moving, eating and chewing. The sleep onset was determined by synchronized EEG activity showing sleep spindles of 10–16 Hz frequencies, theta waves and some slow waves in delta range (2–4 Hz). Deep slow-wave-sleep was characterized by sleep spindles at the beginning which gradually disappear as the ratio of high slow delta waves increased (0.5–4 Hz). In rapid eye movement sleep (REM), continuous theta activity (6–8 Hz) without any motor artifact was observed. Sleep/waking ratios were established in all recording hours in all rats to control for the effect of LPS on sleep.

Experimental paradigms

Repeated application of LPS. WAG/Rij rats (n=5) were recorded for 5 days injecting artificial cerebrospinal fluid (ACSF) as control. Recording was done between 3:30 PM and 8:00 PM when the rats are usually in a drowsy state. Rats were injected by 0.5 ml ACSF then recording was started. Following five control experiments, 350 μg/kg LPS were injected before recording. LPS injection was repeated on five consecutive days then a post-treatment control recording was done. Numbers of SWDs were measured for 270 min of post-injection period. The data of the first 30 min after the injection were not included into the analysis because the stress induced by injection changed the SWD number for 30 min as it was established in pilot studies. Changes in SWD numbers during 240 min (from 4:00 PM to 8:00 PM) were expressed in the percent of average number of SWDs in ACSF injected control and evaluated by ANOVA.

Dose-dependence experiments. SWD activity was investigated as a function of LPS doses. Five WAG/Rij rats were used in the experiments for each dose separately (n=25). We applied 10, 20, 50, 100 and 350 μg/kg LPS i.p. SWD numbers and durations as well as the body temperature changes were measured during 270 min of post-injection time. The first 30 min sample was excluded from the data analysis. First, a 5-day ACSF control section was done. Injecting LPS i.p., rats were recorded for 270 min. Finally, a 270 min long, ACSF control experiment was done on the next day. Data obtained a day after LPS application were used to disclose rebound or long lasting effects of LPS and also demonstrated that SWD number returned to the baseline.

Body temperature was measured in each 10 min. SWDs were separated from data files and they were verified by FFT analysis. The number of SWDs and their duration were measured. Statistical significance of results was obtained by using ANOVA.

Combined application of indomethacin and LPS. The 5-day control section was followed by injection of 2 ml 5% v/v ethanol in saline solution i.p. in WAG/Rij rats (n=5) as solvent control. In solvent control experiments two injections were applied, 2 ml, 5% v/v ethyl-alcohol injection at zero time and a 0.5 ml ACSF injection at 30 min. SWD numbers were measured during 270 min of post-injection time. Indomethacin treatment was 10 mg/kg indomethacin dissolved in 5% v/v ethanol in saline (Kagiwada et al., 2004) i.p. at zero time and 30 min after, 0.5 ml ACSF solution was injected. Recording time was 270 min. On the next day the 10 mg/kg indomethacin injection was followed by injection of 50 μg/kg LPS in 0.5 ml ACSF and rats were recorded for 240 min after LPS injection. The effect of LPS alone was compared with the 50 μg/kg LPS data obtained in dose dependence study. Effect of indomethacin alone was calculated so that data were normalized to the 5% v/v ethanol in saline control while effect of LPS alone was normalized to the saline control.

Combined application of LPS and AP5. Two different dose combinations of LPS and AP5 (Tocris Cookson Ltd., Bristol, UK) were applied to WAG/Rij rats (n=10). The injection (i.p.) and recording paradigms were the same as described for the LPS dose-dependence experiments. The low-dose combination was 20 μg/kg LPS combined with 40 mg/kg AP5; the high-dose combination was 50 μg/kg LPS and 80 mg/kg AP5. Two doses of AP5 were injected without LPS before the LPS and AP5 injection to establish the effect of AP5 on SWD numbers. The first 30 min sample was excluded from the data analysis, measuring SWD numbers and durations in time as it was done in the case of dose-dependence experiments. The results were evaluated by ANOVA.

RESULTS

LPS-induced immune reaction in WAG/Rij rats and its effect on sleep pattern

Checking the LPS sensitivity of WAG/Rij rats, we measured the cytokine release from six rats injected with LPS in either 20 or 350 μg/kg i.p., taking blood samples of 150 μl in each post-injection hour through implanted jugular vein cannula. Two doses of LPS were applied, a low one (20 μg/kg, n=3) and a high one (350 μg/kg, n=3). The experiment was 6 h long. The cytokine release was measured using ELISA for TNF-α, IL-1β and IL-6. The kinetics of cytokine-release was the same both in the case of the low and high dose of LPS. TNF-α release started to increase in the first hour after LPS injection and its peak was in the second hour after LPS-challenge (Table 1). IL-1β level started to rise one hour later than TNF-α and it reached the maximum concentration also in the second hour. We also measured IL-6, which was elevated like

