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TNF α siRNA Reduces Brain TNF and EEG Delta Wave Activity in Rats

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Abstract

Tumor necrosis factor alpha (TNF α) is a pleiotropic cytokine with several CNS physiological and pathophysiological actions including sleep, memory, thermal and appetite regulation. Short interfering RNAs (siRNA) targeting TNF α were incubated with cortical cell cultures and microinjected into the primary somatosensory cortex (SSctx) of rats. The TNF α siRNA treatment specifically reduced TNF α mRNA by 45% *in vitro* without affecting interleukin-6 or gluR1-4 mRNA levels. *In vivo* the TNF α siRNA reduced TNF α mRNA, interleukin-6 mRNA and gluR1 mRNA levels compared to treatment with a scrambled control siRNA. After *in vivo* microinjection, the density of TNF α -immunoreactive cells in layer V of the SSctx was also reduced.

Electroencephalogram (EEG) delta wave power was decreased on days 2 and 3 on the side of the brain that received the TNF α siRNA microinjection relative to the side receiving the control siRNA. These findings support the hypothesis that TNF α siRNA attenuates TNF α mRNA and TNF α protein in the rat cortex and that those reductions reduce cortical EEG delta power. Results also are consistent with the notion that TNF α is involved in CNS physiology including sleep regulation.

Keywords

Sleep; TNF α ; siRNA; gluR; interleukin-6; EEG; cytokine

Classification terms

Theme I: Neural Basis of Behavior; Biological Rhythms and Sleep

INTRODUCTION

Tumor necrosis factor alpha (TNF α) is involved in several brain-regulated physiological processes including sleep, body temperature and appetite (Obal and Krueger 2003). TNF α brain mechanisms are relatively understudied in part due to the difficulty of manipulation of endogenous TNF α levels. TNF α and TNF α mRNA brain levels vary with sleep propensity (Bredow et al., 1997; Floyd and Krueger 1997). Microinjection of TNF into the hypothalamus (Kubota et al., 2001) or locus coeruleus (De Sarro et al., 1997), two areas involved in sleep regulation, promotes non-rapid eye movement sleep (NREMS). TNF α expression in cortical

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neurons, as measured by immunohistochemistry, is enhanced after increased neural activity (Fix et al., 2006). Further, injection of TNF α onto the surface of the cortex enhances the amplitudes of electroencephalogram (EEG) delta (0.5-4 Hz) waves during NREMS (Yoshida et al., 2004). Such data implicate cortical TNF α in sleep regulation although there is a clear need for new tools to study endogenous TNF α .

RNA-mediated interference (RNAi) is a sequence-specific RNA silencing mechanism found in plants and animals (Sioud et al., 2004). The RNAi pathway is triggered in mammalian cells by the presence of double stranded RNA or small interfering RNA molecules (siRNA) (Fire et al., 1998; Elbashir et al., 2001). An RNA-induced silencing complex (RISC) unwinds the siRNA duplex. The specific degradation is guided by the antisense strand and proceeds with the sense strand siRNA binding to the complementary mRNA site for cleavage by Argonaut-2 (Flynn et al., 2004). The cleavage of the sense strand siRNA and target mRNA results in self-amplifying new siRNA intermediates that continue the mRNA target degradation. siRNAs are useful to probe gene function and structure both *in vitro* and *in vivo* and are potential new therapeutic agents (Lewis et al., 2002). siRNA effects are reversible; *in vivo* they begin about 1 day after administration and last 2-3 days (Dillon et al., 2004; Ryther et al., 2005). Compared with the antisense technology the effects of siRNA appear to be more selective and persistent (Hough et al., 2003; Miyagishi et al., 2003). The experimental and therapeutic use of siRNAs is currently expanding rapidly. In this report we use a TNF α siRNA with the goal of determining if TNF siRNA reduces TNF mRNA and protein expressions in brain and whether those actions manifest in one of TNF's biological activities, the sleep electroencephalogram (EEG).

We show herein that a TNF α -siRNA reduces *in vivo* cortical TNF α mRNA and TNF immunoreactivity and *in vitro* TNF α mRNA in cortical cells. We also show that the TNF α siRNA reduces EEG delta wave power during NREMS. Results are consistent with the hypothesis that TNF α is constitutively expressed in brain and has a role in the sleep EEG.

RESULTS

In vitro RT-PCR analyses

An initial screening of TNF α -siRNAs using cultured cells showed that TNF α -siRNA targeting nucleotides 228-247 and 476-494 reduced TNF α mRNA. Therefore these two siRNAs were pooled for all other *in vivo* and *in vitro* experiments. One way ANOVA indicated that TNF α siRNAs induced a significant 45% suppression of TNF α mRNA [$F_{(2, 34)} = 16.9, p < 0.00001$] compared to either scrambled siRNA-treated or saline controls (Fig. 1). In contrast, the *in vitro* levels of interleukin 6, GluR1, 2, 3 and 4 mRNAs were not significantly affected by either the scrambled siRNA or TNF α siRNA treatments (Fig.1).

