DIURNAL VARIATIONS OF INTERLEUKIN-1b mRNA AND b-ACTIN mRNA IN RAT BRAIN

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ABSTRACT

Interleukin-1β (IL-1β) is posited to play an important physiological role in brain functions in addition to its better defined role in pathology. The experiments described herein were performed to determine if IL-1β mRNA and β-actin display diurnal rhythms in various areas of brain. Rats were sacrificed at 4-h intervals across a 12:12 h light/dark cycle. Hypothalamic, hippocampal, and cortical IL-1β mRNA peaked just after lights were turned on, declined slightly during the remaining light period and stayed low in the dark. There were no significant changes in IL-1β mRNA in brain stem or cerebellum samples. β-actin mRNA levels were relatively constant across the day in the hypothalamus, brain stem and cerebellum. However, β-actin mRNA levels were lower during the day than during the night in the hippocampus and cortex.

INTRODUCTION

Interleukin-1β (IL-1β) is constitutively expressed in many tissues (reviewed Krueger and Majde, 1994). There is considerable evidence that IL-1 expression is upregulated during pathology, *e.g.*, during infectious disease (reviewed Dinarello, 1994), after brain injury (Fan et al., 1995; Phelps et al., 1995; Woodroofe et al., 1991), and during local inflammation (Dinarello, 1994). In contrast, the extent of evidence implicating IL-1 in physiological functions is more limited; it is derived primarily from data implicating IL-1β in sleep regulation (reviewed Krueger and Majde, 1994). IL-1β can be detected in a variety of physiological compartments such as plasma (Cannon and Kluger, 1983) and milk (Munoz et al., 1987). Plasma levels of IL-1β are reported to peak at the onset of sleep in humans (Moldofsky et al., 1986) and increase during sleep deprivation (Moldofsky et al., 1989). Further, the ability of circulating monocytes to produce IL-1 also peaks at sleep onset (Uthgenannt et al., 1995). However, sleep is a central nervous system phenomenon and it is thus likely that central IL-1β is the important IL-1

compartment involved in sleep regulation. IL-1β is constitutively expressed in brain; it is found in several areas including the hypothalamus and hippocampus both of which are involved in sleep regulation (Brandtlow et al., 1990; Breder et al., 1988; Lechan et al., 1990). Hypothalamic IL-1β mRNA is upregulated during sleep deprivation (Mackiewicz et al., 1996) as well as by several pathological conditions which also are associated with sleep responses (reviewed Krueger and Majde, 1994), [*e.g.*, brain injury (Fan et al., 1995), systemic LPS injection (Gabellec et al., 1995)]. IL-1β mRNA and IL-1β immuno-like activity are found in both neurons and glia (reviewed Krueger and Majde, 1994). Further, brain IL-1β seems to play a role in normal brain development (reviewed Merrill, 1992).

Considerable evidence supports the notion that IL-1β is a key component in the humoral regulation of sleep. Injections of IL-1β into rats (Opp et al., 1991; Tobler et al., 1984), rabbits (Krueger et al., 1984), mice (Fang et al., 1996), or cats (Susic and Totic, 1989) enhances sleep. Inhibition of IL-1β using either the IL-1 receptor antagonist (Opp and Krueger, 1994a), anti-IL-1β antibodies (Opp and Krueger, 1994b), or the IL-1 soluble receptor (Takahashi et al., 1995) reduces spontaneous sleep. Further, inhibition of IL-1β attenuates sleep responses induced by sleep deprivation (Opp and Krueger, 1994a; Opp and Krueger, 1994b; Takahashi et al., 1995), bacterial products (Takahashi et al., 1996) or mild increases in ambient temperature (Takahashi, unpublished). Cerebrospinal fluid levels of IL-1 activity vary in phase with sleep-wake cycles in cats (Lue et al., 1988). Finally, knockout mice lacking the IL-1 type I receptor sleep less than strain controls (Fang et al., in press). Collectively, these data support the hypothesis that IL-1β is involved in physiological sleep regulation.

