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Cytokine mRNA induction by Interleukin-1 β or Tumor Necrosis Factor α *in vitro* and *in vivo*

Ping Taishi,

Dept. of Veterinary & Comparative Anatomy, Program in Neuroscience, Washington State University, Pullman, WA USA

Lynn Churchill,

Dept. of Veterinary & Comparative Anatomy, Program in Neuroscience, Washington State University, Pullman, WA USA

Alok De,

Dept. of Veterinary & Comparative Anatomy, Program in Neuroscience, Washington State University, Pullman, WA USA

Ferenc Obal Jr., and

Endocrine Unit and Department of Physiology, A. Szent-Gyorgyi Center, Unniversity of Szeged, Szeged, Hungary

James M. Krueger

Dept. of Veterinary & Comparative Anatomy, Program in Neuroscience, Washington State University, Pullman, WA USA

Abstract

Hypothalamic and cortical mRNA levels for cytokines such as interleukin-1 α (IL1 α), tumor necrosis factor alpha (TNF α), nerve growth factor (NGF) and brain derived neurotrophic factor (BDNF) are impacted by systemic treatments of IL1 β and TNF α . To investigate the time course of the effects of IL1 β and TNF α on hypothalamic and cortical cytokine gene expression, we measured mRNA levels for IL1 β , TNF α , interleukin-6 (IL-6), interleukin-10 (IL-10), IL1 receptor 1, BDNF, NGF, and glutamate decarboxylase-67 *in vitro* using hypothalamic and cortical primary cultures. IL1 β and TNF α mRNA levels increased significantly in a dose-dependent fashion after exposure to either IL1 β or TNF α . IL1 β increased IL1 β mRNA in both the hypothalamic and cortical cultures after 2–6 h while TNF α mRNA increased significantly within 30 min and continued to rise up to 2–6 h. Most of the other mRNAs showed significant changes independent of dose *in vitro*. *In vivo*, intracerebroventricular (icv) injection of IL1 β or TNF α also significantly increased IL1 β , TNF α and IL6 mRNA levels in the hypothalamus and cortex. IL1 β icv, but not TNF α increased NGF mRNA levels in both these areas. Results support the hypothesis that centrally active doses of IL1 β and TNF α enhance their own mRNA levels as well as affect mRNA levels for other neuronal growth factors.

Corresponding Author: Dr. James M. Krueger, Sleep and Performance Research Center, Programs in Neuroscience, Veterinary and Comparative Anatomy, Pharmacology and Physiology, College of Veterinary Medicine, Washington State University, Pullman, WA 99164-6520; Telephone: 509-335-8212, FAX: 509-335-4650, **Email:** krueger@vetmed.wsu.edu.

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Keywords

Sleep regulatory substances; hypothalamus; somatosensory cortex; interleukins; tumor necrosis factor; GABA

Introduction

The brain cytokine network is involved in multiple physiological and pathological processes. For example, much evidence supports the hypothesis that interleukin-1 beta (IL1) and tumor necrosis factor alpha (TNF) are key components of the physiological sleep homeostat (reviewed Krueger et al 2007). These cytokines also play a role in memory and cognition (Pickering and O'Connor 2007), the regulation of the hypothalamic neuroendocrine axis (Dunn 2000), temperature regulation (Leon 2004, Conti et al 2004), and feeding (Plata-Salaman 2001). IL1 and TNF also have well-known roles in pathology. For instance, their up-regulation in brain is observed and is likely responsible for, in part, the acute phase response (reviewed Krueger et al 2003) induced by infectious agents. Further, these cytokines seem involved in the pathogenicity of metabolic syndrome including Type II diabetes, chronic inflammatory states, and cardiovascular disease.

IL1, TNF, their receptors and other members of their signalling families are expressed in normal and diseased brain (reviewed Obal and Krueger 2003). For example, there is about a 2 fold change in both IL1 and TNF mRNAs over the course of the day in the hypothalamus and cortex (Taishi et al 1998). Diurnal changes in protein levels can be larger, e.g. TNF cortical and hypothalamic levels vary about 10 fold over the day with highest levels occurring at the onset of daylight hours (Floyd and Krueger 1997). In pathological states, up-regulation of IL1 and TNF can be even larger, and lead to dysfunction including neuronal death (Simi et al 2007, Brabers and Nottet 2006) and at a whole organism level to a condition analogous to endotoxin shock (Lin and Yeh 2005, Dinarello 1991). Cytokines in brain, as well as other organ systems such as the immune system, act within a complex network of other cytokines and hormones including the neurotrophins. IL1 and TNF induce their own synthesis as well as that of each other in monocytes (Bachwich et al., 1986; Philip and Epstein, 1986) and vascular endothelial cells (Libby et al., 1986). Some elements within the cytokine network provide negative feedback signals to dampen the up-regulation of IL1 and TNF, e.g. IL10 (Tu et al 2007, Vitkovic et al 2001), although the time courses of such actions remain unknown. Systemic injections of IL1 β and TNF α also increase each other's mRNA levels as well as BDNF and NGF mRNAs in specific brain regions (Churchill et al., 2006) including the hypothalamus, a component of the central autonomic nervous system, and the somatosensory cortex. For example, inhibition of IL1 prior to TNF treatment attenuates TNF-enhanced sleep. Conversely, inhibition of TNF prior to IL1-treatment attenuates IL1-enhanced sleep (Takahashi et al 1999). The time courses of these two effects are very different suggesting that their inductions of each other have distinct time courses although to our knowledge direct comparisons within a single experiment of their time courses of actions heretofore, has not been reported. To investigate further the regulation of the brain cytokine network, we analyzed the dose- and time-dependency of gene expression of a few cytokines in primary mixed neuronal and glial cultures of the hypothalamus and cortex (De et al., 2005). Since sleep deprivation and afferent input alter GAD-67 mRNA levels in the somatosensory cortex (Churchill et al., 2001), GAD-67 mRNA levels were also evaluated in this study. These same brain regions were analyzed after cerebral intracerebroventricular (icv) injections of IL1 β and TNF α .

