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Research Report

Brain distribution of cytokine mRNA induced by systemic administration of interleukin-1β or tumor necrosis factor α

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ABSTRACT

Brain cytokine mRNA levels are impacted by systemic cytokines. For example, systemic interleukin-1β (IL1β) increases brain IL1β mRNA; subdiaphragmatic vagotomy blocks this effect. To localize which brain regions respond to intraperitoneal cytokines, we measured mRNA levels in selected brain regions for a variety of cytokines and growth factors, IL1β, TNFα, interleukin-6 (IL-6), interleukin-10 (IL10), nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF). Relative to saline administration, IL1β increased IL1β, TNFα and IL6 mRNAs in the nucleus tractus solitarius (NTS), hypothalamus, hippocampus and somatosensory cortex (SSctx), but did not induce any changes in IL10. TNFα also increased TNFα and IL1β mRNAs in the hypothalamus, hippocampus and SSctx. TNFα increased TNFα, IL1β and IL10 mRNAs in the NTS, but did not induce any changes in IL-6 mRNA. In the amygdala, IL1β enhanced IL6 mRNA and TNFα increased IL1β mRNAs. In the insular cortex, IL1β enhanced IL6 mRNA and TNFα increased IL1β mRNA. TNFα administration increased NGF mRNA in the SSctx but decreased NGF and BDNF mRNA levels in the insular cortex. Both IL1β and TNFα decreased BDNF mRNA in the amygdala. We also verified the IL1β-induced increases in TNFα mRNA within the NTS using *in situ* hybridization. These results support the hypothesis that somnogenic doses of IL1β and TNFα enhance their own mRNA levels as well as affect mRNA levels for other sleep-promoting substances.

1. Introduction

The brain cytokine network is involved in the physiological regulation of the hypothalamic neuroendocrine axis (Smith et al., 1999; Spangelo et al., 1995), sleep (Dickstein and Moldofsky, 1999; Obal and Krueger, 2003), appetite (Plata-Salaman, 2001), body temperature (Romanovsky et al., 2005) and both learning and memory (Albensi and Mattson, 2000; Avital et al., 2003; Banks et al., 2002–2003; Vitkovic et al., 2000; Yirmiya et al., 2002). These CNS processes are altered by systemic challenges such as inflammatory diseases. Indeed, during the course of an infectious disease, disturbed sleep, fever, cachexia and cognitive dysfunction are components of the acute phase response. It is likely that these responses are mediated via central cytokines since systemic bacterial and viral products such as lipopolysaccharide (Kakizaki et al., 1999;

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Klir et al., 1994; Morrow and Opp, 2005; Quan et al., 1999) or viral double stranded RNA (Krueger and Majde, 2003) enhance central production of cytokines. Systemic or central administration of cytokines, such as interleukin-1 beta (IL1β) or tumor necrosis factor alpha (TNFα), induces similar pathophysiological responses (Kelley et al., 2003; Majde and Krueger, 2005; Opp, 2005).

Although systemic IL1β or TNFα affect central processes such as sleep, it is posited that brain IL1β and TNFα are important in sleep regulation (reviewed Obal and Krueger, 2003). Intraperitoneal administration of low doses of IL1β or TNFα enhances non-rapid eye movement sleep (NREMS) and EEG slow wave activity in rats. Subdiaphragmatic vagotomy blocks this cytokine induction of NREMS (Hansen and Krueger, 1997; Kubota et al., 2001). The sleepiness associated with several clinical conditions characterized by changes in plasma cytokines also suggests a functional link between systemic and brain cytokines. For example, TNFα is elevated in plasma of sleep apnea, chronic fatigue, chronic insomnia, myocardial infarct, excessive daytime sleepiness, pre-eclampsia, post-dialysis fatigue and cancer patients; all these conditions are characterized by excessive sleepiness (reviewed Majde and Krueger, 2005; Opp, 2005). Furthermore, rheumatoid arthritis patients receiving the soluble TNF receptor report reduced fatigue (Franklin, 1999). Sleep apnea patients treated with the soluble TNF receptor have reduced sleepiness (Vgontzas et al., 2004). Intraperitoneal administration of low somnogenic doses of IL1β increases IL1β mRNA levels in the hypothalamus and cortex 2 h later and vagotomy attenuates these increases in brain IL1β mRNA (Hansen et al., 1998). Such findings suggest that the central effects of peripheral cytokines are dependent, in part, on brain production of cytokines.

TNFα and IL1β are part of a larger brain/systemic cytokine and hormonal network that regulates sleep and other CNS-regulated responses in health and disease (reviewed in Vitkovic et al., 2000; Obal and Krueger, 2003). Nevertheless, very little knowledge is available concerning the influence of systemic cytokines on the expression of cytokines and other sleep regulatory substances in specific brain regions. We describe herein the influence of peripheral administration of IL1β or TNFα on brain levels of mRNAs for the sleep regulatory substances, IL1β, TNFα, interleukin-6 (IL6), interleukin-10 (IL10), nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF). We selected these cytokines and sleep regulatory substances because our previous physiological data demonstrate that IL1β, TNFα, NGF and BDNF meet many of the proposed criteria for sleep regulatory substances and interact with each other (see Krueger and Obál, 1997; Obal and Krueger, 2003; Taishi et al., 2004). Since peripheral administration of lipopolysaccharide increases IL6 and IL10, we also evaluated these cytokines although data linking them to sleep regulation are more limited (Toth and Opp, 2001; Morrow and Opp, 2005; Shearer et al., 2001).