Sleep regulation is often conceptionalized as being composed of two processes (Borbély and Tobler, 1989). A homeostatic process which reflects the common experience that the longer one is awake the more tired one becomes. The second process is dependent on circadian rhythms and is exemplified by the experience that it is easier to fall asleep at certain times of the day. Both processes are influenced by humoral agents and indeed it is posited that brain levels of sleep-promoting substances should be higher just prior to or during sleep than during wakefulness. In rats these processes interact producing a sleep-wake cycle characterized by most of daylight hours being occupied by sleep whereas during the night sleep is considerably less. It was, therefore, of interest to determine if IL-1β gene expression varied across the 24 h light-dark cycle. We now report that there are diurnal variations of IL-1β mRNA content in the hypothalamus, hippocampus and cerebral cortex, but not other areas of brain.

MATERIALS AND METHODS

The RNA extraction, cDNA preparation, PCR amplification and gel electrophoresis of PCR products were described previously in detail (Bredow et al., submitted; Bredow et al., in press; Bredow et al., 1994). Briefly, RNA was extracted from each tissue using RNA STAT-60^{TM}; its integrity was checked using formaldehyde-containing agarose gel electrophoresis and total amount in each sample determined by optical density at 260 nm. First stranded cDNA was synthesized using Superscript^{TM} II RNase H reverse transcriptase (RT) according to the manufacturers instructions with the following modifications. One μg of total RNA was incubated with 0.75 μg oligo$(dT)_{15}$ for 90 min at 42°C. The reaction was terminated by heating to 95°C for 10 min, followed by 1 min of centrifugation at maximum speed at

room temperature using a table top centrifuge. cDNA was stored frozen until further use. The cDNA samples were amplified by PCR in a volume of 50 µl. IL-1β sense (5'GACCTGTTCTTTGAGGCTGAC3') and anti-sense (5'TTCATCTCGAAGCCTGCAGTG3') primers were purchased from Bio-Synthesis, Inc. (Lewisville, TX). To remain in the linear range during PCR amplification 37 cycles were carried out at annealing temperatures of 60°C for 45 s (Fig. 1). In a separate experiment using cortical cDNA samples and the same PCR amplification procedure, the IL-1β PCR product was sequenced by The University of Tennessee Molecular Resource Center the expected IL-1β PCR product was obtained. In another control experiment genomic DNA was amplified by PCR using these primers; a 2.6 kbp product was obtained. In contrast, the RT-PCR product is 330 bp thus indicating that mRNA was amplified. This control is necessary since the genomic structure of rat IL-1β is unknown; primers amplify across a region which in mouse DNA contains an intron. Primers used for β-actin were those previously described and PCR amplification was for 37 cycles (Bredow et al., 1994). The β-actin PCR product was analyzed by restriction analysis followed by Southern blotting to confirm its identity as previously described (Bredow et al., 1994). PCR amplification of IL-1β cDNA and β-actin cDNA were performed separately; aliquots from the separate reaction products were mixed before gel electrophoresis. The mixed PCR products were loaded onto 2% agarose gels containing ethidium bromide. After electrophoresis, gels were washed for 3-6 h depending upon the ethidium bromide background in water then photographed under UV light. The photographic negative were used for densitometric analyses using NIH Image 1.54 for 1-D gels. All materials used were those previously described (Bredow et al., in press). The brain samples used in this study were also used in two previous studies in which different mRNAs were examined (Bredow et al., submitted; Bredow et al., in press).