Results

In vitro mRNA responses to IL1 β treatment

In hypothalamic cell cultures, rat recombinant IL1 β significantly enhanced all of the mRNAs measured at each dose studied (Table 1). IL1 β and TNF α mRNA levels in response to IL1 β -treatment were dose-dependent in that the Newman-Keuls tests indicated the levels of these substances were different from each other after at least two treatment doses. IL1 β -induced IL1 β mRNA levels had a U-shaped dose-response curve whereas IL1 β -induced TNF α mRNA levels increased to a maximal level at 10 ng/ml and did not show a further increase with the higher dose (100 ng/ml). IL1 β -induced IL6 mRNA levels in the hypothalamic cell cultures increased dramatically (about 400 fold) at the two lowest doses of IL1 β tested (1 and 10 ng/ml) but were significantly less, about 300 fold, after 100 ng/ml of IL1 β . IL10, IL1 receptor 1, GAD and BDNF mRNAs significantly increased after the low dose of IL1 but no further increases were observed with the higher doses. IL1 β -induced NGF mRNA was higher after the 1 ng/ml dose than after the 100 ng/ml dose.

Cortical cell mRNA responses to rat recombinant IL1 β were distinct from those observed in hypothalamic cultures (Table 2). Thus, at 1 ng/ml, IL1 β failed to enhance IL1 β , GAD and BDNF mRNAs and the increases in IL6 mRNA were substantially less (only 33 fold). At 10 ng/ml IL1 β -induced IL1 β (1.6 fold), TNF α (12 fold), IL6 (24 fold), IL10 (3 fold), IL1R1 (1.7 fold) and NGF (1.6 fold) mRNA levels. IL1 β mRNA levels were significantly increased at 100 ng/ml relative to those after the 10 ng/ml dose as well as to those after the 1.0 ng/ml IL1 β dose (Table 2). Both TNF α and NGF mRNA levels were significantly higher after the 10 and 100 ng/ml dose of IL1 β compared to the 1 ng/ml dose. In contrast, BDNF mRNA levels decreased after the 100 ng/ml dose relative to the 10 ng/ml dose of IL1 β .

The time courses of IL1 β -induced mRNA expression (10 ng/ml) were similar for both the hypothalamic and cortical cultures (Table 3; Figure 1) although some distinct differences between the two tissues were evident. For example, the IL6 mRNA increases after 2 h of IL1 β were about 200 fold in the hypothalamic cultures and only 40 fold in the cortical cultures. For the hypothalamic cultures, IL1 β -induced IL1 β mRNA increased after 2 h, remained significantly increased at 6 h, dropped back towards control levels by 24 h. For the cortical cultures, IL1 β -induced IL1 β mRNA increased two-fold after 2 h and continued to increase to about 8 fold by 24 h. In the hypothalamic cultures (Fig. 1), IL1 β induced TNF α mRNA within 30 min (1.4 fold), TNF α mRNA continued to increase up to 2 h (3.5 fold) and maintained a 2 fold increase up to 24 h. In contrast, in cortical cultures, IL1 β increased TNF α 8 fold at 30 min and 1 h, and 12 fold by 2 h, 18 fold by 6 h and 30 fold at 12 and 24 h (Table 3). In the hypothalamic cultures, IL1 β also significantly increased NGF mRNA levels at each time point sampled, increasing up to 6 h (4.5 fold) and then returning to lower levels although remaining significantly elevated (1.8–3.3 fold). For the cortical cultures, IL1 β increased NGF mRNA by 2 h (1.3 fold) which continued to increase to 3 fold by 6 and 24 h. In both the hypothalamic and cortical cultures, the increase in IL10, IL1 receptor 1 and BDNF mRNA levels only reached significance by 2 h after IL1 β but returned to control levels by 12 h (Table 3). GAD-67 mRNA levels also increased 2 fold by 1 h after IL1 β in the hypothalamic cultures and 1.2 fold by 2 h in the cortical cultures and increased up to 3 fold by 6 h returning to control levels by 12 h in the hypothalamic cultures (Table 3).

In vitro mRNA responses to TNF α treatment

In both the hypothalamic and cortical cultures rat recombinant TNF α induced significant increases in IL1 β , TNF α and IL6 mRNA levels with 1–100 ng/ml (Table 4 & Table 5 respectively). TNF α dose-dependently increased IL1 β and TNF α mRNA levels in both cell types, although in cortical cells, TNF α -enhanced TNF α mRNA levels reached maximal

increases at 10 ng/ml TNF α . In the hypothalamic cultures (Table 4) TNF α dose-dependently increased GAD, IL10, BDNF and NGF mRNA levels. In contrast, in cortical cultures (Table 5) TNF failed to affect GAD and BDNF mRNAs although NGF mRNA increased.