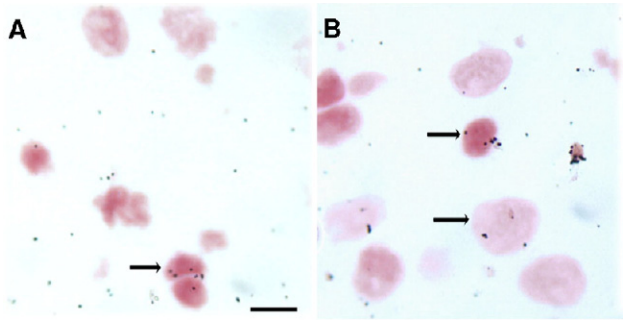

Fig. 1 – *In situ* hybridization of TNFα mRNA in the commissural NTS at 2 h after saline (A) or human recombinant IL1β (0.5 μg/kg) (B) ip injections at dark onset. Note the grains near or overlying the neutral-red-stained cells (arrows). More cells that were associated with 5 or more emulsion grains (dark dots) were evident in the rat that received IL1β than in the rat that received saline. Scale bar=0.010 mm.

2. Results

2.1. *Cytokine mRNA responses to peripheral cytokine injections*

2.1.1. *Nucleus tractus solitarius (NTS)*

Peripheral administration of IL1β significantly increased NTS IL1β mRNA levels by 1.8-fold, IL6 mRNA by 6.4-fold and TNFα mRNA by 1.9-fold compared to values obtained after saline administration (Table 1). IL10 mRNA levels in the NTS were similar after IL1β and saline. TNFα significantly increased

Table 1 – Nucleus tractus solitarius mRNA levels after intraperitoneal interleukin-1β (IL1β) or tumor necrosis factor α (TNFα)

mRNA	ΔCt			Fold change		F ratio	P value
	Saline	IL1β	TNFα	IL1β	TNFα		
IL1β	9.5±0.2	8.8±0.3*	7.3±0.2*	1.8±0.3*	5.0±0.7*	20.38	0.000
IL6	14.9±0.2	12.4±0.3	14.5±0.2	6.40±0.9*	1.40±0.2	32.84	0.000
TNFα	9.6±0.1	8.8±0.2*	8.4±0.1*	1.9±0.2*	2.3±0.2*	18.15	0.000
IL10	3.8±0.3	4.5±0.5	2.8±0.4*	0.8±0.5	2.40±0.4*	6.36	0.006
NGFβ	13.9±0.1	13.9±0.3	13.9±0.1	1.1±0.10	1.10±0.10	0.43	0.655
BDNF	9.8±0.1	9.7±0.1	9.7±0.1	1.10±0.10	1.10±0.10	0.16	0.851

The data are expressed as means for the ΔCt±standard errors of the mean and as the means for the fold change from the control ($2^{-\Delta\Delta Ct}$).
* $p<0.05$, comparing cytokine treatment with saline injection, using ANOVA with a Tukey–Kramer multiple comparison test.

Table 2 – Number of cells positive for the TNF RNA antisense probe

Brain regions	Saline	IL1β	F ratio	P value
Med NTS	1.9±1.0	6.1±01.4*	6.24	0.028
Subpostrema	0.7±0.3	2.3±0.9	3.16	0.101
Area Postrema	1.4±0.5	1.9±0.7	0.24	0.635

Only neutral-red-stained cells with 5 or more emulsion grains nearby were included. The data are expressed as mean number of cells ± standard errors of the mean.
* $p<0.05$, comparing cytokine treatment with saline injection, using ANOVA with a Tukey–Kramer multiple comparison test.

NTS levels of IL1β mRNA (5-fold) above those induced by IL1β activation of itself (1.8-fold). TNFα also enhanced NTS levels of IL10 mRNA by 2.4-fold and TNFα mRNA by 2.3-fold compared to the effects of saline (Table 1). Using *in situ* hybridization for TNFα mRNA, there was an increase in the number of cells with 5 or more emulsion grains over neutral-red-stained cells in the medial NTS after the IL1β injection (Fig. 1 and Table 2). No significant differences were observed in the number of cells in the subpostrema region of the NTS or the area postrema.

2.1.2. *Hypothalamus*

Intraperitoneal administration of either IL1β or TNFα increased hypothalamic IL1β mRNA 4.1-fold, and TNFα mRNA by 1.8- to 2.6-fold (Table 3). IL1β, but not TNFα, enhanced hypothalamic IL6 mRNA levels 3.7-fold ($p<0.001$) and IL10 mRNA 2.6-fold ($p<0.005$).

2.1.3. *Amygdala*

Peripheral administration of IL1β did not significantly increase IL1β or TNFα mRNAs in the amygdala, but did significantly increase IL6 mRNA 1.9-fold (Table 4). Intraperitoneal administration of TNFα significantly enhanced IL1β mRNA 3.0-fold but did not change IL6 or TNFα mRNAs in the amygdala (Table 4). The levels of IL10 mRNA were too low to detect in the amygdala.

2.1.4. *Bed nucleus of stria terminalis (BNST)*

Systemic administration of IL1β increased IL6 mRNA levels by 1.9-fold but did not significantly affect IL1β or TNFα mRNA levels in the BNST compared to saline treatment. In contrast, TNFα administration enhanced IL1β mRNA by 3.9-fold, but failed to affect IL6 or TNFα mRNAs in the BNST (Table 5).