In vivo RT-PCR analyses

Compared to values obtained after scrambled siRNA injections, the TNF α siRNA induced a significant 37% decrease ($t = -4.24; p < 0.0001$) in TNF α mRNA in the somatosensory cortex (SSctx) (Fig. 1). The TNF α siRNA, unlike the *in vitro* results, also significantly reduced other transcripts such as IL6 mRNA (56% less) and GluR1 mRNA levels (12% reduction) (Fig. 1) compared to those found in the opposite SSctx ($t = -5.59; p < 0.001, t = -2.36; p < 0.05$, respectively). The *in vivo* levels of GluR 2, 3 and 4 mRNA did not significantly change after the TNF α siRNA injection (Fig. 1).

TNF α immunoreactivity after TNF α siRNA treatment

TNF α siRNA treatment of the SSctx reduced the number of darkly stained TNF α -immunoreactive cells in layer V within about 1.0 mm of the injection site (Fig. 2). The TNF α -IR cells illustrated in Figure 2 were about 0.7 mm from the TNF α siRNA injected site

(Fig. 2A,C) and they appeared less dense within the cytoplasm than on the scrambled siRNA injected side (Fig. 2B,D). Quantitative evaluation of these TNF α -immunoreactive cells indicated a significant 33% decrease in the density of these cells (density of TNF α siRNA - injected cells = 0.140 ± 0.001 ; density of scrambled siRNA - injected cells = 0.212 ± 0.019 ; $t = -7.6261$; $p < 0.002$).

Effect of TNF siRNA on sleep and EEG slow wave activity (SWA)

The localized microinjection of the TNF α siRNA into the SSctx did not alter duration of either NREMS or rapid eye movement sleep (REMS) across the 5 day recording period (data not shown). The effect of TNF α siRNA on regional EEG SWA during NREMS is illustrated in Fig 3. The first 23 h after injection (day 1) the rats exhibited their normal diurnal rhythms of EEG SWA during NREMS and there were no significant differences between the two sides of the brain (treatment $F_{(1,6)} = 0.60$, $p = 0.47$; time, $F_{(5,30)} = 1.29$, $p = 0.29$) [treatment $\times$ time interaction, $F_{(5,30)} = 1.12$, $p = 0.37$]. In contrast on day 2, there was a significant decrease in EEG SWA during NREMS during the light period on the side that received the TNF α siRNA in comparison with the side that received scrambled siRNA treatment ($F_{(1,6)} = 6.65$, $p = 0.04$; time, $F_{(5,30)} = 0.49$, $p = 0.78$; treatment $\times$ time interaction, $F_{(5,30)} = 0.82$, $p = 0.99$). On day 2 on the side that received the TNF α siRNA, the EEG SWA during NREMS was significantly lower (TK test) in the first 4 h after light onset but was not significantly different at later times. Similar results were obtained on day 3 (treatment $F_{(1,6)} = 6.52$, $p = 0.04$; time, $F_{(5,30)} = 1.19$, $p = 0.38$; treatment $\times$ time interaction, $F_{(5,30)} = 3.04$, $p = 0.02$). On day 3 the significant decrease in EEG SWA during NREMS was sustained during the first 8 h of the light period (TK test). The EEG SWA returned to control levels by 8 h after light onset on day 3. On day 4, there was also a significant decrease in EEG SWA during NREMS on the side that received the TNF α siRNA compared to the side that received scrambled siRNA (treatment $F_{(1,6)} = 2.47$, $p = 0.17$; time, $F_{(5,30)} = 0.98$, $p = 0.30$; treatment $\times$ time interaction, $F_{(5,30)} = 3.08$, $p = 0.02$). On Day 4, as on Day 2, the significant difference occurred during the first 4 h after light onset (TK test). The TNF α siRNA did not significantly affect EEG SWA during NREMS during the dark period. Using time blocks corresponding to the 12 h light and 11 h dark periods there were no significant differences during the dark periods (Fig. 3, right). In contrast, EEG SWA was significantly reduced by TNF α siRNA on days 2 and 3 ($t = -2.52$; $p < 0.05$; and $t = -2.42$; $p < 0.05$, respectively) during the light period. By day 4 there were no significant differences in EEG SWA during NREMS during the light period (Fig. 3 right). There were no differences in EEG SWA during wakefulness or REMS at any time after TNF α siRNA treatment. Finally, EEG power in higher frequencies (>4 Hz) was not altered by the TNF α siRNA (data not shown).

DISCUSSION

The major findings reported herein are that the TNF α siRNA, when injected directly into the cortex, locally reduced endogenous TNF α mRNA levels and reversibly reduced EEG SWA during NREMS unilaterally. These findings suggest that TNF α is involved in spontaneous EEG SWA and that EEG delta activity is generated in part within the cortex. Similar conclusions were reached previously after the demonstration that injection of TNF α onto the SSctx enhanced EEG SWA regionally whereas injection of the TNF soluble receptor reduced EEG SWA that had been enhanced by sleep loss (Yoshida et al., 2004). EEG SWA is posited to be indicative of sleep intensity (Borbely and Tobler 1989) because it is enhanced during the deep sleep following sleep deprivation (Pappenheimer et al., 1975). Microinjection of TNF α onto the surface of the SSctx also increases the amplitude of surface evoked potentials induced by whisker stimulation (Churchill et al., 2005a) in a fashion similar to the increase in surface-evoked potentials during quiet sleep (Rector et al 2005), suggesting that increases in extracellular TNF can alter the physiological responses to neural stimulation. Such enhanced potentials are characteristic of a cortical column sleep-like state (Rector et al., 2005).