The time courses of gene expression in the hypothalamic cultures after TNF α treatment (Table 6; Figure 1) differed substantially from those observed after IL1 β treatment. In the hypothalamic cultures, TNF α treatment had a rapid effect; it significantly increased IL1 β (56 fold), TNF α (43 fold) and IL6 (5 fold) mRNA levels at 0.5 h. At the 2 h time point, another increase in these transcripts above that observed at the 1 h time point occurred although these increases were not as high as those observed at 0.5 h. However, for TNF α -enhanced IL1 β mRNA levels an additional increase (54 fold) occurred by 24 h. TNF α treatment increased the IL6 mRNA levels up to 450 fold by 2 h and the levels subsided to only 8 fold increases by 24 h. TNF α -enhanced increases in IL10, IL1 receptor1, GAD, BDNF and NGF mRNA levels were observed after 2 h in the hypothalamic cultures (Figure 1, Table 6). TNF α -enhanced increases continued for BDNF and NGF mRNA levels up at 12 h and 24 h.

In the cortical cultures (Figure 1, Table 6), the time courses of TNF α -enhanced expression of transcripts were different from those induced by the IL1 β treatment. After 30 min of incubation with TNF α , the increases were large (30–50 fold) for IL1 β , TNF α and IL6 mRNA levels and a significant decrease (0.3 fold) occurred for IL10 mRNA (Table 6; Figure 1). By 1 h, IL1 β mRNA continued to increase (42 fold) and IL10 mRNA increased by 3 fold, while TNF α and IL6 mRNA levels, although still elevated, decreased to 17 and 23 fold respectively. After 1 h of TNF α treatment, NGF mRNA levels decreased significantly to 0.75 fold. In contrast, by 2 h IL1 β , TNF α and IL6 mRNA levels were about 20 fold higher and IL10, IL1 receptor1 and NGF mRNAs about 2 fold higher (Figure 1; Table 6). After 6 h of TNF α treatment, further mRNA increases, 34 fold for IL1 β , 107 fold for TNF α and 65 fold for IL6 occurred. By 12 h, another subsiding of these transcripts to roughly 20 fold increases occurred (Figure 1). By 24 h, IL1 β and TNF α mRNA levels, increased again (60–80 fold respectively). In contrast to these changes in cytokine mRNA levels, GAD mRNA levels showed a decrease (0.5–0.8 fold) for 6–24 h after TNF α treatment (Table 6).

In vivo RNA responses to IL1 β or TNF α treatment

In vivo human recombinant IL1 β and TNF α also significantly increased IL1 β , TNF α and IL6 mRNA levels in both the hypothalamus and the somatosensory cortex (Table 7). IL1 β icv increased IL1 β mRNA 10 to 15 fold, TNF α mRNA 4 to 9 fold and NGF mRNA 3 to 1.5 fold in the hypothalamus and somatosensory cortex, respectively. IL1 β icv increased IL6 mRNA levels 40 fold in both of these regions but increased IL10 mRNA levels (7 fold) and IL1 receptor (3 fold) only in the hypothalamus. TNF α icv increased IL1 β , TNF α and IL6 mRNA levels 6 to 13 fold in both the hypothalamus and the somatosensory cortex. TNF α icv also increased IL1 receptor 1 mRNA levels (1.8 fold) in the hypothalamus and GAD mRNA in the somatosensory cortex (1.3 fold).

Discussion

Results presented clearly indicate the capacity of both IL1 and TNF to induce themselves, each other and an array of other cytokines and neurotrophins in mixed neuronal and glial cultures as well as *in vivo*. Results also suggest that specific patterns of induction are tissue-dependent since differences in mRNA expressions between hypothalamic and cortical cell cultures and expressions in these tissues *in vivo* were evident. Results also indicate that there are wide variations in magnitude and time course of individual cytokine/neurotrophin mRNA responses to IL1 or TNF. It is difficult to know how the magnitude and timing of changes of one cytokine to another observed *in vitro* manifest physiologically *in vivo* because the microanatomy, *internal milieu*, and dynamic influences such as neural and hormonal inputs differ in the two

conditions. Nevertheless, the *in vitro* results are important because they are likely to reflect general IL1- and TNF-induced response patterns. For example, TNF inductions of itself and IL1 were relatively rapid compared to those induced by IL1; and this result is consistent with prior published *in vivo* results (Churchill et al 2006). The magnitudes of IL6 responses were large both *in vivo* and *in vitro*, although the relative tissue-specific increases differed in the two conditions. Finally, the *in vitro* time courses of expressions are likely indicative of important *in vivo* cytokine dynamics although the exact timing of magnitudes of *in vitro* responses are probably different. For example, several cytokines exhibited two peaks of enhanced expression; the second peak likely resulted from the secondary induction by itself and other cytokines. Such patterns, although currently not ascribed to specific physiological changes *in vivo* because of the difficulty of conducting such studies, are very likely important for dynamic cyclic processes. Regardless of such issues, current results clearly illustrate the richness and complexity of cytokine dynamics and expression in brain tissue.

It is difficult to know if the doses of exogenous cytokine applied either *in vivo* or *in vitro* are physiological doses because both IL1 and TNF act in autocrine, juxtacrine, paracrine and endocrine fashions. For example, local concentrations of these cytokines could be relatively high if they are acting in an autocrine fashion and the concept of concentration is not relevant for juxtacrine signaling mechanisms. Nevertheless, some of the responses observed *in vitro* showed clear dose-response relationships and the *in vivo* doses were chosen because those doses of IL1 and TNF used previously were shown to induce physiological sleep responses (Gemma et al., 1997; Yoshida et al., 2004).