2.1.5. *Insular cortex*

Peripheral administration of IL1β significantly increased IL6 mRNA but not IL1β mRNA or TNFα mRNA levels in the insular cortex (Table 6). TNFα, in contrast, significantly enhanced IL1β mRNA 3-fold but not TNFα or IL6 mRNAs.

2.1.6. *Prefrontal cortex*

Intraperitoneal administration of IL1β significantly increased IL6 mRNA levels 2.7-fold in the prefrontal cortex but not IL1β or TNFα mRNAs (Table 7).

TNFα significantly increased IL1β mRNA 3.5-fold but failed to affect either TNFα or IL6 mRNAs in this area.

2.1.7. *Somatosensory cortex*

IL1β significantly increased IL6 mRNA by 2.9-fold and TNFα mRNA by 1.6-fold compared to results obtained after saline administration (Table 8). TNFα enhanced IL1β mRNA by 4.1-fold, and TNFα mRNA by 2.0-fold in the SSctx but it did not alter IL6 mRNA levels. The levels of IL10 mRNA were too low to detect in the somatosensory cortex.

2.1.8. *Hippocampus*

Hippocampal cytokine mRNAs responded to systemic IL1β and TNFα (Table 9) similarly to those responses observed in the NTS. IL1β injections increased IL1β mRNA levels by 2.2-fold but TNFα injections increased IL1β mRNA by 5.4-fold. IL1β and TNFα significantly increased TNFα mRNA levels by 2.0- to 2.6-fold. IL1β enhanced IL6 mRNA levels, but TNFα reduced IL6 mRNA levels.

2.1.9. *Neurotrophin responses to peripheral cytokine injections*

No differences were observed in BDNF or NGF-β subunit mRNA levels in the NTS after either IL1β or TNFα administration (Table 1), but slight significant decreases in BDNF mRNA were observed in the amygdala after either IL1β or TNFα administration (Table 4). No differences were observed in BDNF or NGFβ mRNA levels in the hypothalamus, BNST, prefrontal cortex, insular cortex or hippocampus after IL1β administration (Tables 3, 5, 6, 7, 9). However, intraperitoneal administration of TNFα in comparison with saline induced significant decreases in NGF and BDNF mRNAs in the insular

Table 3 – Hypothalamic mRNA changes induced by IL1β or TNFα

mRNA	ΔCt			Fold change		F ratio	P value
	Saline	IL1β	TNFα	IL1β	TNFα		
IL1β	10.2±0.2	8.1±0.2	8.0±0.1	4.1±0.5*	4.1±0.4*	20.09	0.000
IL6	17.1±0.2	15.4±0.3	16.7±0.2	3.7±0.7*	1.4±0.2	12.10	0.000
TNFα	9.3±0.1	8.5±0.1	8.0±0.1	1.8±0.2*	2.6±0.2*	16.95	0.000
IL10	12.8±0.4	11.4±0.3	12.6±0.4	3.0±0.6*	1.5±0.4	6.66	0.005
NGFβ	13.3±0.2	13.4±0.1	13.4±0.1	0.9±0.1	1.0±0.1	0.53	0.596
BDNF	8.6±0.1	8.6±0.1	8.6±0.1	1.0±0.1	1.0±0.1	0.05	0.955

The data are expressed as means ± standard errors of the mean.
* $p<0.05$, comparing cytokine treatment with saline injection, using ANOVA with a Tukey–Kramer multiple comparison test.

Table 4 – Amygdala changes in mRNAs induced by IL1β or TNFα

mRNA	ΔCt			Fold change		F ratio	P value
	Saline	IL1β	TNFα	IL1β	TNFα		
IL1β	9.9±0.2	9.6±0.2	8.5±0.2	1.4±0.3	3.0±0.6*	6.57	0.005
IL6	10.1±0.2	8.9±0.1	10.1±0.2	1.9±0.2*	0.8±0.1	19.06	0.000
TNFα	9.3±0.2	9.0±0.2	9.0±0.1	1.3±0.20	1.3±0.1	1.45	0.252
NGFβ	4.2±0.1	4.5±0.2	4.4±0.1	0.9±0.1	0.9±0.04	0.50	0.614
BDNF	1.4±0.1	1.8±0.1	1.8±0.1	0.8±0.1*	0.8±0.003*	7.81	0.002

The delta Ct is reported for each of the injections and then the fold change relative to the saline averages are reported with the *F* ratio and *P* values. The data are expressed as means±standard errors of the mean.
* $p<0.05$, comparing cytokine treatment with saline injection, using ANOVA with a Tukey–Kramer multiple comparison test.

cortex by 0.5- to 0.8-fold. TNFα administration also increased NGF mRNA by 1.5-fold in the somatosensory cortex (Table 8).