TNF α plays a role in physiological sleep (Obal and Krueger, 2003). Systemic or central injection of TNF α enhances NREMS and EEG delta wave power during NREMS (Obal and Krueger, 2003; Krueger et al., 1998). In contrast, inhibition of TNF α reduces spontaneous sleep and attenuates sleep rebound after sleep loss (Takahashi et al., 1996). In humans, circulating levels of TNF α correlate with sleepiness in health and disease (Dreisbach et al., 1998; Imagawa et al., 2004; Moss et al., 1999; Darko et al., 1995; Obal and Krueger 2003). The soluble TNF receptor reduces fatigue in rheumatoid arthritis patients (Franklin et al., 1999) and in sleep apnea patients reduces sleepiness (Vgontzas et al., 2004). Collectively, such data implicate TNF α in sleep regulation and other brain processes. Current data are consistent with this conclusion because the TNF siRNA inhibited cortical TNF α expression and EEG SWA.

After TNF α siRNA treatment, the unilateral reductions in EEG SWA during NREMS occurred during daylight hours. The reason for this time-of-day effect remains unknown. However, a similar time-of-day-dependent reduction in duration of spontaneous NREMS occurs in mice lacking the TNF 55 kD soluble receptor (Fang et al., 1997). While EEG SWA activity during NREMS is in part regulated independently from duration of NREMS, the two parameters often covary with each other (Fang et al., 1999). For instance, after sleep loss both are enhanced (Pappenheimer et al., 1975). Further, there is now a relatively large literature indicating that localized sleep intensity (EEG SWA) is dependent upon neural activity within the region during prior wakefulness and that activity enhances neuronal expression of TNF α in the activated areas. Thus, for example, stimulation of the right hand enhances EEG delta wave power in the left somatosensory cortex during the subsequent first NREMS period (Kattler et al., 1994). Stimulation of a facial whisker enhances the number of TNF α - and Fos-immunoreactive cells in the activated somatosensory cortex barrel but not in the adjacent unstimulated barrel columns (Fix et al., 2006). In rats spontaneous levels of cortical TNF mRNA and protein are highest at the beginning of the light period (Floyd and Krueger 1997; Bredow et al., 1997). This is likely the end result of greater activity levels during the night time hours. Further, spontaneous EEG SWA also is highest at the beginning of daylight hours when rat NREMS duration is greatest, likely a manifestation of higher TNF α levels. Thus, the current finding that the TNF α siRNA reduced EEG SWA during NREMS during daylight hours is consistent with prior data. Current data also strongly suggest that TNF α plays a role in the local regulation of the sleep EEG and sleep intensity.

There are several methodological issues that limit interpretation of the *in vivo* EEG SWA results. Thus, because the TNF α siRNA was injected into brain parenchyma the effective dose would decrease the further the distance from the injection site. There could be differential effects of reducing endogenous TNF by different magnitudes. High and low doses of exogenous TNF α have differential effects on neuroprotection (see below). Further, the time courses of TNF α siRNA-induced effects could also be dependent on dose because TNF α induces its own production (Churchill et al., 2006). In this study we did not localize the topographical distribution of the TNF α siRNA-induced changes in EEG SWA beyond comparing the two cerebral hemispheres. Studies are underway to determine if the actions of the TNF α siRNA on EEG delta power are localized only to the small region of TNF α knockdown.

There are several possible mechanisms by which TNF α siRNA could decrease EEG SWA. First, TNF α enhances AMPA receptor expression and activity (Beattie et al., 2002; De et al., 2003; Furukawa and Mattson 1998; reviewed Pickering et al., 2005) and TNF α siRNA injections into the cortex *in vivo* reduce the AMPA receptor subunit, GluR1, mRNA (Fig. 2). Thus, the TNF α siRNA may decrease AMPA receptor expression and these receptors are posited to be involved in the generation of EEG synchronization (Bazhenov et al., 2002). Second, the reduction of TNF α immunoreactivity was mainly in large pyramidal neurons in layer V. These neurons show a burst firing mediated long-term depression and endocytosis of AMPA receptors, which may provide a mechanism by which synaptic weights may be

reconfigured during NREMS (Czarnecki et al., 2006). Cortical pyramidal neurons also provide an excitatory cortical output and influence the cortical-subcortical circuitry important in generating EEG synchronization (Steriade and Amzica, 1998). Finally, although topical application of TNF α enhances cerebral blood flow (Shibata et al., 1996), a reduction in cerebral blood flow due to reduced levels of TNF α induced by TNF α siRNA cannot explain the decreases in EEG slow wave activity, because cerebral blood flow is negatively correlated with EEG SWA (Kjaer et al., 2002; Dang-Vu et al., 2005).

The lack of change in mRNA levels for interleukin-6 and the AMPA receptor subunit mRNAs after TNF α siRNA treatment *in vitro* suggests that the TNF α siRNA was somewhat specific in silencing TNF α mRNA levels. However, this specificity is likely an artifact of cell culture because TNF α activates nuclear factor kappa B and other transcription factors which in turn could secondarily enhance expression of multiple genes. For instance, TNF α enhances IL6 expression (Ghezzi et al., 2000), and in our hands, the TNF α siRNA reduced IL6 mRNA. Further, the dynamics of the effects of TNF α siRNA on expression of the mRNAs examined or others is unknown; it seems likely that after the primary effect on TNF α mRNA there would be multiple secondary or tertiary effects, each with their own time course. Finally, the specific effects of the TNF α siRNA likely would depend on TNF α levels. Thus, for example, high levels of TNF α are associated with apoptosis and neuronal death while lower levels of TNF α are neuroprotective (De et al., 2005; Pickering et al., 2005).