Our interest in IL1-TNF interactions stems from our studies showing their involvement in sleep regulation. Both IL1 and TNF seem to be key components of the physiological sleep homeostat (Krueger et al 2007; Krueger et al., 1995; Krueger and Obal, 1997; Vitkovic et al., 2000; Obal and Krueger, 2003). Consistent with current results, they affect each other's production and inhibition of one attenuates the sleep induced by the other (Takahashi et al., 1999, Churchill et al., 2006). Low doses of IL1 β or TNF α enhance non rapid eye movement sleep (NREMS) (Kapas et al., 1992; Krueger et al., 1984; Shoham et al., 1987; Tobler et al., 1984). Conditions associated with increases in endogenous levels of IL1 β or TNF α , e.g., time-of-day (Floyd and Krueger, 1997; Taishi et al., 1998), excessive food intake (Hansen et al., 1998) or infectious disease (Toth and Krueger, 1988) promote NREMS. In contrast, inhibition of endogenous IL1 β or TNF α , using antibodies or endogenous inhibitors such as their soluble receptors, decreases spontaneous NREMS (Opp and Krueger, 1994; Takahashi et al., 1995a–c, 1996 a,b). Administration of IL1 β or TNF α into the anterior hypothalamus increases NREMS (Alam et al., 2004; Kubota et al., 2002; Terao et al. 1998). IL1 β increases the firing rate of a subpopulation of anterior hypothalamic, sleep-active neurons while it suppresses the firing rate in most of the wake-active neurons in this region (Alam et al., 2004). Other cytokines, such as interleukin-6 and interleukin-10 (Alberti et al., 2003; Hogan et al., 2003; Toth and Opp, 2001; Shearer et al., 2001; Smith et al., 1999) as well as NGF (Kapas et al., 1996) and BDNF (Kushikata et al., 1999), also affect sleep and interact with TNF α and IL1 β . Current results have bearing on these findings to the extent that we demonstrate that both IL1 and TNF have broad-ranging actions on other cytokines; each with distinct time courses of mRNA expression. Further, we show that these actions occur *in vivo* within a time and dose framework that is reasonable to propose their influence physiologically and pathologically.

The cytokines and neurotrophic factors, i.e. IL1 β , TNF, BDNF and NGF, are activity-dependent (Brandt et al., 2001; Fix et al., 2006; Guan et al., 2007). For example, ATP, co-released during neurotransmission, stimulates IL1, TNF, NGF and BDNF release from glia (Mingam et al 2008, Bianco et al 2005, Sanz and DiVirgilio 2000, Liu et al 2000, Domercq et al 2006, Suzuki et al 2004, Inoue 2002, Rathbone et al 1999). These substances are also expressed in somatosensory cortical pyramidal neurons in response to whisker-stimulation

(Brandt et al 2001, Fix et al 2006, Guan et al 2007). We have posited that activity within cortical column neuronal circuits increases production of cytokines and neurotrophic growth factors and these factors in turn initiate localized state changes (Krueger et al 2007). Recently Faraguna et al. (2008) demonstrated that a unilateral microinjection of BDNF into the cortex increases slow wave activity locally, which supports this hypothesis.

NF- κ B is a hetero-dimeric gene transcription factor that influences multiple regulatory and effector genes (Kaltschmidt et al., 1993) including the cytokines and neurotrophic factors. The dephosphorylation of I- κ B and the nuclear translocation of the p65 subunit of NF- κ B may be one of the signaling pathways important in the interaction between the cytokines and neurotrophins (Zampieri and Chao, 2006). NF- κ B also acts as a pivotal mediator of sleep (Borbely and Tobler, 1989; Krueger and Toth, 1994; Krueger and Obal, 1997). Sleep deprivation increases the translocation of NF- κ B into cortical (Chen et al., 1999) as well as the hypothalamic cells (Brandt et al., 2004). Further, the *Drosophila* homologue of NF- κ B, relish, is implicated in sleep regulation in that species (Williams et al 2007). Inhibition of NF- κ inhibits spontaneous NREMS and IL1 β -induced sleep in rats (Kubota et al., 2000). However, loss of the p50 subunit of NF- κ B in mice increases slow wave sleep and increases the homeostatic response to sleep loss (Jhaveri et al., 2006). Since the p50 subunit acts to inhibit the translocation of the p65 subunit, the removal in the knockout mice may result in more translocated p65 to upregulate the sleep promoting substances. Regardless, IL1 β , TNF α and NGF upregulate NF- κ B and NF- κ B induces IL1 β , TNF α and NGF in a positive feedback loop. Such interactions likely reflect some of the dynamics of cytokine expression observed *in vitro* in the current study.

There are multiple interactions between the cytokines and neurotrophic factors in addition to those related to sleep. At an expression level of analysis, microinjection of IL-1 β into the somatosensory cortex increases the number of NGF-immunoreactive cells in layer V near the injection site as well as in the horizontal diagonal band (Yasuda et al., 2007). Other studies also support an interaction between IL-1 β and NGF. For example, NGF expressing cells are mainly found in brain regions, i.e. hippocampus, olfactory bulb and prefrontal cortex that synthesize IL-1 β as shown by *in situ* hybridization (Bandtlow et al., 1990). Further, intrastriatal injection of IL1 β results in a robust increase in NGF protein and mRNA levels in adult rats (Otten et al., 1994). IL-1 induces the synthesis of NGF in astrocytes and microglia *in vitro* and also increases the NGF mRNA levels in the hippocampus after intracerebroventricular injections (Spranger et al., 1990; Heese et al., 1998; Gadiant et al., 1990). Such findings are consistent with the results reported herein to the extent that all these studies, whether physiological or molecular in nature clearly indicate the involvement of these complex interactions in neurobiological processes.