3. Discussion

The major finding reported here, that systemic IL1β or TNFα enhances central mRNA levels of these and other cytokines and sleep regulatory substances, is consistent with prior literature. For example, Hansen et al., (1998) showed that intraperitoneal IL1β promoted IL1β mRNA expression in the hypothalamus, hippocampus and cortex in addition to the liver. If the rats were given a subdiaphragmatic vagotomy, the intraperitoneal IL1β failed to upregulate hippocampal and cortical expression of IL1β mRNA although the liver response remained (Hansen et al., 1998). Other data also suggest that the biological activities of systemic IL1β (Hansen and Krueger, 1997) or TNFα (Kubota et al., 2001) are dependent upon vagal afferents; e.g., the sleep-promoting activity and febrile responses induced by low doses of these substances are blocked by vagotomy. Although such results suggest that IL1β or TNFα-induced action potentials within vagal afferents induce IL1β and TNFα mRNA in the brain, other routes by which systemic cytokines affect the brain also are likely (Dantzer et al., 2000; Maier et al., 1998; Watkins and Maier, 2005). Other routes likely involved include cytokine transport mechanisms at the blood–brain barrier (Banks et al., 1995), passage through leaky areas of the blood–brain barrier, such as the vascular organ of the lamina terminalis (Saper and Breder, 1994), and induction of smaller more lipid soluble molecules such as prostaglandins or nitric oxide at the blood–brain barrier and their subsequent passage into the brain (Cao et al., 1998; Van Dam et al., 1993; Beasley et al., 1991; Schini et al., 1991). Current experiments did not distinguish between these possibilities, although they clearly indicate that systemic IL1β or TNFα have the capacity to alter central mRNA levels of these and other cytokines.

The current studies only analyzed brain mRNA levels in samples harvested at 2 h after the peripheral injection of the cytokines; thus, the dynamics of the cytokine mRNA responses and their translation into protein would be missed. For example, although both IL1β and TNFα mRNAs increase within 2 h after a cerebral ischemia, IL10 does not show a significant mRNA increase until 6 h later (Zhai et al., 1997). The increases in IL10 mRNA levels observed at 2 h after TNFα in the NTS suggest that TNFα-activated vagal afferents are able to induce IL10 mRNA quickly. Although IL1β injection enhances IL6 mRNA and TNFα injection increases IL1β mRNA within 2 h, TNFα injection does not increase IL6 mRNA via the increases in IL1β. Perhaps there was insufficient time for the TNFα enhanced IL1β mRNA to affect IL6 mRNA. Regardless, we chose the 2 h time point for mRNA levels because after either intraperitoneal TNFα or IL1β there are clear sleep responses at this time. Protein levels of cytokines in the brain are low, rendering study of the translation of proteins in the central nervous system after peripheral injections difficult. However, cortical injections of TNFα increased cortical IL1β-immunoreactive protein within 2 h (Churchill et al., 2005), therefore suggesting that immunohistochemical approaches may be useful.

A second major finding reported here is that the response profile induced by systemic IL1β is different from that induced by TNFα. This difference is expected since the biological activities of IL1β and TNFα are not identical. Nevertheless, the

Table 5 – Bed nucleus of stria terminalis; relative fold changes in mRNAs

mRNA	ΔCt			Fold change		F ratio	P value
	Saline	IL1β	TNFα	IL1β	TNFα		
IL1β	9.4±0.4	8.5±0.5	7.8±0.4	2.1±0.6	3.9±0.9*	4.52	0.021
IL6	10.6±0.3	9.6±0.3	10.5±0.2	1.9±0.2*	1.2±0.2	3.85	0.034
TNFα	7.9±0.1	7.8±0.2	7.7±0.2	1.2±0.2	1.2±0.1	0.29	0.748
NGFβ	12.5±0.2	12.5±0.2	12.8±0.2	1.1±0.2	0.9±0.1	0.68	0.516
BDNF	8.6±0.2	8.8±0.2	8.6±0.2	0.9±0.1	1.1±0.1	0.48	0.623

The data are expressed as means±standard errors of the mean.
* $p<0.05$, comparing cytokine treatment with saline injection, using ANOVA with a Tukey–Kramer multiple comparison test.

Table 6 – Insular cortex; relative fold changes in mRNAs

mRNA	ΔCt			Fold change		F ratio	P value
	Saline	IL1β	TNFα	IL1β	TNFα		
IL1β	10.0±0.2	9.2±0.4	8.9±0.2	1.8±0.5	2.5±0.2*	5.07	0.015
IL6	10.3±0.1	9.5±0.2	10.4±0.2	1.9±0.2*	1.0±0.2	7.28	0.003
TNFα	9.3±0.1	9.1±0.2	9.6±0.1	1.3±0.2	0.9±0.1	3.42	0.049
NGFβ	5.3±0.2	5.0±0.1	5.7±0.2	1.3±0.1	0.8±0.1*	5.88	0.008
BDNF	9.2±0.1	9.2±0.1	9.7±0.1	1.0±0.1	0.7±0.1*	4.72	0.020

The data are expressed as means±standard errors of the mean.
* $p<0.05$, comparing cytokine treatment with saline injection, using ANOVA with a Tukey–Kramer multiple comparison test.

biological activities of IL1β and TNFα are often related. For example, IL1β-induced sleep responses are attenuated if animals are pretreated with a TNF inhibitor (Takahashi et al., 1999); conversely TNFα-induced sleep responses are attenuated if IL1β is inhibited. However, the time courses of these two inhibitory effects are very different. Other independent data clearly indicate differential time courses of brain cytokines. Thus, lipopolysaccharide induces distinct hypothalamic temporal profiles in IL1β, IL6 and TNFα (Kakizaki et al., 1999). Second, a mechanical injury induced by *in vivo* microdialysis induces a rapid increase in IL6 but a much slower (days) increase in IL1 (Woodroofe et al., 1991). Nevertheless, the single time point examined in the current study was sufficient to illustrate unique brain mRNA profiles induced by systemic IL1β and TNFα.