The motivation for pursuing this line of research is to understand and develop a mechanism to transiently silence TNF α mRNA *in vivo* and *in vitro* and show that by doing so a sleep phenotype, EEG SWA, is altered. The current data show the effectiveness of using the TNF α siRNA. Previously, another sleep phenotype, duration of REMS was altered by an siRNA. Thus, Chen et al., (2006) showed using an orexin siRNA a reduction in orexin mRNA and an enhancement in REMS selectively in the dark period after microinjection into the lateral hypothalamus. Those data coupled with the current results, clearly indicate that selected sleep phenotypes can be targeted via use of siRNAs and thereby offer potential new experimental and therapeutic tools. Regardless, current data support the hypothesis that TNF α siRNA will silence TNF α mRNA and thereby reduce the levels of TNF α immunoreactivity in the rat cortex and that TNF α plays an important role in EEG synchronization in localized regions of cortex.

MATERIALS AND METHODS

Somatosensory cortical (SSctx) cell culture and transfection: To validate that the TNF α siRNA affects TNF α mRNA cultured rat cortical cells were used. Primary cultures of rat fetal cortical cells were prepared via a non-enzymatic dissociation as described previously for the hypothalamus with slight modifications (De et al., 2005). For the cortical cultures, $2-4 \times 10^5$ cells/well were plated in 6 well plates coated with 100 μ g/ml of poly-L-ornithine in 0.15 M borate buffer (pH 8.4). The cells were grown in Dulbecco's modification of Eagle's medium (DMEM) with 10% fetal bovine serum for two days in a humidified atmosphere of CO₂: air (5:95) at 37°C. Then the medium was replaced every other day after plating the cells with serum-free DMEM supplemented with 1 μ M human transferrin, 5 μ g/ml insulin, 100 μ M putrescine, and 60 nM sodium selenite (sf-HDMEM). At 13 days after plating, the medium was replaced with 0.8 ml antibiotic free basic DMEM medium for 3 h before transfection. A real-time reverse transcriptase polymerase chain reaction (RT-PCR) assay was used to screen different TNF α siRNAs. Treatment of the cells was performed in 3-4 different culture preparations from 1-2 mother rats. The treatment conditions were 1) without siRNA control (C), 2) with a Silencer Negative Control #1 called scrambled siRNA (Ambion, Austin, TX) or 3) a pool of two different TNF α siRNA sense: 5'-GACCCUCACACUCAGAUCAtt-3', antisense: 5'-UGAUCUGAGUGUGAGGGUCtg-3' (228-247) and sense: 5'-GCCGAUUUGCCACUUCAUAtt-3', antisense: 5'-UAUGAAGUGGCAAAUCGGCtg-3

(476-494) (Ambion). Both of these TNF α siRNAs were used in subsequent experiments. The cells were transfected using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Briefly, 2 μ l of 20 pmol siRNA was mixed with 100 μ l of Opti-MEM (Invitrogen). In a second tube, 2 μ l of Lipofectamine was mixed with 100 μ l of Opti-MEM and incubated at room temperature for 5 min. These two solutions were gently mixed then incubated for 20 min. This lipid-siRNA formulation was taken to a final volume of 1 ml with DMEM. After a 4 h incubation, the cells were replated into the growth medium. Twenty four hours post-transfection, TNF α , interleukin-6 (IL6), cyclophilin and glutamate receptors (gluR) 1-4 mRNA levels were measured by real-time RT-PCR.

Animals and surgery

Male Sprague-Dawley rats weighing (300–350g) were obtained from Taconic Farms, Inc. (Germantown, NY). Rats ($n = 7$) were housed individually with a 12:12 h light-dark cycle (lights on at 0900 h; about 300 lux) with an ambient temperature of $23 \pm 1^\circ\text{C}$. Food and water were available ad libitum. Surgical implantations of the guide cannulae and EEG electrodes were performed using ketamine and xylazine anesthesia, 87 and 13 mg/kg, respectively, as previously described (Yasuda et al., 2005b). The stainless steel jewelry screw EEG electrodes (Plastics One Inc, Roanoke, VA) were placed bilaterally just under the dura above the somatosensory cortex (Bregma coordinates: 2.5 mm AP, 5.5 mm ML) and bilateral microinjection guide cannulae were placed in layers V-VI as previously described (Yasuda et al., 2005b). Another EEG electrode used as the common reference was implanted 10 mm posterior to bregma on the midline over the cerebellum. Electromyogram (EMG) electrodes were implanted in the dorsal neck muscles. Lead wires were fitted into a miniature plug and attached to the skull with dental cement (Duz-All; Coralite Dental Products, Skokie, IL). Post-surgery recovery was monitored for ten days with rats in their home cages placed inside environmental chambers (Hotpack 352600, Philadelphia, PA). Rats had relatively unrestricted movements during the recovery period.