Materials and Methods

Pharmacological agents

Recombinant human IL-1 β and rat TNF α were purchased from R&D Systems (Minneapolis, MN) and reconstituted in sterile physiological saline (0.9% NaCl). In the *in vivo* studies, each dose of human recombinant IL-1 β (2.5 ng/rat) and TNF α (200 ng/rat) was dissolved in sterile phosphate-buffered physiological saline and injected in a volume of 4 μ l. Rat recombinant IL1 β and TNF α were dissolved in sterile phosphate-buffered saline prior to dilution of the doses in the tissue culture media.

Primary culture of fetal hypothalamic and cortical cells

Primary cultures of fetal hypothalamic and cortical cells were prepared according to methods described previously with slight modifications (De et al., 1994). Briefly, mediobasal

hypothalamic or cortical tissues from fetal brains obtained from rats on gestation days 18–21 day were collected in ice cold Hank's balanced salt solution (HBSS), containing 0.1% bovine serum albumin (BSA, pH 7.4). The tissues were washed 2–3 times with HBSS and once with Hepes buffered Dulbecco's Modified Eagle's Medium (DMEM) and then incubated in DMEM for 10–15 min at 37°C. After incubation tissues were dissociated mechanically using 20 G and 22 G needles fixed to a 20 cc syringe. Cells ($4-5 \times 10^6$ cells/flask) were plated in 25 cm² flasks (Corning, MA) previously coated with 100 µg/ml of poly-L-ornithine in 0.15 M borate buffer (pH 8.4). The cells were grown in DMEM with 10% heat-inactivated fetal calf serum (Hyclone Laboratories, Logan, UT) in a humidified atmosphere of CO₂:air (5:95) at 37°C. Two days after cell plating the medium was replaced with serum free DMEM containing serum supplement (1 µM human transferrin, 5 µM insulin, 20 nM progesterone, 100 µM putrescine (a growth factor for cell division; Smith, 1990) and 30 nM sodium selenite (a trace element used in cell proliferation; Zhu et al., 1992). Thereafter, the medium was replaced every 2 days. The neuronal and glial cells were grown 14 days then treated with IL1β or TNFα at 3 different doses (0, 1, 10 and 100 ng/ml) for 2 h and at 10 ng/ml at 6 different times (0.5, 1, 2, 6, 12 and 24 h). Each group was analyzed in triplicate.

Animals

Male Sprague-Dawley rats (300–350g) obtained from Taconic Farms (Germantown, NY) were implanted with icv cannulae during ketamine-xylazine (87 mg/kg–13 mg/kg)-induced anesthesia. Guide cannulae were inserted into the left lateral cerebral ventricle at 1.4 mm lateral and 2.5 mm posterior to Bregma; placement was determined during implantation by a drop in resistance against inflow of physiological saline. The guide tube was cemented in place with dental acrylic (Duz-All, Coralite Dental Products, Skokie, Ill.). Animals were given one day to recover from surgery, before testing the cannulae placement by a functional test using angiotensin II administration (40 ng, icv). If the cannulae was placed in the lateral ventricle, angiotensin II elicited a drinking response (Epstein et al. 1970). Data from only those animals that showed an appropriate drinking response were used.

Rats were housed individually and kept on a 12:12-h light-dark cycle with an ambient temperature of $23 \pm 1^\circ\text{C}$. Food and water were available ad libitum. Seven days after surgery the rats were habituated to handling and icv injections by injecting physiological saline (4 µl) icv at 1 h after light onset for two subsequent days. The rats were divided into three groups. Group 1 (n=10) was injected with physiological saline; Group 2 (n=10) and group 3 (n=10) were injected with human recombinant IL-1β or TNFα respectively. Two h after injections, the animals were killed, brains were removed and dissected as previously described (Churchill et al., 2006), and the brain regions were quickly frozen in liquid nitrogen then stored at -70°C .

Isolation of RNA and cDNA preparation

Total RNA was extracted from either cells grown *in vitro* or from dissected brain by using Trizol reagent according to the manufacturer's protocol (Gibco BRL Rockville, MD). Briefly, tissue samples were homogenized in Trizol® reagent (2 ml for cortex and 1 ml for hypothalamus tissue) on ice. After centrifugation, the aqueous phase from each sample was collected with the addition of 200 µl of chloroform for every one ml Trizol®. After isopropanol precipitation, the RNA pellets were washed twice with 75% ethanol, air-dried and resuspended in RNase-free water. The samples were then treated with 2–4 U RNase-free DNase 1 and 20 U SuperRNase-In (Ambion Austin, TX) and incubated at 37°C for 1.5 h. DNase inactivation reagent was then added. After two min the samples were centrifuged at 10,000g for 1 min at 4°C and the supernatants collected. RNA concentration was quantified by spectrophotometry at 260 nM. RNA integrity was verified by an electrophoresis on a 1% agarose gel and subsequent visualization.

The synthesis of first stranded cDNA was described in detail previously (Taishi et al., 2001). Briefly, aliquots of RNA (2 µg/4 µl), 0.5 µg oligodT (12–18) and 1 µl of 10 mM dNTPs were incubated at 65°C for 5 min. After chilling on ice, 200U of Superscripts III RNase H reverse transcriptase (RT)) (Gibco BRL Rockville, MD), 2 µl of 0.1M DTT, 4 µl of 25 mM MgCl₂, 1 µl RNase inhibitor and buffer were added to a final volume of 20 µl and incubated at 55°C for 60 min. Then the mixture was heated at 70°C for 15 min and digested with 1 µl of RNase H at 37°C for 20 min. Finally, cDNA was cooled to room temperature and stored at –20°C until further analysis.