Current data support the hypothesis that cytokine mRNA levels are induced in many localized brain regions in response to systemic somnogenic doses of NREMS-inducing agents. Many of the areas showing change in cytokine levels were within the central autonomic nervous system. Although our studies did not analyze every anatomically connected region from the NTS to the cortex, all of the selected brain regions did show enhancement in mRNA levels in some of the cytokines although for some, such as NGF and BDNF, decreases were observed in the central autonomic nervous system while increases were observed in the neocortex. The increase in NGF mRNA in the somatosensory cortex in response to sleep-inducing doses of TNF peripherally is consistent with the previous findings of enhanced NGF immunoreactive cells in the somatosensory cortex after sleep deprivation and whisker stimulation (Brandt et al., 2001). Current data also show that many cytokines induce their own mRNA levels as well as others within the cytokine network of the hypothalamus, a sleep regulatory center (Saper et al., 2005).

The neuroanatomical pathways by which peripheral cytokines influence the brain cytokines are still unknown. Perhaps the activation of the cytokine network within the central autonomic nervous system (NTS, hypothalamus, amygdala, insular cortex, BNST) (Saper, 2004) induces homeostatic regulation of other growth factors involved in sleep regulation (Obal and Krueger, 2003; Obal and Krueger, 2004). The NTS sends visceral information from the vagus either directly or through a relay to the parabrachial nucleus, hypothalamus, central amygdala, BNST, medial prefrontal cortex and insular cortex (Saper, 2004). The somatosensory cortex receives inputs mainly from the thalamus but also from the basal forebrain, which receives inputs from the prefrontal cortex (Golmayo et al., 2003). In general, the similar responses in the cytokine mRNA levels to the peripheral administration of cytokines within these various brain regions suggest that the peripheral cytokines induce changes throughout the central autonomic nervous system as well as other regions such as the somatosensory cortex and hippocampus that locally induce changes in the brain cytokine levels.

The induction of the cytokine network throughout the central autonomic nervous system may be an important mechanism by which peripheral cytokines modulate neuronal functions such as sleep or appetite. Nevertheless, local application of cytokines into specific brain regions induces state-specific changes in the EEG (Yasuda et al., 2005; Yoshida et al., 2004) as well as immunohistochemical changes in other cytokines (Churchill et al., 2005). Furthermore, microinjection of low doses of TNFα or IL1β into the anterior hypothalamus induces NREMS (Alam et al., 2004; Kubota et al., 2002). Such results indicate that direct application of cytokines to the brain also results in biological actions. Whether such central injections enhance production of cytokine mRNAs throughout the central autonomic nervous system remains unknown.

Table 7 – Prefrontal cortex, relative fold changes in mRNA levels

mRNA	ΔCt			Fold change		F ratio	P value
	Saline	IL1β	TNFα	IL1β	TNFα		
IL1β	8.0±0.3	6.7±0.7	6.2±0.2	1.7±0.3	3.4±0.5*	12.66	0.000
IL6	8.8±0.4	7.6±0.3	8.5±0.2	2.7±0.5*	1.4±0.2	4.09	0.029
TNFα	8.1±0.3	7.6±0.3	7.8±0.2	1.6±0.3	1.4±0.2	0.97	0.393
NGFβ	9.9±0.3	9.4±0.2	10.0±0.3	1.6±0.3	1.1±0.2	1.21	0.315
BDNF	7.4±0.3	7.6±0.3	7.4±0.3	1.1±0.3	1.1±0.3	0.06	0.943

The data are expressed as means±standard errors of the mean.
* $p<0.05$, comparing cytokine treatment with saline injection, using ANOVA with a Tukey–Kramer multiple comparison test.

Table 8 – Somatosensory cortex; relative fold change in mRNA levels

mRNA	ΔCt			Fold change		F ratio	P value
	Saline	IL1β	TNFα	IL1β	TNFα		
IL1β	15.4±0.2	14.6±0.1	12.9±0.2	1.4±0.1	4.1±0.4*	51.27	0.000
IL6	15.1±0.2	13.8±0.3	14.9±0.3	2.9±0.5*	1.3±0.2	8.69	0.001
TNFα	10.1±0.2	9.5±0.2	9.2±0.1	1.6±0.2*	2.0±0.2*	9.68	0.001
NGFβ	7.1±0.2	6.8±0.1	6.5±0.1	1.2±0.1	1.5±0.1*	4.01	0.030
BDNF	9.4±0.1	9.7±0.1	9.4±0.2	0.9±0.1	1.1±0.1	1.35	0.275

The data are expressed as means±standard errors of the mean.
* $p<0.05$, comparing cytokine treatment with saline injection, using ANOVA with a Tukey–Kramer multiple comparison test.

Regardless, electrical stimulation of the NTS as well as regions within the anterior hypothalamus or basal forebrain produces EEG synchronization (Benedek et al., 1979; Chase et al., 1967; Clemente and Sterman, 1963), thereby suggesting that local neuronal activation is the proximate cause of cytokine-induced biological activity such as EEG delta power.

In conclusion, peripheral cytokines that induce NREMS when administered at dark onset also amplify increases in other cytokine mRNA levels within specific brain regions particularly within the central autonomic nervous system. Current data suggest that these localized changes in mRNA levels for sleep regulatory substances are involved in coordinating the brain responses to peripheral inflammatory signals including sleep.