Table 1. The kinetics of the proinflammatory cytokine responses in jugular vein blood samples of WAG/Rij rats upon LPS 20 μg/kg, i.p. injection

Time after LPS inj. (h)	IL-1β		TNF-α		IL-6	
	Mean	SD	Mean	SD	Mean	SD
0	4.56	1.97	0.61	0.12	8.79	0.18
1	7.45	4.24	651.77	68.23	304.99	65.19
2	44.53	9.10	2754.40	155.22	3096.96	266.01
3	23.42	9.41	86.02	17.47	2747.62	84.79
4	16.43	9.63	31.32	17.53	1980.95	390.56
5	17.71	4.31	12.43	12.19	1137.75	357.86
6	22.63	1.78	8.65	3.45	566.03	252.49

All values are pg/ml, were determined by ELISA per the manufacturer's instructions. Abbreviation: inj.: injection.

TNF-α but remained in high concentration in the blood longer than IL-1β or TNF-α. We did not find significant differences in the kinetics or levels of cytokine release using the low and high dose of LPS so the data of high dose are not shown.

It is known that increased levels of proinflammatory cytokines could induce sleep and may extend synchronized EEG periods in rats. We performed a sleep stage evaluation on the basis of EEG recordings in control and LPS-treated experiments. By visual evaluation of EEG and correlating it with behavioral observations, we found no significant change in sleep and waking periods ratio induced by LPS in contrast to what was reported in the literature. The average ratio of active waking in the 4-h recording period was 3.5%, which remained the same (3.3%) after LPS injection. It must be noted that this very low ratio of active waking is due to the careful selection of recording hours from the circadian sleep waking spectra of our rats. During that period (from 3:30 to 8:00 PM), food intake drive and sleep inducing effect of cytokines can compensate each other.

Effects of LPS on SWD electrogenesis

SWD activity is about 7–11 Hz rhythmic series of high amplitude waves (Fig. 1A) which can be recorded in the drowsy state in WAG/Rij rats with fronto-parietal electrode combination, furthermore SWD could also be recorded on the occipital electrodes and on many other places of the brain.

Administration of LPS resulted in fragmentation of SWDs activity (Fig. 1A), which in turn, changed the power spectra (Fig. 1B). Power spectra of typical SWDs with and without LPS application show a decrease and splitting in dominant power peak at 8 Hz (Fig. 1B) as compared with

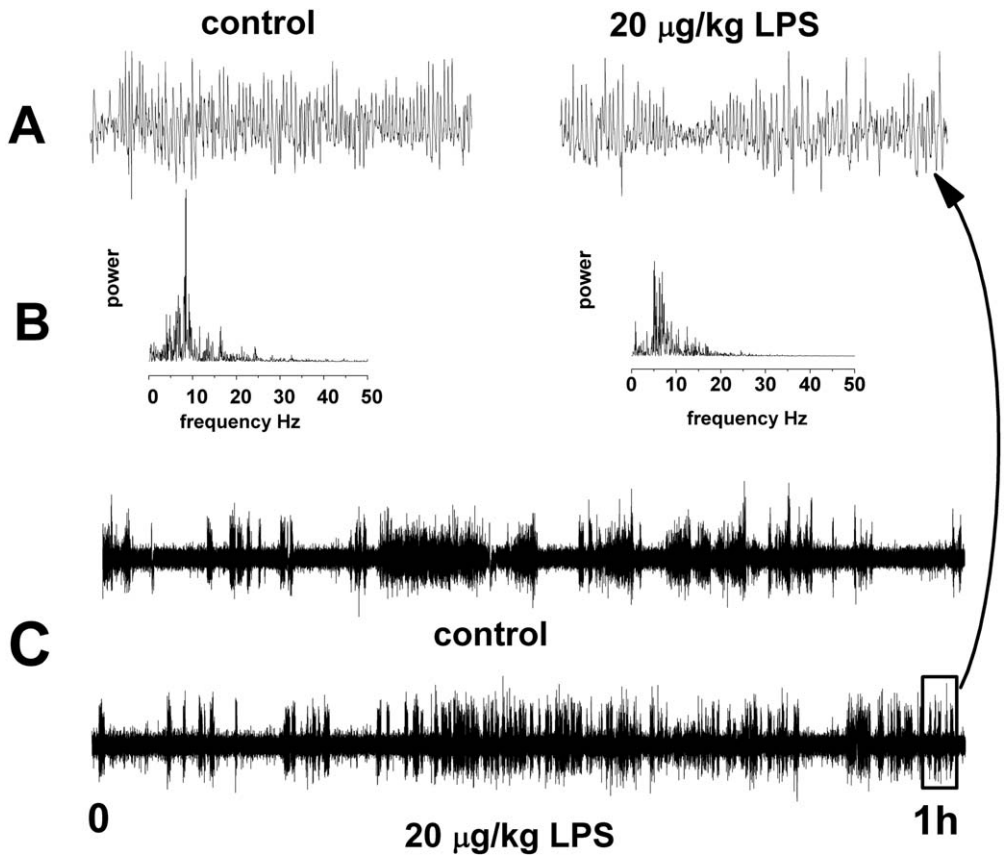

Fig. 1. Wave-shapes and power spectra of SWDs after application of LPS (20 μg/kg). The figure shows the morphology (A) and power spectra (B) of SWDs in control and LPS application experiments. In part C, we show a typical 1 h section of EEG recording after ACSF (control) and LPS injection to illustrate that there was no change in sleeping behavior of the rats, a finding supported by almost evenly distributed SWDs in both records.