Relative quantitative Real-time PCR

Real-time PCR was performed as previously described (Taishi et al., 2001). The PCR reaction mixture (25 µl) contained 5 µl of the diluted cDNA (25 ng total RNA), 12.5 µl of 2x PLATINUM Quantitative PCR SuperMix-UDG (Gibco BRL), 0.25 µl of each SYBR Green (1:1000 dilution) and Fluorescein (1: 1000 dilution) and 0.5 µl of the primers at 10 µM. Primer sequences for IL1R1 mRNA were (F) AGATGACAGCAAGAGGGACAGACC and (R) CCATTCCACTTCCAGTAGACAAGG, and primers for the other mRNAs measured including GAD-67 were published (Churchill et al 2006). The RT-reaction conditions were 3 min at 50°C and 2 min at 95°C to activate uracil DNA glycosylase (UDG) followed by 40 cycles of 15 sec each at 94°C, 58°C and 72°C. 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 during the melt curve analyses the data were not used. At the end of each cycle, the fluorescence emitted by the SYBR Green (threshold cycle –C_t) was measured. All of the reactions of the samples were performed in duplicate or triplicate. Each C_t value was an average of the values obtained from each reaction. The mean of the control C_t values were computed and the ΔC_t values were determined by subtracting the average Cyclophilin A C_t value from control and experimental C_t values. Then gene expression was evaluated using a comparative C_t method (User Bulletin #2 ABI PRISM 7700 sequence detection system, PE Applied Biosystems) using the formula $2^{-(\Delta C_t \text{ for exp from the control mean}) - (\Delta C_t \text{ for con from the control mean})}$.

Statistics

Data are presented as means ± standard error. Statistical analysis of mean values between groups was performed with one-way analysis of variance (ANOVA). When significant variations were found by ANOVA, the group or treatment causing the difference was identified by Newman–Keuls multiple-comparison tests. In all tests, an α level of P < 0.05 was considered statistically significant.

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Figure 1.

Time course of changes in IL1 β , TNF α , IL6, BDNF, and NGF mRNA levels in hypothalamic (HT, left) and cortical (CT, right) cell cultures after treatment with IL1 β (10 ng/ml; open circles) or TNF α (10 ng/ml; closed circles). The legend to the left of each graph illustrates the fold change after TNF α treatment (light grey, dashed line), while the legend to the right illustrates the fold change after IL1 β treatment (bold, solid line).

Dose response to rat recombinant IL1 β for cytokine and neurotrophin mRNA levels in primary hypothalamic cells *in vitro* Data are expressed as mean $\pm$ SEM for 10 isolations.

Table 1

Genes	F(3,44-68)/prob	Dose (ng/ml)			
		0	1	10	100
IL-1 β	14.65 0.0001	1.02 $\pm$ 0.04	1.52 $\pm$ 0.13 [*]	2.03 $\pm$ 0.15 ^{*+}	1.31 $\pm$ 0.09 [^]
TNF α	7.38 0.001	1.03 $\pm$ 0.12	1.98 $\pm$ 0.29 [*]	3.10 $\pm$ 0.61 [*]	3.35 $\pm$ 0.35 ^{*+}
IL6	296.2 0.0001	0.99 $\pm$ 0.09	439.2 $\pm$ 13.8 [*]	419.0 $\pm$ 30.2 [*]	300.1 $\pm$ 10.5 ^{*^+}
IL10	4.97 0.005	1.06 $\pm$ 0.12	1.93 $\pm$ 0.23 [*]	1.62 $\pm$ 0.16 [*]	1.64 $\pm$ 0.12 [*]
IL1RI	33.2 0.0001	0.98 $\pm$ 0.02	2.12 $\pm$ 0.10 [*]	1.85 $\pm$ 0.10 [*]	2.10 $\pm$ 0.10 [*]
GAD	3.42 0.03	1.03 $\pm$ 0.04	2.61 $\pm$ 0.54 [*]	2.24 $\pm$ 0.45 [*]	2.65 $\pm$ 0.43 [*]
BDNF	7.44 0.0005	1.01 $\pm$ 0.07	1.43 $\pm$ 0.09 [*]	1.45 $\pm$ 0.09 [*]	1.36 $\pm$ 0.05 [*]
NGF β	21.57 0.0001	1.03 $\pm$ 0.08	3.36 $\pm$ 0.29 [*]	2.69 $\pm$ 0.18 [*]	2.70 $\pm$ 0.24 ^{*+}

* p<0.05, using a one-way ANOVA and Newman-Keuls Multiple-Comparison. Significant at alpha = 0.05
comparison with 0ng/ml (*)
comparison with 1ng/ml (+)
comparison with 10ng/ml (^)

Dose response to rat recombinant IL1 β of cytokine and neurotrophin mRNA levels in primary cortical cells *in vitro*. Data are expressed as mean $\pm$ SEM for 10 isolations.