Male Sprague-Dawley rats (320-350 g) were acclimated to housing conditions [12:12 hour (h) light/dark cycle (lights on at 08:00 h) at 25°C] for 10 days prior to sacrifice. Rats were sacrificed every 4 h beginning 1 h after light onset; thus, samples taken at 09:00, 13:00, and 17:00 h were during daytime h, whereas samples taken at 21:00, 01:00, and 05:00 h were taken at nighttime. Four rats were sacrificed at each time point then the experiment was repeated on a separate day resulting in a total of 8 rats for each of the 6 time points. Rats were decapitated and their brains removed within 1-2 minutes (min) after opening the door of their environmental cage. The hypothalamus, hippocampus, cerebral cortex, brain stem and cerebellum were dissected as previously described (Bredow et al., in press) and individual brain samples frozen in liquid N_2 within 5 min of removal of rats from their cages. RNA extraction and subsequent cDNA synthesis was done separately for each rat and area of brain. Each of the total of 240 cDNA samples (hypothalamus, hippocampus, cortex, brain stem and cerebellum, from each of 8 rats for each of 6 time points) was subjected to at least two separate PCR amplifications. For each brain area samples from 4 rats for each time point were processed together; samples from the other 4 animals at each time point were processed separately in an identical manner. The resulting PCR products (from 4 rats, one area of brain, 6 time points; n = 24) were loaded onto a single gel (each PCR product was subjected to gel electrophoresis twice). To compare values between gels, normalized values within each gel were first calculated. Densitometric values of each individual band were divided by the mean of the 4 densitometric values obtained from the 4 samples from the 21:00 h samples then multiplied by 100. Therefore, from each gel the normalized values from 4 rats for one area of brain for each of the 6 time points were obtained. On a separate day another PCR and gel were run for each sample in the identical manner. The normalized values from each of the 2 gels for each brain area and time point were averaged

to yield one value representing one area of brain and time point for each rat. Thus, for each area of brain and time point 8 values; (each representing a mean of 2 PCRs from 2 gels and each normalized to 21:00 h values) were used to calculate group means. Data were subjected to one-way ANOVA followed by Student-Newman Keuls (SNK) multiple comparison test for statistical analyses. If the data failed on normality the Kruskal-Wallis method was used. For comparisons of daylight and dark period samples the Students' *t*-test was used. An alpha level of $p < 0.05$ was accepted.

RESULTS

The IL-1β mRNA PCR product was found in all areas of brain, *e.g.*, Fig. 2. The sequence of the PCR product obtained was determined and it corresponded to the appropriate 330 base pair in IL-1β mRNA. Further, the 330 base pair PCR product has the appropriate electrophoretic mobility (Fig. 2). The β-actin 764 base pair PCR product was not sequenced but it also had the appropriate electrophoretic mobility (Fig. 2).

The relative levels of IL-1β mRNA had significant diurnal variations in the hypothalamus [$H(5) = 13.1$, $p = 0.02$], hippocampus [$F(5,41) = 4.42$, $p = 0.003$] and cerebral cortex [$H(5) = 14.8$, $p = 0.01$] but not the brain stem [$F(5,41) = 0.79$, $p = 0.56$] or cerebellum [$F(5,43) = 1.88$, $p = 0.12$]. Peak values of IL-1β mRNA occurred 1 hour after lights were turned on (09:00 h) in the hypothalamus, hippocampus and cortex; they remained elevated, though decreasing slightly, during the remaining two times at which samples were taken during daylight hours (13:00 and 17:00 h) (Fig. 3). During the nighttime relative amounts of IL-1β mRNA were lower than those found during the day and remained relatively constant (Fig. 3). In the brain stem and cerebellum the patterns of the abundance of IL-1β mRNA observed in hypothalamus, hippocampus and cortex were not observed (data not shown). The pooled values obtained during the daylight period (09:00, 13:00 and 17:00 h) for hypothalamus, hippocampus and cortex IL-1β mRNA were significantly higher than values obtained during the dark period (21:00, 01:00 and 05:00 h) (Fig. 4). Similar values obtained for the brain stem and cerebellum were not significantly different.

Relative levels of β-actin also had significant variations in the hippocampus and cortex [$H(5) = 22.8$, $p = 0.0004$] [$H(5) = 17.3$, $p = 0.004$] but not in the other areas of brain examined (Fig. 3). In contrast to IL-1β mRNA, values of β-actin were lower during the day and higher during the night in both cortex and hippocampus (Fig. 3). The pooled values obtained during the daylight period for hippocampal and cortical β-actin mRNA were significantly less than the corresponding nighttime values (Fig. 4). Pooled daytime and nighttime values for the hypothalamus, brain stem and cerebellum were not significantly different.