ACSF control. However, the overall character of SWD activity did not change allowing the same definition for SWD in control and LPS-treated rats, respectively, which improved the accuracy of determination of SWD number and duration.

LPS-induced changes in body temperature of WAG/Rij rats

The core body temperature changes of rats were measured at 10, 20, 50, 100, 350 μg/kg LPS dose injected i.p. (Fig. 2). We observed a typical fever response at 10 and 20 μg/kg doses of LPS. At low doses, there was an initial temperature decrease for 40 min followed by a marked elevation of body temperature reaching its maximum of 1–1.2 °C above basal level at 140–150 min after LPS injection (Fig. 2). At a medium dose of LPS (50 μg/kg), the body temperature showed a typical biphasic curve of an initial temperature drop followed by a slight elevation of body temperature (Fig. 2). Application of 100 μg/kg LPS i.p. caused a biphasic temperature change curve in which an initial temperature elevation was followed by a small drop in temperature. High doses of LPS (350 μg/kg) induced only a temperature drop after LPS injection (Fig. 2).

At low doses of LPS we observed less active behavior than usual, in accordance with the description of the "sickness syndrome induced by endotoxin" by Dantzer (2001). Rats were standing still or lay on the floor of the box and moved very little in general. Head bobbing, flat body postures, trying to stay close to the floor of the cage, and a significant reduction in motor activity were observed. At high doses of LPS, continuous flat body posture and sweating, sometimes shivering was observed. All behavioral effects, however, disappeared on the next day.

Dose dependent changes in SWD number and duration

Numbers of SWD episodes recorded in control experiments varied in a wide range from 9 to 24 in an hour from individual to individual. Because of the relatively large dispersion of control data, we normalized our results to the average number of SWD episodes. It has to be noted that in the first 30 min after ACSF injection, we observed an effect of injection by itself because there were more SWD episodes recorded. Thus we eliminated the data of the first 30 min from the further evaluation.

LPS increased the number of SWD episodes in a dose dependent manner but in the case of 10 and 20 μg/kg dose of LPS the increase in SWD number was significant only in 90–150 and 210–270 min periods after injection. From 50–350 μg/kg doses, the SWD numbers increased in each