4. Experimental procedures

All animal use was in compliance with NIH standards published in the *Guide for Care and Use of Laboratory Animals* and was approved by our Institutional Animal Care and Use Committee. Male Sprague–Dawley rats (280–350 g) were acclimatized to a light/dark cycle of 12 h light and 12 h dark at 24 °C for 7 days prior to experimentation. The rats were housed in groups of 4 and fed standard rat chow and water *ad libitum*. Rats were handled for 3 subsequent days for 2.5 min each day to habituate them to the handling process prior to the intraperitoneal injections. Rats were injected intraperitoneally just prior to dark onset with saline or a dose of a cytokine that previously was shown to be somnogenic. Three groups of rats were used for RNA/cDNA determinations. Group I received pyrogen-free saline (PFS), Group II received 0.5 μg/kg human recombinant IL1β (R&D Systems, Minneapolis, MN) and Group III received 100 μg/kg rat recombinant TNFα (R&D Systems). Rats were sacrificed 2 h after intraperitoneal injection. At this time, NREMS is enhanced after either IL1β (Opp et al., 1991) or TNFα (Kubota et al., 2001) at the doses used. Rats were then anesthetized with isoflurane and decapitated. Brains were removed from the skull and cooled on ice for 20 s followed by coronal sectioning into 4 regions. During dissection, three individuals quickly dissected 2 forebrain regions (Figs. 2A and B), the midbrain section (Fig. 2C) and brainstem region (Fig. 2D) as demarcated in the atlas drawings. The brain regions were snap frozen in liquid nitrogen within 5 min of the kill. The NTS was dissected from the dorsal brainstem below and caudal to the cerebellum (Fig. 2D). The amygdala and the hypothalamus were dissected from a coronal midbrain section that included the anterior (above the chiasm of the optic tract) to posterior hypothalamus (mammillary body) (Figs. 2B and C). The hippocampus and the primary somatosensory cortex were also dissected from this midbrain section. The coronal forebrain section anterior to the optic tract was used to dissect the BNST and the piriform cortex (see Fig. 2B). Finally, the coronal section anterior to the caudate–putamen was used to dissect the prefrontal cortex, which included the prelimbic and infralimbic prefrontal cortex and anterior cingulate cortex (Fig. 2A).

Using a Tissue Tearor (Biospec Products, Inc; Bartlesville, OK), each brain region was individually homogenized using 2 ml of TRIzol (Invitrogen; Carlsbad, CA) on ice and the RNA was purified according to the manufacturer's instructions. After isopropanol precipitation, the RNA pellet was hydrated in 50 μl DNase–RNase free water (Gibco; Grand Island, NY). The samples were then treated with 2–4 U DNase–RNase free and

Table 9 – Hippocampus; relative fold change in mRNA levels

mRNA	ΔCt			Fold change		F ratio	P value
	Saline	IL1β	TNFα	IL1β	TNFα		
IL1β	10.4±0.1	9.4±0.2	8.2±0.3	2.2±0.3[a]	5.4±1.0[a,b]	13.46	0.000
IL6	11.4±0.2	9.7±0.3	10.8±0.3	3.8±0.7[a,b]	1.7±0.2[a]	8.59	0.001
TNFα	9.9±0.2	9.0±0.2	8.7±0.2	2.0±0.3[a]	2.6±0.4[a]	6.80	0.004
IL10	11.1±0.3	10.7±0.2	10.9±0.2	1.4±0.1	1.3±0.2	0.44	0.649
NGFβ	10.0±0.1	10.0±0.1	9.9±0.1	1.0±0.1	1.1±0.1	0.04	0.961
BDNF	6.3±0.2	6.4±0.2	6.1±0.1	1.0±0.1	1.2±0.1	1.10	0.348

The data are expressed as means±standard errors of the mean.
[a] $p<0.05$, comparing cytokine treatment with saline injection, using ANOVA with a Tukey–Kramer multiple comparison test.
[b] $p<0.01$, comparing IL1β with TNFα, using ANOVA with a Tukey–Kramer multiple comparison test.