Recording and analyses of EEG delta activity

A flexible tether (Plastics One) connected the EEG and EMG electrodes to an electronic swivel as described previously (Yasuda et al., 2005a) and rats were given three days to acclimate to this experimental condition. During each of the acclimation days, the rats were handled for 10 min during the 40 min period just prior to light onset. Recordings began at light onset (0900 h). The vigilance states NREMS, rapid eye movement sleep (REMS) and wake were determined off line in 10 sec epochs using criteria published previously (Zhang et al., 1999). On the first day, the EEG baseline was recorded and on the following day microinjections were made on each side of the brain. One side received TNF α siRNA (0.025 nmol of each siRNAs in 2 μ l total) while the opposite side received 2 μ l of scrambled siRNA (0.050 nmol). Each injection took about 10 min. Lateralization was counterbalanced such that four rats were injected with TNF α siRNA on the right side and three rats on the left side. Each rat was removed from the tether and its environmental chamber and exposed to lights during the microinjection. Experiments were performed in series using 3-4 rats to minimize the time between treatment and light onset (0820 and 0900 h). After injection, EEG and EMG were recorded for 5 days (23 h/day with 1 h/day for cage cleaning and data backup). The average power of EEG slow wave activity (SWA) (1/2 – 4 Hz) during NREMS throughout the 23 h control-recording period was normalized to 100% for each animal and EEG SWA data are expressed as percent of control determined on the baseline day as previously described (Yasuda et al., 2005a).

In vivo mRNA expression

Seven days after completion of the EEG recordings described above, the same rats ($n = 7$) were again microinjected at the same site and with the same doses of TNF α siRNA or scrambled

siRNA as were previously used in the EEG studies. EEG SWA responses to the initial TNF α siRNA were over by day 4 after the initial injection (Fig. 3, right side). This second injection took place one week later. Rats were sacrificed 24 h after the injection and the SSctx was dissected and immediately frozen in liquid nitrogen. The dissection of the SSctx was 2 mm anterior to posterior and 2 mm medial to lateral and 1 mm dorsal to ventral. Whole tissues were stored at -80°C until preparation for RT-PCR analyses.

RT-PCR

Total RNA was extracted, cDNA synthesized and RT-PCR performed as previously described (Taishi et al 2004). Briefly, DNase I-treated total RNA (2 μ g) was reverse transcribed with oligo (dt) 18 primer using superscript III for first-strand cDNA synthesis (Invitrogen) according to the manufacturer's instructions. Primers were designed using Primer3 (www.genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) and if possible, an intron was spanned. A standard curve and efficiency analysis of the primers were performed and the primers efficiencies were above 90%. The sequences of primers used are listed in Table 1. The real time PCR reaction was performed in triplicate using the PLATINUM Quantitative PCR SuperMix-UDG (Invitrogen) with 5 μ l of diluted cDNA (5 ng, total RNA) in a final reaction volume of 25 μ l. The amplification was followed by 40 cycles of denaturation at 94°C for 15 s, annealing at 58°C for 15 s and extension at 72°C for 15 s. Finally, a melting curve was generated by stepwise increases in temperature (0.5°C increase every 10 s) for 80 cycles starting at 55°C. The threshold cycle (C_t) was determined using SYBR Green fluorescence and gene expression was evaluated by means of a comparative C_t method (User Bulletin #2 ABI PRISM 7700 sequence detection system, PE Applied Biosystems). The fold-change between the mRNA expression levels of the experimental and the control samples was calculated as previously described (Churchill et al., 2006). The dissociation curves of each primer pair used showed a single peak and the samples tested after the PCR reactions had a single expected DNA band in an agarose gel analyses.

Immunohistochemistry and quantitative analyses

Another group of five rats were used for immunohistochemical analyses of TNF α . These rats also received bilateral microinjection cannulae positioned just above the SSctx as described above for the EEG/mRNA experiments. TNF α siRNA and scrambled siRNA were injected into opposite sides of the cerebral cortex just prior to 0900 as described above. Forty eight hours after the microinjections, the rats were sleep deprived for 6 h in order to enhance TNF α expression (Bredow et al., 1997). Twenty four hours later, the rats were anesthetized and cardiac-perfused with isotonic saline, followed by 4% paraformaldehyde. The brains were removed and post-fixed for 6 h, and then sunk in 20% sucrose overnight. The brains were frozen in crushed dry ice and stored at -80°C until sectioning. Brains were sectioned coronally with the sliding microtome at 30 μ m thickness and tissue sections were treated for immunohistochemistry using a goat polyclonal anti-rat TNF α (0.5 μ g/ml, R&D Systems, Inc Minneapolis, MN) and a biotinylated secondary antibody as previously described (Churchill et al., 2005b). Avidin-biotin-peroxidase reagent (ABC kit at 1:200) and diaminobenzidine tetrahydrochloride (DAB) were from Vector Labs (Burlingame, CA). Negative controls were performed by omitting the primary antibody or pre-incubating the primary antibody (250 ng) with 750 ng recombinant TNF α (R&D Systems) for 24 h prior to the 3-day incubation with the sections. No specific IR-labeling was observed in the sections incubated without primary antibodies and a significant reduction in the labeling was observed when the primary antibody was preabsorbed with the recombinant TNF α . Western analyses of rat brain extracts indicated that the anti-rat TNF α antibody reacted with the 17 kD and 26 kD forms of the TNF α (data not shown).