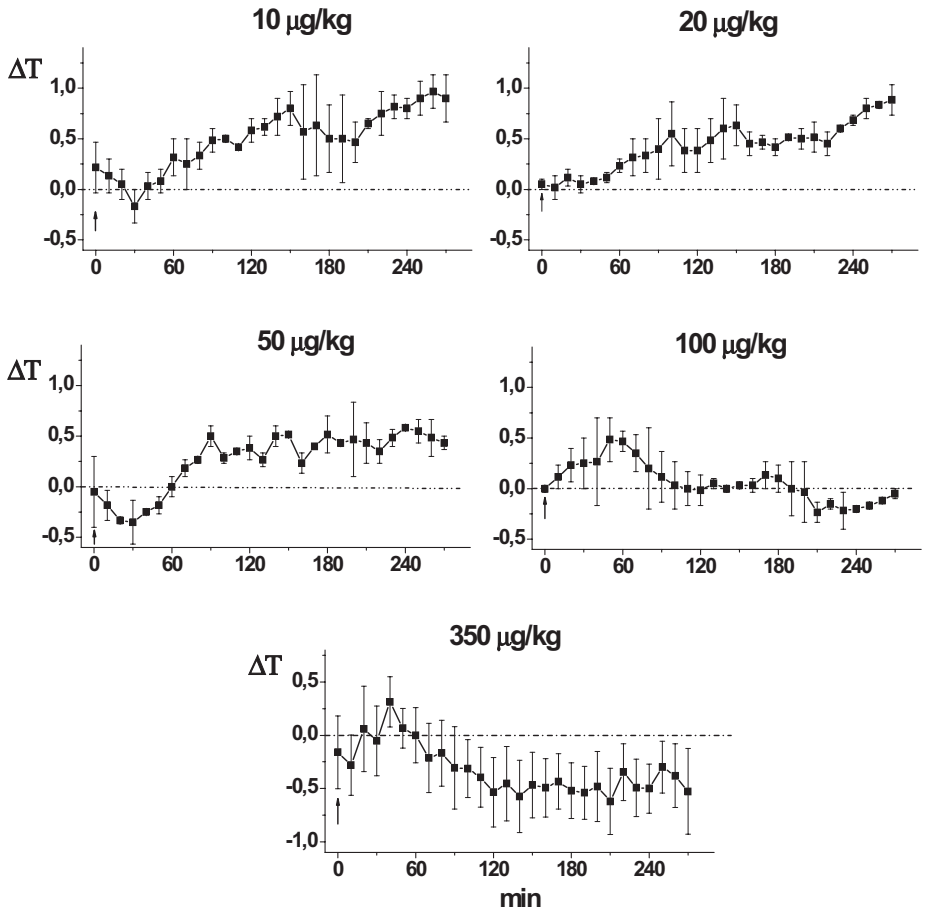

Fig. 2. LPS-induced body temperature changes in WAG/Rij rats at different doses of LPS. Arrows indicate the i.p. injection (0 min.).

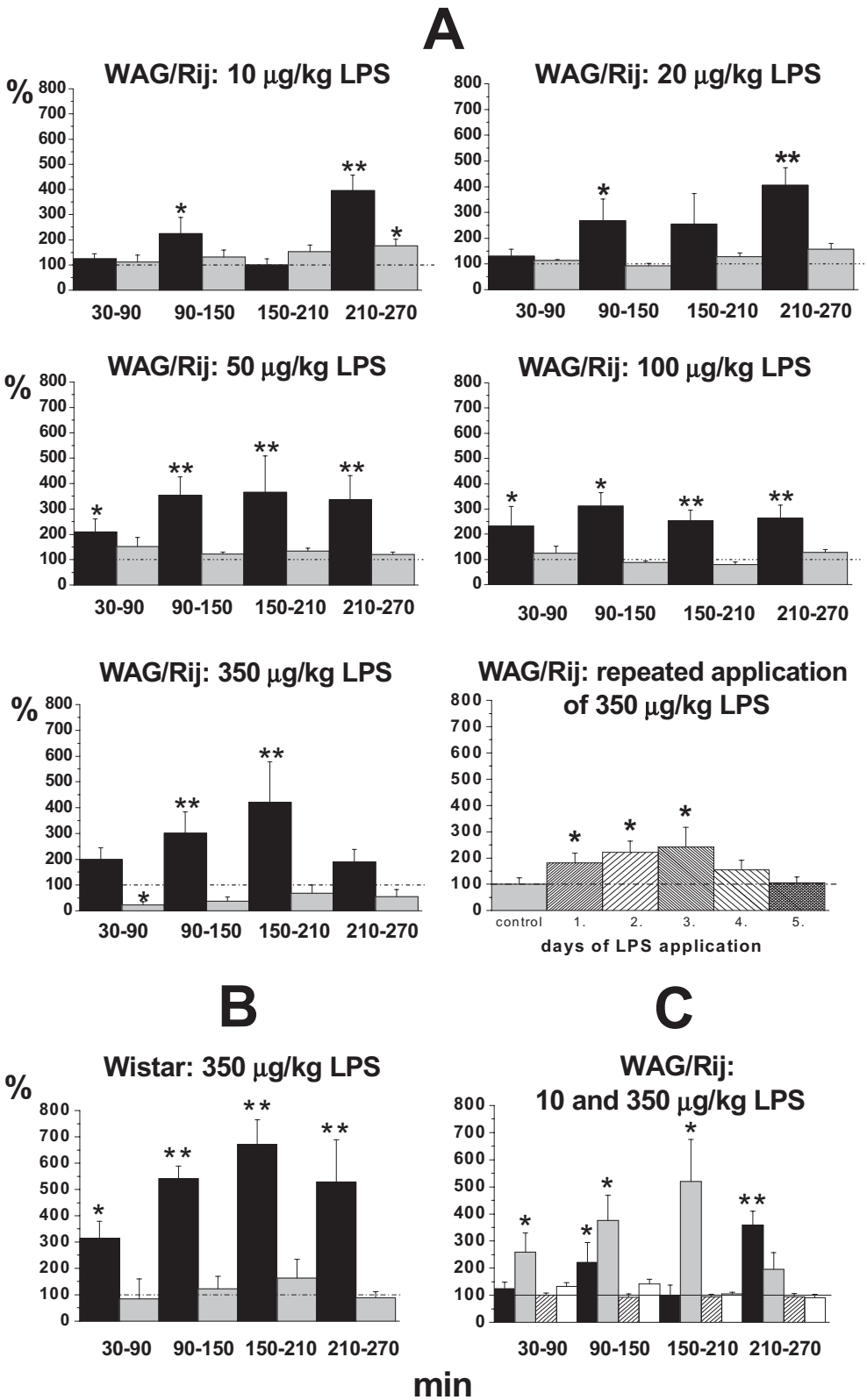

Fig. 3. Changes in SWD numbers after injection of different doses of LPS in WAG/Rij rats (A, from first to fifth panels, black columns) and on the post-treatment day (gray columns). Last panel of A shows changes in SWD numbers in the course of repetitive application of LPS (350 μg/kg) for five consecutive days. Changes in SWD numbers after injection of the highest dose of LPS (350 μg/kg) in Wistar rats (B, black columns) and on the post-treatment day (gray columns). In C, we show LPS-induced changes in total SWD time during recording (black columns: 10 μg/kg LPS; gray columns: 350 μg/kg LPS) and average duration of SWDs (striped columns: 10 μg/kg LPS; open columns: 350 μg/kg LPS) compared with the ACSF control. * $P<0.05$ and ** $P<0.005$ level of significance.