DISCUSSION

Current results confirm and extend previous findings demonstrating constitutive expression of IL-1β mRNA in normal brain. The primary contribution of this study is the finding that there is a diurnal variation of the amount of IL-1β mRNA in the hypothalamus, hippocampus and cortex but not in the brain stem and cerebellum. This result constitutes the first demonstration of physiological variation of

IL-1β mRNA in brain; they are consistent with a previous result showing a sleep cycle-dependency of IL-1 bioactivity in cerebrospinal fluid (Lue et al., 1988). Current results are also consistent, in part, with those of Mackiewicz, *et al.* (1996), who showed that 24 h of sleep deprivation induced an increase in hypothalamic and brain stem IL-1β mRNA expression. In that study IL-1β mRNA levels did not change in the thalamus or cerebral cortex after sleep deprivation. The current study extends the Mackiewicz study by reporting values for hippocampal IL-1β mRNA. The very first study of the distribution of IL-1β mRNA in brain (Farrar et al., 1987) and many subsequent studies (reviewed Krueger and Majde, 1994) demonstrated clear staining for IL-1β mRNA in the hippocampus. Further, the hippocampus has a role in sleep regulation. The Mackiewicz, *et al.* study failed to find differences in IL-1β mRNA in free-funning rats in any brain area in samples taken 2 h before the onset of expected running activity and samples taken 2 h after the onset of running activity. Although the amount of sleep occurring in those rats at those times was not determined, in rats kept on a 12 h light/dark cycle the amount of sleep occurring 2 h before lights are turned off (the beginning of activity) is roughly equal to the amount of sleep occurring 2 h after lights are turned off. In our studies we chose to sacrifice animals 1 h after lights were turned on (maximum activity) and 1 h after lights were turned off (maximum sleep); maximum IL-1 mRNA levels occurred 1 h after lights were turned on.

It is difficult to demonstrate that changes in IL-1β or other putative sleep regulatory substances are specific for sleep since all these substances thus far identified have multiple biological activities (reviewed Krueger and Majde, 1994). For example, prolonged wakefulness is often considered a stressor and IL-1β expression is enhanced under stressful conditions and activates the glucocorticoid axis (Berkenbosch et al., 1987; Shintani et al., 1995). Nevertheless, the Mackiewicz, *et al.* study showing a sleep deprivation induction of IL-1β mRNA was very well controlled by including yoked control rats. These animals were subjected to the identical procedures as the sleep-deprived rats, except they were allowed to sleep at will. Current results are consistent with their interpretation that changes in IL-1 mRNA are linked, in part, to changes in sleep rather than stress since we found that IL-1β mRNA levels are higher during the sleep period (daytime) than during the waking period. Regardless, there are many other activities that are incompatible with sleep and/or sleep deprivation or are altered by sleep that also are affected by IL-1β; *e.g.*, eating, sex and body temperature. Unfortunately, these activities are also regulated by the hypothalamus and thus limitations are placed on the interpretation of current data as to the specificity of changes in IL-1β mRNA for sleep. Additional approaches in which sleep is varied and IL-1β mRNA expression measured are needed to clarify the issue of sleep specificity.

The strongest evidence suggesting that sleep is regulated, in part, by humoral agents comes from experiments in which cerebrospinal fluid (CSF) from sleep-deprived animals was transferred to normal recipient animals. Several independent laboratories, over a 70 year period, have shown that animals receiving CSF from sleep-deprived animals sleep more than those receiving CSF from control animals (reviewed Krueger and Majde, 1994). The general idea envoked by the humoral regulation of sleep hypothesis is that the humoral agents are upregulated by waking activity. The subsequent increase in their concentration induces an increase in the probability of sleep occurring. It is visualized that their actions would be on those circuits and transmitters systems responsible for sleep. Indeed, IL-1β affects several neuronal transmitter systems implicated in sleep-wake regulation including serotonin (Gemma et al., 1991; Grazie de Simoni et al., 1995; Palazzolo and Quadri, 1992), histamine (Kang et al., 1995),

acetylcholine (Sawada et al., 1992), GABA (Miller et al., 1991), glutamate, glutamine (Bianchi et al., 1995), and norepinephrine (Shintani et al, 1995; Terao et al., 1995; reviewed Plata-Salaman, 1991). Further, IL-1β alters activity of hypothalamic (Shibata, 1990) and hippocampal (Plata-Salaman and Ffrench-Mullen, 1994) neurons. Nevertheless, how the brain is organized to produce sleep remains unknown; in fact, the minimal component of brain capable of sleep is not known (reviewed Krueger et al., 1995).