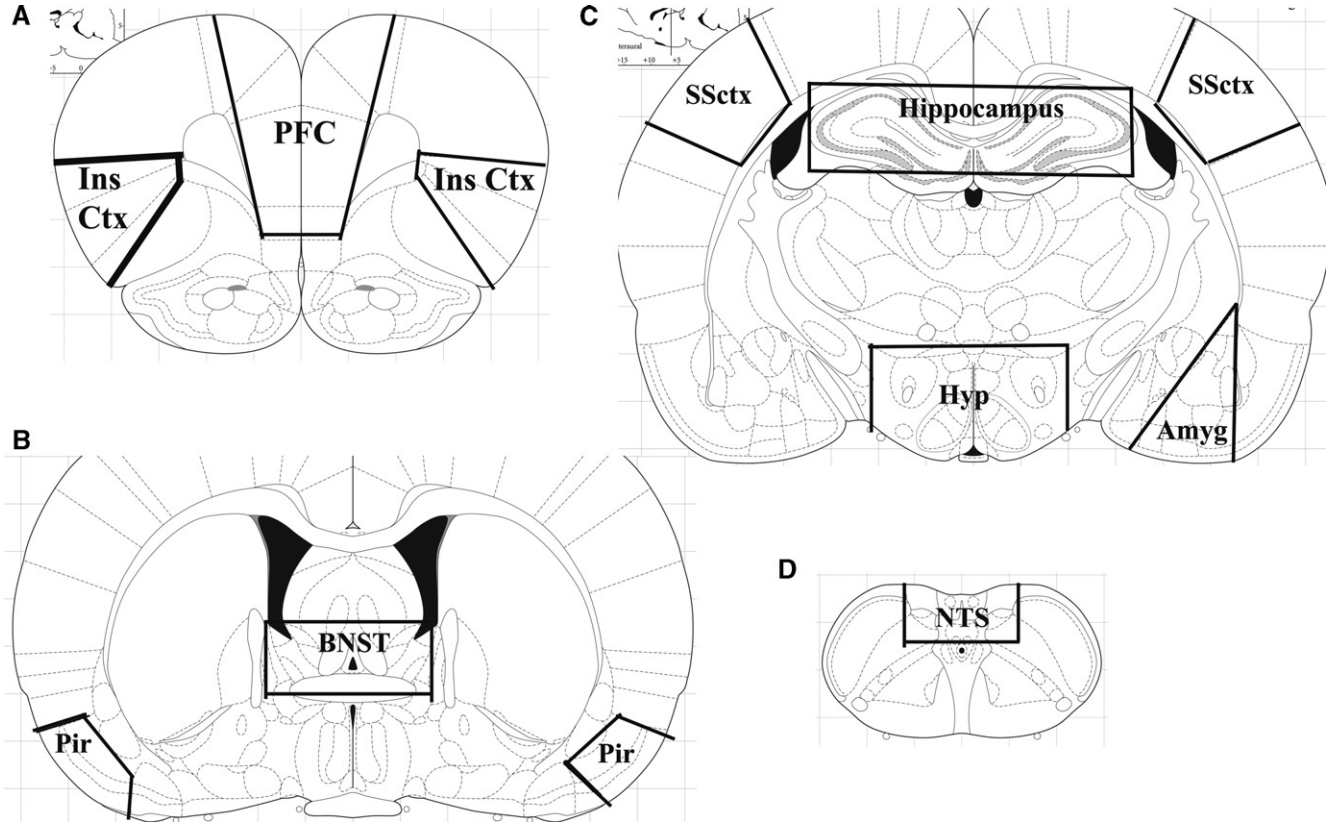

Fig. 2 – The dissections of the brain analyzed for cytokine and other sleep regulatory substance mRNA levels. The inside of the bars indicates the areas dissected for analyses. (A) The coronal section for the dissection of the prefrontal (PFC) and insular (Ins) cortex (ctx) at Bregma +3.72 mm. (B) The coronal section for the dissection of the bed nucleus of stria terminalis (BNST) and piriform cortex at Bregma −0.36 mm. (C) The coronal section for dissection of the somatosensory cortex (SSctx), hippocampus, amygdala (Amyg) and hypothalamus (hyp) at Bregma −2.64 mm. (D) The coronal section for dissection of the nucleus of the solitary tract (NTS) at Bregma −15.00 mm. These diagrams were reprinted from the CD provided with Paxinos and Watson (2004).

1 μl SuperRNase In (Ambion; Austin, TX) according to the manufacturer's instructions. RNA was quantified by spectrophotometry at 260 nm. RNA integrity was verified by electrophoresis run on a denaturing agarose gel and subsequent visualization under ultraviolet light. First-strand cDNA was synthesized by priming with oligo-dT using 1 μg total RNA. Reverse transcriptase (RT)-PCR was performed using the Thermoscript RT system (Invitrogen). Aliquots (5 μl) of a 1:20 dilution of cDNA (12 ng of total RNA) were amplified by real time RT-PCR. The primers were designed for rats using Affymetrix software. The primers used are listed in Table 10; the primers were designed with PCR efficiencies of 100± 10%. An example of an efficiency curve is illustrated in Taishi et al., 2004. Real time RT-PCR was performed using an iCycler IQ multi-color real time RT-PCR detection system (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions. Platinum® qPCR Supermix-UDG (Invitrogen), SYBR Green I mix (Molecular Probes; Eugene, OR) and Fluorescein were added. Reactions were run in triplicate. The initial amplification conditions were carried out with one cycle at 50 °C for 2 min and 95 °C for 2 min. The subsequent amplifications were: 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. for IL6, IL10, TNFα, BDNF and NGFβ (NGF) mRNA. Finally, a melting curve was generated by stepwise increasing temperature (0.5 °C increase every 10 s) for 80 cycles starting at 55 °C. If multiple peaks were observed in the melt curve analysis, the data were not used.