hour after injection, only the levels of significance changed by the applied dose of LPS (Fig. 3A, from first to fifth panels). The highest elevation in SWD numbers was about 400%, observed in 210–270 and 150–210 min after injection of 10, 20 and 350 μg/kg LPS. The dose dependence of SWD numbers was not clear for all post-injection periods. Principally it seemed true that a small dose of LPS (10–20 μg/kg) induced a slightly smaller average elevation of SWD incidence. Data obtained a day after LPS application demonstrated that SWD number returned to the baseline but at the lowest (10 μg/kg) and at the highest (350 μg/kg) LPS doses between 210 and 270 and 30–90 min after injection the number of SWDs was significantly different from ACSF control (Fig. 3A).

The average duration of SWD episodes was also calculated and it did not change significantly after LPS injection (Fig. 3C). The total time of SWD episodes during the 4 h of the recording period, increased in parallel with the increase in number of SWD (Fig. 3C), namely the SWD number but not the duration of SWD changed after LPS injection.

Repeated application of LPS and decay of increase in SWD number

To confirm that LPS-induced increase in the SWD generation in five WAG/Rij rats is a direct consequence of the LPS induction of immune and inflammatory response we applied 350 μg/kg LPS on five consecutive days. It is known that fever response and cytokine response decline upon repeated application of LPS (Chen et al., 2005).

The effect of LPS on SWD numbers increased on the first, second and third days of repeated application. SWD number decreased after the 4th injection and returned to the control level after the 5th application (Fig. 3A, last panel). LPS-induced body temperature decrease followed the time course of SWD number in the repeated injection experiment showing that the hypothermic and SWD effects of LPS are desensitized similarly.

LPS affects SWDs in old Wistar rats

To demonstrate that the LPS changes in the SWDs are not exclusively observable only in the inbred WAG/Rij strain we selected three, 1 year-old Wistar rats from ordinary Wistar breeding stock which were generating SWDs, and subjected these to LPS treatment.

The average SWD number in old Wistar rats during the recording period was 38 ± 9. Upon injection of 350 μg/kg LPS i.p. the number of SWDs significantly increased as it is shown on Fig. 3B. The average duration of SWDs did not change. All results in Wistar rats were identical to the WAG/Rij rats' data.

Interaction of LPS and NMDA receptor blocker AP5 in control SWD number

We applied a potent, reversible, competitive blocker of NMDA receptors (AP5) i.p. in two different doses, the low dose was 40 mg/kg and the high dose was 80 mg/kg. When the low dose of AP5 was applied we used a low dose of LPS as well (20 μg/kg) to examine interactions between the effects of these substances. Using the high dose of AP5 (80 mg/kg), we applied a high dose of LPS (50 μg/kg; Fig. 4A and B). In the case of the low dose of AP5 combined with low dose of LPS, the application of AP5 alone showed no significant effect on SWD number over 4 h but decreased the number of SWD between 30 and 90 min. LPS application alone produced an increase in SWD number. This increase was not significant in 30–90 min post-injection time and it was only barely significant in 90–150 min time interval and it was not significant in 150–210 min after injection. A highly significant increase in SWD number was observed only after 210 min following LPS injection. Applying low doses of LPS (20 μg/kg) and AP5 (40 mg/kg) together, the increase in SWD number in all examined post-injection times became highly significant as compared with AP5 alone (Fig. 4B), to saline control ($P<0.005$). A significant difference was also observed comparing the SWD numbers of 30–210 min post injection periods recorded at low dose of LPS and low dose of AP5 and 20 μg/kg LPS injection alone ($P<0.05$).

Application of a high dose of AP5 (80 mg/kg) and a high dose of LPS (50 μg/kg) resulted in no interaction and the high dose of LPS alone and high dose of AP5 both increased the number of SWDs significantly during the entire experimental period (Fig. 4A and B).

Indomethacin inhibits the effect of LPS on SWDs

Indomethacin can be dissolved in 5% v/v ethanol solution. Therefore the solvent of indomethacin containing 5% v/v ethanol in saline was used as control. This control solution did not change SWD numbers.

Comparison of effects of indomethacin alone, indomethacin and LPS in combination as well as LPS application alone is shown on Fig. 4C. Injecting indomethacin (10 mg/kg) 30 min before LPS (50 μg/kg) application completely abolished SWD number increasing the effect of LPS. Interestingly, indomethacin alone also significantly decreased the number of SWDs in the first hour (Fig. 4C). The same tendency can be observed when indomethacin was applied together with LPS but the decrease in SWD numbers did not reach the significance level.