Table 2

Genes	F(3,36-51)/prob	Dose (ng/ml)			
		0	1	10	100
IL-1 β	6.01 0.01	0.98 $\pm$ 0.16	1.17 $\pm$ 0.09	1.62 $\pm$ 0.21 [*]	2.58 $\pm$ 0.48 ^{*+^}
TNF α	31.43 0.0001	1.0 $\pm$ 0.08	7.11 $\pm$ 0.47 [*]	12.34 $\pm$ 1.05 ^{*+}	10.27 $\pm$ 0.97 ^{*+}
IL6	10.57 0.0001	1.02 $\pm$ 0.05	32.63 $\pm$ 5.30 [*]	28.73 $\pm$ 4.15 [*]	44.23 $\pm$ 7.86 [*]
IL10	5.33 0.004	1.00 $\pm$ 0.11	2.22 $\pm$ 0.31 [*]	1.96 $\pm$ 0.27 [*]	2.43 $\pm$ 0.26 [*]
IL1RI	6.4 0.0001	1.08 $\pm$ 0.08	1.51 $\pm$ 0.08	1.68 $\pm$ 0.15 [*]	1.78 $\pm$ 0.14 [*]
GAD	2.76 0.056	0.98 $\pm$ 0.03	1.09 $\pm$ 0.11	1.21 $\pm$ 0.11	0.88 $\pm$ 0.04 [^]
BDNF	5.0 0.01	1.01 $\pm$ 0.04	1.10 $\pm$ 0.03	1.13 $\pm$ 0.03	0.86 $\pm$ 0.09 ^{+^}
NGF β	5.57 0.003	1.03 $\pm$ 0.03	1.01 $\pm$ 0.06	1.28 $\pm$ 0.09 ^{*+}	1.34 $\pm$ 0.09 ^{*+}

* p<0.05, using a one-way ANOVA and Newman-Keuls posthoc analyses. Significant at alpha = 0.05
comparison with 0ng/ml (*)
comparison with 1ng/ml (+)
comparison with 10ng/ml (^)

Table 3

Time course of rat recombinant IL1 β (10 ng/ml) on cytokine and GAD-67 mRNA levels in the primary cortical (CT) and hypothalamic (HT) cultures. Data is expressed as mean $\pm$ SEM for 10 isolations.

Cells	Genes	F(6,63).... P<	Time (h)	0.5	1	2	6	12	24
HT	IL10	2.16 0.06	1.04 $\pm$ 0.08	1.15 $\pm$ 0.14	2.59 $\pm$ 0.61	2.33 $\pm$ 0.42*	1.47 $\pm$ 0.26	1.57 $\pm$ 0.38	1.36 $\pm$ 0.20
	IL1RI	1.02 0.05	1.02 $\pm$ 0.05	1.06 $\pm$ 0.04	1.15 $\pm$ 0.02	1.66 $\pm$ 0.13*	1.34 $\pm$ 0.03*	0.97 $\pm$ 0.07	1.00 $\pm$ 0.04
	GAD-67	5.52 0.001	1.04 $\pm$ 0.05	1.14 $\pm$ 0.06	2.27 $\pm$ 0.11*	2.53 $\pm$ 0.42*	2.98 $\pm$ 0.11*	1.38 $\pm$ 0.07	1.32 $\pm$ 0.01*
CT	IL10	4.17 0.002	1.09 $\pm$ 0.10	0.97 $\pm$ 0.24	3.77 $\pm$ 1.23	1.75 $\pm$ 0.32*	1.13 $\pm$ 0.09	1.9 $\pm$ 0.33	1.25 $\pm$ 0.31
	IL1RI	7.17 0.0001	1.02 $\pm$ 0.04	1.03 $\pm$ 0.13	1.72 $\pm$ 0.18	1.55 $\pm$ 0.170*	2.01 $\pm$ 0.26*	1.79 $\pm$ 0.29	1.99 $\pm$ 0.11*
	GAD	2.46 0.034	1.03 $\pm$ 0.03	1.10 $\pm$ 0.07	1.25 $\pm$ 0.17	1.23 $\pm$ 0.08	0.94 $\pm$ 0.08	0.92 $\pm$ 0.18	1.65 $\pm$ 0.39

* p<0.05, comparing IL1 β to no treatment at each time point using a one way ANOVA and Newman-Kuels posthoc test.

Table 4

Dose response to rat recombinant TNF α for cytokine and neurotrophin mRNA levels in primary hypothalamic cells *in vitro*. Data is expressed as mean $\pm$ SEM for 10 isolations.

Genes	F(3,44-68)/prob	Dose (ng/ml)			
		0	1	10	100
IL-1 β	19.8 0.0001	1.02 $\pm$ 0.05	22.96 $\pm$ 2.4 [*]	40.65 $\pm$ 6.09 ^{*+}	52.85 $\pm$ 7.48 ^{*+}
TNF α	11.2 0.001	1.03 $\pm$ 0.04	2.96 $\pm$ 0.73 [*]	3.66 $\pm$ 0.31 [*]	5.37 $\pm$ 0.71 ^{*+^}
IL6	41.99 0.0001	0.99 $\pm$ 0.08	19.62 $\pm$ 1.68 [*]	176.2 $\pm$ 25.6 ^{*+}	219.2 $\pm$ 21.9 ^{*+}
IL10	7.99 0.001	1.06 $\pm$ 0.12	1.67 $\pm$ 0.31	1.91 $\pm$ 0.14 [*]	2.7 $\pm$ 0.31 ^{*+^}
IL1RI	9.66 0.001	0.98 $\pm$ 0.05	1.38 $\pm$ 0.19 [*]	1.74 $\pm$ 0.07 [*]	1.92 $\pm$ 0.16 ^{*+}
GAD	5.19 0.01	1.03 $\pm$ 0.44	1.40 $\pm$ 0.19	2.45 $\pm$ 0.42 ^{*+}	2.03 $\pm$ 0.30 [*]
BDNF	5.95 0.01	1.01 $\pm$ 0.07	1.20 $\pm$ 0.08	1.40 $\pm$ 0.07 [*]	1.70 $\pm$ 0.21 ^{*+}
NGF β	10.71 0.0001	1.03 $\pm$ 0.08	1.28 $\pm$ 0.10	1.49 $\pm$ 0.15 [*]	1.93 $\pm$ 0.13 ^{*+^}

* p<0.05, using a one-way ANOVA and Newman-Keuls posthoc analyses. Significant at alpha = 0.05

comparison with 0ng/ml (*)

comparison with 1ng/ml (+)

comparison with 10ng/ml (^)

Table 5

Dose response to rat recombinant TNF α for cytokine and neurotrophin mRNA levels in primary cortical cells *in vitro*. Data is expressed as mean $\pm$ SEM for 10 isolations.