In the current studies we used PCR to amplify signals so they could be detected. This method and the associated densitometric analyses of gels are inherently variable. Several approaches have been used to control this variation though all only allow statements about amounts relative to another molecule or relative to the same mRNA at another time or location. One approach is to express the amount of PCR product relative to the amount of PCR product of a housekeeping gene. We started these experiments using that approach and chose β-actin mRNA to compare to IL-1β mRNA. However, β-actin mRNA varied with the time of day and location in brain; this marker was, therefore, unsuitable as a reference. In another study using the same cDNA samples two other substances, α-tubulin mRNA and tumor necrosis factor-α (TNFα) mRNA also varied with the time of day and location in brain (Bredow et al., submitted). The diurnal variations of each of these substances was unique, for example, α-tubulin mRNA increased during the day in the hypothalamus and hippocampus but not in cortex. In contrast, β-actin mRNA levels in the current study were less during the day than during the night in the hippocampus and cortex and did not change in the hypothalamus. The method used in the current study relies upon statistical analyses of a large number of samples and comparing the relative amounts of the same PCR product from samples taken from different areas and times. Confidence in the data is derived from the pattern of expression observed and statistical significance.

IL-1β is only one of several cytokines implicated in sleep regulation; others include: TNF, interferon-α, fibroblast growth factor, and IL-10 (reviewed Krueger and Majde, 1994). Indeed, TNFα mRNA is higher in the hypothalamus and hippocampus, but not cortex during the day than during the night (Bredow et al., submitted). There is no information on the diurnal variation in brain of the other cytokines mentioned. Nevertheless, it is likely that a redundant cytokine network, analogous perhaps to the cytokine network involved in immune cell regulation, is involved in sleep regulation. Regardless of such speculation, current results clearly indicate that IL-1β mRNA has a diurnal variation in the brain.

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FIGURE LEGENDS

Figure 1: The effects of increasing the number of PCR cycles for amplification of IL-1β cDNA on amount of product obtained as determined by relative density. PCR was performed with cDNA samples corresponding to 14 ng RNA of original material. PCR product was subjected to gel electrophoresis as described in Methods. Relative density was obtained by densitometric scanning of the ethidium bromide-stained agarose gel.

Figure 2: Electrophoresis of RT-PCR-assisted amplification of IL-1β and β-actin mRNA from five brain areas of normal rat at 09:00 h. An ethidium bromide-stained agarose gel (2%) is shown: the top band (764 bp) correspond to β-actin the bottom band (330 bp) to IL-1β cDNA. Lane 1 = hypothalamus, Lane 2 = hippocampus, Lane 3 = cortex, Lane 4 = cerebellum, and Lane 5 = brain stem.

Figure 3: Diurnal variation in the relative amounts of IL-1β mRNA and β-actin mRNA in the hypothalamus, hippocampus and cerebral cortex. Data from one 24-h experimental period are double plotted. Mean ± SEM (n = 8) is

shown for each time point. Black bars indicate the dark period. Data for hypothalamic β-actin mRNA were previously published (Bredow et al., submitted); they are reproduced here for comparative purposes.

Figure 4: Daytime and nighttime levels of IL-1β mRNA (top panel) and β-actin mRNA (bottom panel) in normal rat brain. The mRNA levels for IL-1β and β-actin in all areas of brain analyzed [hypothalamus (HT), hippocampus (HC), cerebral cortex (CT), brain stem (BS) and cerebellum (CB)] during the light and dark phases are shown. Since rats sleep mostly during the day, the means of all values from individual rats sacrificed during the day (n = 24) were compared to values from rats sacrificed at night (n = 24). The means ± SEM for the percent deviation from the values obtained at 21:00 h are shown. Statistical significance Students' *t*-test; ($p < 0.05$) between daytime and nighttime samples is indicated by an asterisk.

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