DISCUSSION

The major finding in this study is that, LPS-induced inflammatory response can increase the number of SWDs in genetically epileptic WAG/Rij rats and in old Wistar rats in a dose dependent manner (Fig. 3A and B). WAG/Rij is an inbred rat strain derived from one breeding pair (Khattab et al., 1989; Solleveld et al., 1986; van Putten and Zwieten, 1988), therefore checking of their immune and inflammatory response dynamics to LPS was needed. The LPS-induced serum IL-1β, IL-6 and TNF-α release pattern was normal in WAG/Rij rats (Table 1) supporting that inflammation reaction in WAG/Rij strain is the same as in ordinary Wistars rats. One of the main characteristics of immune reactions is that repeated application of LPS causes desensitization of the response: LPS tolerance (Chen et

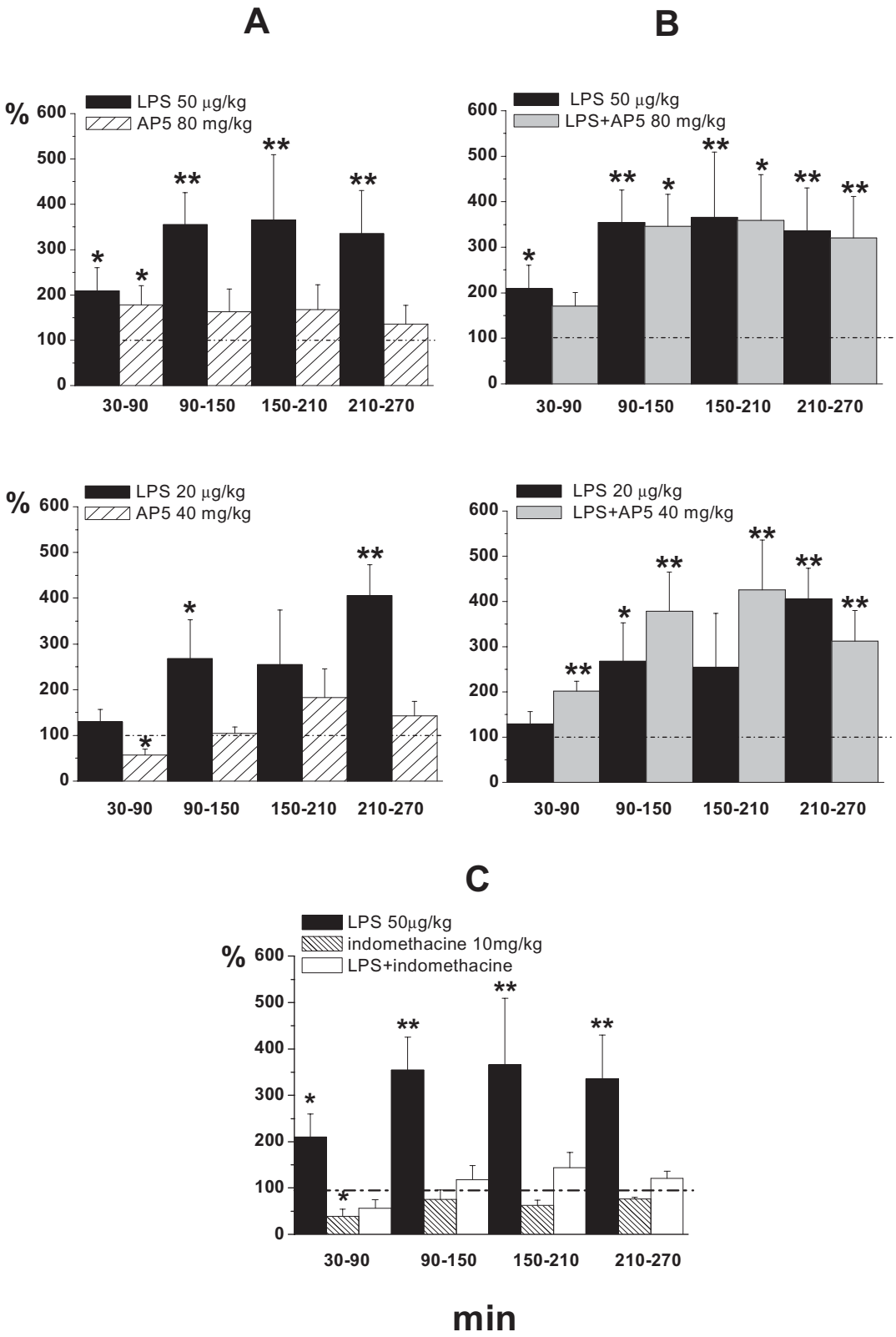

Fig. 4. (A) Effects of LPS (top 50 μg/kg and bottom 20 μg/kg, black columns) and AP5 (top 80 mg/kg and bottom 40 mg/kg, striped columns) on SWD numbers. (B) Increase in SWD numbers after application of AP5 and LPS in combination (top 50 μg/kg LPS+80 mg/kg AP5 and bottom 20 μg/kg LPS+40 mg/kg AP5, gray columns) as compared with the effect of AP5 alone (striped columns). * $P<0.05$ and ** $P<0.005$ level of significance. (C) Effects of indomethacin on LPS-induced increase of SWD numbers. Black columns: LPS alone (50 μg/kg) normalized to ACSF solution; striped columns: indomethacin injection alone (10 mg/kg) normalized to 5% v/v ethanol in ACSF control; open columns: injection of LPS plus indomethacin normalized to 5% v/v ethyl alcohol. Statistical significance of results (LPS/ACSF solution; indomethacin/5% v/v ethyl alcohol; LPS+indomethacin/5% v/v ethyl alcohol) was marked. * $P<0.05$ and ** $P<0.005$ level of significance.

al., 2005; Fan and Cook, 2004). That disappearance of SWD number increase in the course of repetitive LPS injection supports that it is subject to tolerance development as other TLR4-mediated LPS responses (Beutler et al., 2001; Chen et al., 2005; Fan and Cook, 2004). This idea is further supported by the finding that inhibition of LPS-induced inflammatory reaction by indomethacin efficiently blocked LPS-induced SWD genesis enhancement via blocking prostaglandin synthesis (Fig. 4C). The time courses of release of cytokines and the desensitization of the cytokine response in the repeated LPS application experiment were similar to all the reported data on LPS effects and on development of endotoxin tolerance. Thus we can conclude that LPS initiates normal inflammatory response in WAG/Rij rats with respect to cytokine composition, amplitude, duration and tolerance development of the cytokine response and it is the inflammatory response leading to prostaglandin E2 (PGE2) synthesis that enhances SWD generator mechanisms.