Photomicrographs of the SSctx adjacent to the injection sites from 2 different sections were prepared using a Leica DMLB microscope with a Spot digital camera. Quantitation of the uncalibrated optical density for each layer V neuron within each figure was determined using Image J (NIH). The counts of 40 neurons/per section were averaged on each side of the brain and two sections in the region of the injection sites were averaged for each rat. The data for each of the rats ($n=5$) was statistically evaluated using a paired Students *t*-test. The comparison was evaluated between TNF α siRNA and scrambled siRNA-injected sides of the same rat brains.

Statistical analysis

Data are presented as means $\pm$ standard error. Two-way repeated measures analysis of variance (ANOVA) was used to compare 4 h block values of NREMS, REMS, and EEG SWA among the various groups. For the EEG SWA, treatment effect (between scrambled siRNA and TNF α siRNA injection on two sides of each rat) and time effect were the two factors of the two-way ANOVA. When significant differences were detected using the Tukey-Kramer multiple-comparison test (TK test), individual differences were evaluated between TNF α siRNA and scrambled siRNA injected. The time blocks corresponding to the 12 h light and 11 h dark periods for EEG SWA were compared by means of the paired *t*-test between TNF α siRNA- and scrambled siRNA-injected sides. Since variation in sleep parameters with time of day are well known, only the group or treatment effects are reported herein. The changes in levels of mRNA were also analyzed using of the paired Students' *t*-test for *in vivo* data or a one-way ANOVA for *in vitro* data. An α -level of $P < 0.05$ was considered to be significant in all tests.

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References

- Bazhenov M, Timofeev I, Steriade M, Sejnowski TJ. Model of thalamocortical slow-wave sleep oscillations and transitions to activated States. *J Neurosci* 2002;22:8691–8704. [PubMed: 12351744]
- Beattie EC, Stellwagen D, Morishita W, Bresnahan JC, Ha BK, Von Zastrow M, Beattie MS, Malenka RC. Control of synaptic strength by glial TNF α . *Science* 2002;295:2282–2285. [PubMed: 11910117]
- Borbely AA, Tobler I. Endogenous sleep-promoting substances and sleep regulation. *Physiol Rev* 1989;69:605–670. [PubMed: 2564687]
- Bredow S, Guha-Thakurta N, Taishi P, Obal F Jr, Krueger JM. Diurnal variations of tumor necrosis factor- α mRNA and α -tubulin mRNA in rat brain. *Neuroimmunology* 1997;4:84–90.
- Chen L, Thakkar MM, Winston S, Bolortuya Y, Basheer R, McCarley RW. REM sleep changes in rats induced by siRNA-mediated orexin breakdown. *Eur J Neurosci* 2006;24:2039–2048. [PubMed: 17067300]
- Churchill L, Taishi P, Wang M, Brandt J, Cearley C, Rehman A, Krueger JM. Brain distribution of cytokine mRNA induced by systemic administration of interleukin-1 β or tumor necrosis factor α . *Brain Res* 2006;1120:64–73. [PubMed: 17022949]
- Churchill L, Rector DM, Yasuda K, Rojas MJ, Schactler S, Sanderson V, Yasuda T, Krueger JM. Tumor necrosis factor increases surface somatosensory evoked potentials to whisker deflection. *Soc Neurosci Abstract* 2005a;63:13.
- Churchill L, Yasuda K, Yasuda T, Blindheim KA, Falter M, Garcia-Garcia F, Krueger JM. Unilateral cortical application of tumor necrosis factor alpha induces asymmetry in Fos- and interleukin-1beta-immunoreactive cells within the corticothalamic projection. *Brain Res* 2005b;1055:15–24. [PubMed: 16098952]
- Czarnecki A, Birtoli B, Ulrich B. Cellular mechanisms of burst-firing mediated long-term depression in rat neocortical pyramidal cells. *J Physiol*. 2006(Epub ahead of print) PMID: 17082228 PubMed