For all groups, the data were analyzed with the iCycler iQ software and statistical differences were calculated using a

Table 10 – List of gene primers

Gene	Gene bank	Primer	Sequence 5′–3′
IL6	NM_012589	Sense	ggagtgctaaggaccaagacca
		Antisense	aggtttgccgagtagacctca
IL10	NM_012854	Sense	gccatcactctgcaaccact
		Antisense	aaacaatacgccattcccaag
TNFα	X66539	Sense	gacaaggctgccccgactatgtgctc
		Antisense	tgatggcggagaggaggctgactttc
GluR1	X17184	Sense	acttcctcgtacacagccaacc
		Antisense	agacaccatcctctccacagtca
BDNF	NM_012513	Sense	gatgaggaccagaaggttcg
		Antisense	tccagcagaaagagcagagg
NGFβ	XM_227525	Sense	acttcctcgtacacagccaacc
		Antisense	agacaccatcctctccacagtca

one-way analysis of variance (using the statistical program NCSS). Gene expression was calculated using a comparative threshold cycle (Ct) method (User Bulletin 2 ABI PRISM 7700 sequence detection system, PE Applied Biosystems) (www.ukl.uni-freiburg.de/core-facility/taqman/user_bulletin_2.pdf). Each Ct value was an average of the values obtained from each reaction. Triplicate measurements were made to strengthen the accuracy of these measurements. The differences in the threshold cycles between the gene of interest and cyclophillin, ΔCt values were determined by subtracting the cyclophillin Ct values from the gene of interest Ct values. The differences between the mean of the threshold cycles for the saline group and each of the other individual data points were computed in order to normalize the data (similar to a percent of control value) to obtain ΔΔCt values. The relative fold changes were obtained by computing the 2 to the power of the negative of this number (−ΔΔCt). The data are expressed as means ± standard errors of the mean. This exponential calculation allowed us to the compare the threshold cycles between the experimental groups using analysis of variance (ANOVA). As warranted by ANOVA test results, Tukey–Kramer multiple comparison tests were performed and were considered significant for <0.05.

4.1. *In situ hybridization*

A fourth group of rats was used for *in situ* hybridization. Seven rats received IL1β (0.5 μg/kg) 2 h before dark onset. A control group ($N=8$) received saline at this time. Rats were killed 2 h later and brain harvested and prepared as described below. To generate sense and antisense probes for *in situ* hybridization, the *Xho*I–*Sac*I fragment of rat TNFα cDNA (gb: X66359) was cloned in *Xho*I–*Sac*I digested pGEMZ-11f. To clone the rat TNFα, 2 μg total RNA that was isolated from rat spleen was primed with oligo-dT in a reverse transcription reaction as described in Superscript RT-PCR system (Invitrogen, Inc). After diluting the reverse transcription reaction to 50 μl, a 5 μl aliquot was then amplified using sense (5′-GTGTCTGTGCCTCAGCCT-3′ that binds to 87–105 bp of rat TNFα, gb: X66359) and antisense (5′-CTCCTCCCAGGTACATG-3′ that binds to 584–601 bp or rat TNFα, gb: X66359) primers in a PCR protocol according to vendor's instructions. The PCR product was subjected to agarose gel electrophoresis, and the DNA from the 515 bp band was eluted and subjected to restriction digest with *Xho*I and *Sac*I restriction enzymes according to the vendor's instructions (Promega Inc., Madison, WI). This PCR product contains *Xho*I and *Sac*I sites that exist in the rat TNFα cDNA sequence (gb: X66359). The *Xho*I and *Sac*I digested PCR product, a 308 bp band, was then gel purified and ligated into *Sac*I and *Xho*I digested pGEM-11Zf(+) vector with T4 DNA ligase according to instructions (Promega Inc). The ligation mixture was then used to transform competent cells (JM109) and subjected to blue/white screening on an Luria–Bertani (LB)-agar plate containing ampicillin (100 μg/ml) in addition to X-gal and isopropylthio-D-β-galactoside (IPTG). The insert containing white colonies was isolated and grown in LB medium containing ampicillin. The isolated plasmid DNA from these white colonies was subjected to restriction digest with *Xho*I and *Sac*I to confirm orientation of the insert and subsequently subjected to cyclic sequencing on both strands. The sequence was subjected to a BLAST search that was identical to the reported sequence (gb: X66359). The plasmid containing the TNFα insert was diluted to 1 ng/ml and amplified using T7 and SP6 primers in a PCR amplification protocol. The amplified PCR product was gel purified and used for synthesis of the radioactive sense or antisense RNA using either T7 for the antisense or SP6 polymerase for the sense, respectively.

The protocol for *in situ* hybridization was modified from Mansour et al. (1990). The radioactive cRNA probes were diluted in hybridization buffer to a final concentration of 2×10^6 dpm/30 μl. Volumes of 30 μl were applied to coronal sections that had been previously thaw-mounted onto gelatin-subbed slides, were post-fixed for 30 min in 4% paraformaldehyde in PBS, treated with a mixture of 0.1 triethanolamine, pH 8.0 and acetic anhydride for 10 min, rinsed with 2× SSC (300 mM NaCl, 30 mM sodium citrate, pH 7.2) for 5 min and dehydrated through graded alcohols and air-dried. After hybridization (overnight, 55 °C), the slides were rinsed in 2× SSC (5 min) and rinsed with RNase A (200 μg/ml in 100 mM Tris pH 8.0 and 0.5 M NaCl) for 30 min at 37 °C to remove unhybridized, radioactive RNA. Subsequently, the sections were rinsed in a series of SSC concentrations and dehydrated in alcohol and air dried. The sections were dipped in Kodak NTB-2 emulsion and stored at 4 °C for 2 months prior to development. After development, the sections were counter-stained with 1% neutral red for 20 min and dehydrated through a series of ethanol concentrations. A control was performed by incubating sections with the radioactive sense probe prepared using the SP6 polymerase.

The number of cells with 5 or greater grains above or close to the neutral-red-stained cell body within the medial NTS, subpostrema and area postrema was manually counted by an investigator blinded to the experimental conditions.

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