It has to be noted here that SWD genesis is active in drowsiness and light slow-wave-sleep which is a very unstable and sensitive period of sleeping behavior. Taking blood samples from the jugular vein of a freely moving rat needs entering the soundproof recording chamber which by itself is enough to wake up the animal and abolish SWD genesis for as long as 20–30 min. It was the reason why we were not able to get reliable data on correlation of SWD number and blood cytokine levels. Number of SWDs settles after several days of recovery so measuring of brain cytokines by microdialysis is also impossible because of the gliosis producing a physical barrier around the probe within a day. Inserting a new dialysis probe just before the experiment seriously changes the number of SWDs as we found in preliminary studies, thus we also have no studies on brain cytokine levels and SWD number because of this technical reason. It is known, however, that EEG synchronization and sleep enhance IL-1β level in the brain (Krueger et al., 1998), thus we believe that we can reasonably assume that brain cytokine concentration in WAG/Rij rats is also higher during light slow-wave-sleep and drowsiness as in normal rats.

Genesis of SWDs is based on pathologically synchronous pattern of neuronal excitation in thalamo-cortical or/and cortico-thalamic neurons due to calcium spike genesis, hyper-synchronization and in turn, high amplitude waves in EEG and local field potentials (Buzsáki et al., 1990; Danober et al., 1998; Meeren et al., 2002, 2005). Hyper-synchronous neuronal activity in epileptic rats appears in correlation with transitional phases of sleep cycle when animals wake up or fall asleep (Coenen et al., 1991; Drinkenburg et al., 1991, 1995; Gralewicz and Luczak, 1994). Sleep scoring in our experiments showed no change in sleep–waking ratio during the late afternoon recording sections in spite of the fact that LPS or exogenously applied cytokines are able to enhance sleep (Schiffelholz and Lancel, 2001). So, our observation is in disagreement with earlier studies showing a marked increase in sleep and change in sleep pattern after LPS injection (Lancel et al., 1995; Schiffelholz and Lancel, 2001). This contradiction is due to the fact that we carried out the experiments in a relatively short period of circadian activity of rats characterized by the highest incidence of SWDs. In fact, the extremely small and LPS insensitive ratio of active waking we obtained and the very large 400% increase in SWD number after LPS injection confirms this idea. Thus we suggest that LPS-induced increase in SWD number is not the consequence of decrease in waking state as it was suggested by several authors (Coenen et al., 1991; Drinkenburg et al., 1991, 1995). Our data suggest that when a circadian period is chosen with little sleep LPS effects on SWD still can be observed. These facts clearly support that thalamic and cortical SWD generator mechanisms in WAG/Rij rats are directly influenced by LPS-induced inflammation response like increased expression and release of IL-1β, and subsequent synthesis and release of PGE. Our suggestion is supported by the facts that interleukin-1 receptor (IL-1R) is expressed in thalamic neurons (Yabuuchi et al., 1994) and thalamic IL-1β mRNA transcription can be induced by application of LPS (Ban et al., 1992).