- Dang-Vu TT, Desseilles M, Laureys S, Degueldre C, Perrin F, Phillips C, Maquet P, Peigneux P. Cerebral correlates of delta waves during non-REM sleep revisited. *Neuroimage* 2005;28:14–21. [PubMed: 15979343]
- Darko DF, Miller JC, Gallen C, White J, Koziol J, Brown SJ, Hayduk R, Atkinson JH, Assmus J, Munnell JT. Sleep electroencephalogram delta-frequency amplitude, night plasma levels of tumor necrosis factor alpha, and human immunodeficiency virus infection. *Proc Natl Acad Sci USA* 1995;92:12080–12084. [PubMed: 8618848]
- De A, Krueger JM, Simasko SM. Tumor necrosis factor alpha increases cytosolic calcium responses to AMPA and KCl in primary cultures of rat hippocampal neurons. *Brain Res* 2003;981:133–142. [PubMed: 12885434]
- De A, Krueger JM, Simasko SM. Glutamate induces the expression and release of tumor necrosis factor- α in cultured hypothalamic cells. *Brain Res* 2005;1053:54–61. [PubMed: 16040010]
- De Sarro G, Gareri P, Sinopoli VA, David E, Rotiroti D. Comparative, behavioural and electrocortical effects of tumor necrosis factor-alpha and interleukin-1 microinjected into the locus coeruleus of rat. *Life Sci* 1997;60:555–64. [PubMed: 9042390]
- Dillon CP, Sandy P, Nencioni A, Kissler S, Robinson DA, Van Parijs L. RNAi as an experimental and therapeutic tool to study and regulate physiological and disease processes. *Ann Rev Physiol* 2004;67:47–173.
- Dreisbach AW, Hendrickson T, Beezhold D, Riesenberger LA, Sklar AH. Elevated levels of tumor necrosis factor alpha in postdialysis fatigue. *Int J Artif Organs* 1998;21:83–6. [PubMed: 9569129]
- Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T. Duplexes of 21 nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* 2001;411:494–498. [PubMed: 11373684]
- Fang J, Wang Y, Krueger JM. Mice lacking the TNF 55kDa receptor fail to sleep more after TNF α treatment. *J Neurosci* 1997;17:5949–5955. [PubMed: 9221791]
- Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* 1998;391:806–811. [PubMed: 9486653]
- Fix C, Churchill L, Hall S, Krueger JM. The number of tumor necrosis factor alpha-immunoreactive cells increases in layer IV of the barrel field in response to whisker deflection in rats. *Sleep* 2006;28:A11.
- Floyd RA, Krueger JM. Diurnal variations of TNF α in the rat brain. *NeuroReport* 1997;8:915–918. [PubMed: 9141064]
- Franklin CM. Clinical experience with soluble TNF p75 receptor in rheumatoid arthritis. *Semin Arthritis Rheum* 1999;29:172–81. [PubMed: 10622681]
- Furukawa K, Mattson MP. The transcription factor NF-kappaB mediates increases in calcium currents and decreases in NMDA- and AMPA/kainite-induced currents induced by tumor necrosis factor-alpha in hippocampal neurons. *J Neurochem* 1998;70:1876–1886. [PubMed: 9572271]
- Ghezzi P, Sacco S, Agnello D, Marullo A, Caselli G, Bertini R. Lps induces IL-6 in the brain and in serum largely through TNF production. *Cytokine* 2000;12:1205–1210. [PubMed: 10930297]
- Hough SR, Wiederholt KA, Burrier AC, Woolf TM, Taylor MF. Why RNAi makes sense. *Nat Biotechnol* 2003;21:731–732. [PubMed: 12833082]
- Imagawa S, Yamaguchi Y, Ogawa K, Obara N, Suzuki N, Yamamoto M, Nagasawa T. Interleukin-6 and tumor necrosis factor-alpha in patients with obstructive sleep apnea-hypopnea syndrome. *Respiration* 2004;71:24–29. [PubMed: 14872107]
- Kattler H, Dijk D-J, Borbely AA. Effect of unilateral somatosensory stimulation prior to sleep on the sleep EEG in humans. *J Sleep Res* 1994:1599–1604.
- Kjaer TW, Law I, Wiltschiotz G, Paulson OB, Madsen PL. Regional cerebral blood flow during light sleep—a H(2)(15)O-PET study. *J Sleep Res* 2002;11:201–207. [PubMed: 12220315]
- Krueger JM, Fang J, Taishi P, Chen Z, Kushikata T, Gardi J. Sleep: A physiologic role for IL-1 beta and TNF-alpha. *Ann N Y Acad Sci* 1998;856:148–159. [PubMed: 9917875]
- Kubota T, Li N, Guan Z, Richard RA, Krueger JM. Intrapreoptic microinjection of TNF α enhances non-REM sleep in rats. *Brain Res* 2001;932:37–44. [PubMed: 11911859]
- Lewis LD, Hagstrom JE, Loomis AG, Wolff JA, Herweijer H. Efficient delivery of siRNA for inhibition of gene expression in postnatal mice. *Nat Genet* 2002;32:107–108. [PubMed: 12145662]