Genes	F(3,36-51)/prob	Dose (ng/ml)			
		0	1	10	100
IL-1 β	7.26 0.001	1.03 $\pm$ 0.14	27.19 $\pm$ 6.15 [*]	25.90 $\pm$ 5.22 [*]	40.87 $\pm$ 7.74 [*]
TNF α	25.31 0.0001	1.02 $\pm$ 0.10	10.93 $\pm$ 1.01 [*]	19.64 $\pm$ 2.19 ^{*+}	24.85 $\pm$ 3.03 ^{*+}
IL6	7.24 0.0005	1.29 $\pm$ 0.37	34.99 $\pm$ 8.58 [*]	20.92 $\pm$ 3.33 [*]	29.68 $\pm$ 5.46 [*]
IL10	4.47 0.02	1.05 $\pm$ 0.1	2.43 $\pm$ 0.4 [*]	2.42 $\pm$ 0.38 [*]	2.35 $\pm$ 0.3 [*]
IL1RI	2.3 0.09	1.06 $\pm$ 0.09	1.36 $\pm$ 0.18	1.75 $\pm$ 0.25 [*]	1.49 $\pm$ 0.15 [*]
GAD	1.32 0.27	0.99 $\pm$ 0.03	0.93 $\pm$ 0.03	0.98 $\pm$ 0.05	1.04 $\pm$ 0.03
BDNF	0.98 0.41	1.12 $\pm$ 0.17	1.01 $\pm$ 0.10	1.28 $\pm$ 0.15	1.26 $\pm$ 0.10
NGF β	35.81 0.0001	1.02 $\pm$ 0.09	1.85 $\pm$ 0.30 [*]	2.20 $\pm$ 0.28 [*]	4.08 $\pm$ 0.21 ^{*+^}

* p<0.05, using a one-way ANOVA and Newman-Keuls posthoc analyses. Significant at alpha = 0.05
 comparison with 0ng/ml (*)
 comparison with 1ng/ml (+)
 comparison with 10ng/ml (^)

Table 6

Time course of rat recombinant TNF α treatment (10 ng/ml) on cytokine and GAD mRNA levels in the primary cortical (CT) and hypothalamic (HT) cultures. Data is expressed as mean $\pm$ SEM for 10 isolations.

		Time (h)							
Cells	Genes	F(6,63).... P<	0	0.5	1	2	6	12	24
HT	IL10	7.51 0.0001	1.09±0.06	1.02±0.22	1.35±0.41	2.12±0.28*	1.54±0.28	9.92±2.10*	9.95±2.99
	IL1RI	3.3 0.005	1.05±0.04	0.88±0.06	0.87±0.10	1.54±0.10*	0.85±0.08	2.94±0.82	2.24±0.54
CT	GAD-67	4.61 0.0001	1.04±0.05	0.87±0.11	1.54±0.11	2.34±0.36*	1.64±0.16*	1.20±0.09	1.24±0.10
	IL10	18.96 0.0001	1.04±0.05	0.87±0.11	1.54±0.11	2.34±0.36*	1.64±0.16*	1.20±0.09	1.24±0.10
	IL1RI	32.2 0.0001	1.02±0.06	1.02±0.15	1.07±0.35	1.53±0.14*	4.30±0.38*	1.61±0.11	1.29±0.24
	GAD	17.1 0.0001	1.01±0.03	0.94±0.01	1.04±0.01	0.98±0.03	0.82±0.02*	0.82±0.02*	0.46±0.02*

* p<0.05 comparing TNF α treatment with no treatment at each time point using repeated measures ANOVA and Newman-Keuls posthoc analyses

In vivo cytokine and neurotrophin mRNA levels in the hypothalamus or SSctx 2h after icv injection of saline, human recombinant IL1β (25 ng) or human recombinant TNFα (200 ng). Data is expressed as mean ± SEM for 10 rats in each group.

Table 7

Gene	Treatment/Hypothalamus			Treatment/SSctx		
	Saline	IL1β	TNFα	Saline	IL1β	TNFα
IL1β	2.91±1.31	14.66±4.90*	8.11±1.26*	1.19±0.23	11.30±3.43*	12.80±1.27*
TNFα	1.77±0.75	8.92±2.13*	7.98±0.85*	0.92±0.11	4.46±0.87*	8.19±1.27*
IL6	1.38±0.28	38.3±10.95*	11.05±2.49*	1.41±0.26	40.78±12.00*	6.61±0.70*
IL10	1.21±0.29	7.11±2.51*	2.00±0.88	0.93±0.06	1.74±0.36	2.25±0.51
IL1R1	1.10±0.17	2.69±0.43*	1.84±0.21*	0.98±0.21	1.19±0.18	1.10±0.12
GAD	1.10±0.13	1.37±0.09	1.42±0.09	1.02±0.06	1.10±0.09	1.33±0.07*
BDNF	1.02±0.08	1.06±0.07	1.00±0.08	1.02±0.08	1.06±0.07	1.00±0.08
NGFβ	1.10±0.17	2.94±0.68*	1.61±0.29	1.04±0.11	1.5±0.16*	1.25±0.14

* p<0.05 comparing icv injected treatment with saline using a one-way ANOVA and Newman-Keuls posthoc analyses