In conjunction with the increase in SWD number, LPS caused changes in core body temperature (Fig. 2) in a dose dependent manner as earlier described (Doğan et al., 2000; Takahashi et al., 1997). A low dose of LPS increased the body temperature 1–1.2 °C. A medium dose of LPS resulted in a biphasic change in body temperature and a high dose of LPS decreased the body temperature as has been observed earlier (Doğan et al., 2000, 2002; Linthorst et al., 1995; Sayyah et al., 2003; Yirmiya et al., 2001). We were able to reproduce the known core body temperature changes at different LPS doses in WAG/Rij rats.

The observed increase in SWD number after LPS injection was independent from inflammation-induced changes in body temperature because each applied dose of LPS increased the number of SWDs in spite of the fact that high doses of LPS decreased the body temperature while low doses increased it (Figs. 2 and 3). It is an important observation since it suggests that LPS activates thalamic and cortical synchronization generators independently from LPS action on temperature control mechanisms generating fever, most likely at the hypothalamic thermoregulatory center. The site of action of PGE in enhancing febrile seizures (Bender et al., 2004) and in enhancing SWDs must be partly different as non febrile LPS does also enhance SWDs, but also common components are involved as suggested by human genomics studies that revealed that febrile seizures and absence seizures have closely related genetic background and molecular mechanisms, as represented by mutations in the subunits of $GABA_A$ receptor (Kang and Macdonald, 2004; Marini et al., 2003).

The fact that SWD genesis enhancement by LPS was abolished by indomethacin (Fig. 4C), a potent inhibitor of prostaglandin synthesis, a cyclooxygenase1/2 inhibitor indicates a need for studying LPS/toll signaling (Beutler et al., 2001) involving activation of cyclooxygenase-2 and biosynthesis and release of PGE2 as a first step in the

search for intracellular mechanisms of the SWD enhancing effect by LPS.

Cytokine effects in the nervous system are reported to enhance or synergize with NMDA receptor-mediated events but the data are model dependent and somewhat contradictory. IL-1β could increase NMDA receptor function (Viviani et al., 2003) facilitating excitotoxic, NMDA receptor-mediated hippocampal neuronal death in cell cultures (Ma et al., 2003). In contrast, IL-1β binding to its receptor, IL-1R type 1, inhibits NMDA receptor-mediated neurotransmission and reduces long-term potentiation (LTP) in hippocampal slices (Coogan and O'Connor, 1997). However, it has been shown that endogenous IL-1β is involved in the maintenance of LTP (Schneider et al., 1998), and that TNF-α plays a role in synaptic plasticity (Beattie et al., 2002; Pickering et al., 2005). Applying NMDA receptor blocker AP5 in combination with LPS (Fig. 4A and B), we observed an additive interaction between the low dose of LPS (20 μg/kg) and the low dose of AP5 (40 mg/kg) but a lack of interaction was found in the case of the high dose of LPS (50 μg/kg) combined with the high dose of AP5 (80 mg/kg). The SWD enhancing interaction of the low dose of LPS and AP5 was observed perhaps because the SWD generating thalamo-cortical system was not close to its limit. In the case of high doses of either LPS or of AP5, the thalamic SWD generator was saturated. The lack of additivity and synergy between the supramaximal doses of AP5 and LPS suggests that the final targets of their action in enhancing SWD numbers may be common. The mechanism of facilitation of absence seizure genesis by LPS combined with AP5 can be based either on inhibitory or excitatory processes. Absence seizure genesis requires a sustained hyperpolarization in the thalamus and opening of T-type calcium channels (Steriade et al., 1993; Steriade, 2001). NMDA receptors expressed on glutamatergic neurons of the thalamus and the cortex could enhance sustained inhibition by decrease of the excitatory input. Consequently, when AP5 was applied we suggest that more and more neurons reached the hyperpolarization range necessary for activation of T-type calcium channels. On the other hand, NMDA receptors expressed on the GABAergic cells are able to control GABA release from these neurons suggesting a reduction of GABA release by AP5 due to the decrease in excitation of GABAergic cells in the thalamus and the cortex, with lowered hyperpolarization, which inhibits T-type calcium channel activation. Whatever the main component of NMDA receptor blocker action is changing in excitation/inhibition balance is the major factor of genesis of any kind of seizures in the brain (Snead, 1995; Danober et al., 1998) and it appears that both LPS and AP5 are able to modulate that balance.

CONCLUSION

In conclusion, our report on modulation of SWDs in WAG/Rij rats by LPS-induced inflammation response reveals a direct connection between cortico-thalamic SWD generator mechanism and inflammatory reaction and PGE synthesis by LPS induction. Our results show a novel effect of inflammatory mechanisms on cortico-thalamic generation of brain waves, the pathological significance of this finding may affect our views on absence seizures and on the importance of controlling even low grade inflammation and infection. In addition we established a freely moving animal model for studying effects of activation of the inflammatory system on the excitability and synchronization of neuronal networks of the CNS.

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