- Miyagishi M, Hayashi M, Taira K. Comparison of the suppressive effects of antisense oligonucleotides and siRNAs directed against the same targets in mammalian cells. *Antisense Nucleic Acid Drug Dev* 2003;13:1–7. [PubMed: 12691531]
- Moss RB, Mercandetti A, Vojdani A. TNF- α and chronic fatigue syndrome. *J Clin Immunol* 1999;19:314–316. [PubMed: 10535608]
- Obal F Jr, Krueger JM. Biochemical regulation of sleep. *Front Biosci* 2003;8:520–550.
- Pappenheimer JR, Koski G, Fencel V, Karnovsky ML, Krueger JM. Extraction of sleep-promoting Factor S from cerebrospinal fluid and from brains of sleep-deprived animals. *J Neurophysiol* 1975;38:1299–1311. [PubMed: 1221075]
- Pickering M, Cumiskey D, O'Connor JJ. Actions of TNF- α on glutamatergic synaptic transmission in the central nervous system. *Exp Physiol* 2005;90:663–670. [PubMed: 15944202]
- Rector DM, Topchii IA, Carter KM, Rojas MJ. Local functional state differences between rat cortical columns. *Brain Res* 2005;1047:45–55. [PubMed: 15882842]
- Ryther RC, Flynt AS, Phillips JA III, Patton JG. siRNA therapeutics: big potential from small RNAs. *Gene Ther* 2005;12:5–11. [PubMed: 15496962]
- Shibata M, Parfenova H, Zuckerman SL, Leffler CW. Tumor necrosis factor- α induces pial arteriolar dilation in newborn pigs. *Brain Res Bull* 1996;39:241–247. [PubMed: 8963690]
- Sioud M. Therapeutic siRNAs. *Trends Pharmacol Sci* 2004;25:22–28. [PubMed: 14723975]
- Steriade M, Amzica F. Coalescence of sleep rhythms and their chronology in corticothalamic networks. *Sleep Res Online* 1998;1:1–10. [PubMed: 11382851]
- Taishi P, De A, Alt J, Gardi J, Obal F Jr, Krueger JM. Interleukin-1 β stimulates growth hormone-releasing hormone receptor mRNA expression in the rat hypothalamus *in vitro* and *in vivo*. *J Neuroendocrinol* 2004;16:113–118. [PubMed: 14763997]
- Takahashi S, Kapas L, Seyer JM, Wang Y, Krueger JM. Inhibition of tumor necrosis factor attenuates physiological sleep in rabbits. *Neuroreport* 1996;7:642–646. [PubMed: 8730848]
- Vgontzas AN, Zoumakis E, Lin H-M, Bixler EO, Trakada G, Chrousos GP. Marked decrease in sleepiness in patients with sleep apnea by etanercept, a tumor necrosis factor- α antagonist. *J Clin Endocrinol Metab* 2004;89:4409–4413. [PubMed: 15356039]
- Yasuda T, Yasuda K, Brown RA, Krueger JM. State-dependent effects of the light/dark cycle on the somatosensory and the visual cortex EEG in rats. *Am J Physiol* 2005a;289:1083–1089.
- Yasuda T, Yoshida H, Garcia-Garcia F, Kay D, Krueger JM. Interleukin-1 β has a role in cerebral cortical state-dependent electroencephalographic slow-wave activity. *Sleep* 2005b;28:177–184. [PubMed: 16171241]
- Yoshida H, Peterfi Z, García-García F, Kirkpatrick R, Yasuda T, Krueger JM. State specific asymmetries in EEG slow wave activity induced by local application of TNF α . *Brain Res* 2004;1009:129–136. [PubMed: 15120590]
- Zhang J, Obal F Jr, Zheng T, Fang J, Taishi P, Krueger JM. Intrapreoptic microinjection of GHRH or its antagonist alters sleep in rats. *J Neurosci* 1999;19:2187–2194. [PubMed: 10066272]

Abbreviations

TNFα	tumor necrosis factor alpha
SSctx	somatosensory cortex
EEG	electroencephalogram
siRNA	small interfering RNA
NREMS	non rapid eye movement sleep

Figure 1.

RT-PCR for TNFα mRNA, IL6 mRNA and GluR1, 2, 3, 4 mRNA levels in the rat fetal cortex primary cell cultures (right, *in vitro*) after transfection with saline or 20 pmol scrambled siRNA (Con) or 20 pmol TNFα siRNA, see Methods Section for details. Means ± SE were determined from four independent transfections each measured in triplicate. Left; *in vivo*: one side of the brain received scrambled siRNA (Con) and the other side received TNFα siRNA. Results are expressed as relative change compared with cyclophilin; 7 rats were used. Asterisks indicate statistical significance using the Students' paired t-test ($p < 0.05$)

Figure 2.

Microinjection of TNF α siRNA into layer V of the somatosensory cortex reduced the immunoreactivity in TNF α -IR pyramidal neurons in layer V (bar below indicates affected region) (A) relative to the opposite side that was injected with a scrambled siRNA control (B). The darkly stained TNF α -IR cells are evident throughout layer V on the side receiving the scrambled siRNA control injection but are reduced in number within 1 mm of the injection of TNF α siRNA (A above the bar relative to B above the bar). The photograph A illustrates the region between 0.7 and 1.3 mm from the injection site. Bar in A and B = 0.3 mm. C and D: Higher power magnification of layer V neurons within the 1 mm region affected by the siRNA injection illustrates the differences in the TNF-IR staining between the siTNF treated (C) and the scrambled siRNA treated side (D) of the brain sections. Bar = 0.025 mm.

Figure 3.

Time course of EEG SWA during NREMS expressed as a percentage of baseline EEG SWA determined the day before Day 1. A significant decrease in EEG SWA was observed on Days 2-4. Left graphs: On Day 1, the TNF α siRNA was injected at time 0 on one side of the cortex (open circles) and a scrambled siRNA was injected onto the other side (closed circles). Right graphs: EEG SWA during NREMS calculated for the 12 h light period and the 11 h dark period. The significant decreases only occur in the light phase on Days 2 and 3 after microinjection of the TNF α siRNA (grey bar) relative to the scrambled siRNA (black bar) only.

Table 1

A list of gene, primer and sequence used to detect mRNA levels of rat

Gene	Gene bank	Primer	Sequence 5'-3'
IL6	NM_012589	Sense Antisense	ggagtgctaaggaccaagacca aggtttgccgagtagacctca
TNF α	X66539	Sense Antisense	gacaaggctgccccgactatgtgctc tgatggcggagaggaggtgactttc
GluR1	NM_031608	Sense Antisense	acttcctcgtacacagccaacc agacaccatcctctccacagtca
GluR2	M85035	Sense Antisense	Gtcctggcatgggaatgaat atcggtatgcctctcaccact
GluR3	M85036	Sense Antisense	caatgtggcaggcgtgttctat tgtgagtttcacgctttgg
GluR4	NM_017263	Sense Antisense	tcagtgaggcaggcgtctta cgagtccttgggtccacattc
Cyclophilin A	NM_008907	Sense Antisense	aaatgctggaccaaacacaaa ctcatgccttctttcaccttc