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Interleukin-1β enhances non-rapid eye movement sleep when microinjected into the dorsal raphe nucleus and inhibits serotonergic neurons *in vitro*

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Abstract

Interleukin-1 (IL-1) and IL-1 receptors are constitutively expressed in normal brain. IL-1 increases non-rapid eye movements (NREM) sleep in several animal species, an effect mediated in part by interactions with the serotonergic system. The site(s) in brain at which interactions between IL-1 and the serotonergic system increase NREM sleep remain to be identified. The dorsal raphe (DRN) is the origin of the major ascending serotonergic pathways to the forebrain, and it contains IL-1 receptors. This study examined the hypothesis that IL-1 increases NREM sleep by acting at the level of the DRN. IL-1β (0.25 and 0.5 ng) was microinjected into the DRN of freely behaving rats and subsequent effects on sleep–wake activity were determined. IL-1β 0.5 ng increased NREM sleep during the first 2 h post-injection from 33.5 ± 3.7% after vehicle microinjection to 42.9 ± 3.0% of recording time. To determine the effects of IL-1β on electrophysiological properties of DRN serotonergic neurons, intracellular recordings were performed in a guinea-pig brain stem slice preparation. In 26 of 32 physiologically and pharmacologically identified serotonergic neurons, IL-1β superfusion (25 ng/mL) decreased spontaneous firing rates by 50%, from 1.6 ± 0.2 Hz (before IL-1β superfusion) to 0.8 ± 0.2 Hz. This effect was reversible upon washout. These results show that IL-1β increases NREM sleep when administered directly into the DRN. Serotonin enhances wakefulness and these novel data also suggest that IL-1β-induced enhancement of NREM sleep could be due in part to the inhibition of DRN serotonergic neurons.

Introduction

Although interleukin-1 (IL-1) was originally described as a product of the peripheral immune system, there is now ample evidence that IL-1, IL-1 receptors and the IL-1 receptor antagonist are constitutively expressed in normal brain (Vitkovic *et al*., 2000). IL-1 modulates behaviours such as feeding, sexual behaviour, social exploration, locomotor activity and sleep (Opp & Imeri, 1999). IL-1 consistently has been shown to enhance non-rapid eye movement (NREM) sleep and inhibit rapid eye movement (REM) sleep in several animal species (Terao *et al*., 1998; Opp & Imeri, 1999; Krueger & Fang, 2000). Moreover, central administration of the IL-1 receptor antagonist (Opp & Krueger, 1991) or of antibodies directed against IL-1 reduces spontaneous NREM sleep in normal animals and inhibits the physiological NREM sleep rebound that follows sleep deprivation (Opp & Krueger, 1994a, b). IL-1 mRNA expression in rat brain exhibits diurnal variation with greater levels during the light (rest/sleep) period than during the dark (active) period (Taishi *et al*., 1997) and increases during sleep deprivation (Mackiewicz *et al*., 1996). IL-1 is detected more frequently in plasma samples taken from humans during sleep than during waking (Gudewill *et al*., 1992). IL-1-like activity in cerebrospinal fluid of cats varies in phase with the sleep–wake cycle (Lue *et al*., 1988) and IL-1 plasma levels in humans peak at sleep onset (Moldofsky *et al*., 1986).

Extensive experimental data and clinical observations indicate that brain serotonin (5-hydroxytryptamine; 5-HT) plays a pivotal role in the regulation of many physiological processes and complex behaviours (Jacobs & Azmitia, 1992), including waking and sleep (Jouvet, 1999; Pace-Schott & Hobson, 2002). Specifically, it has been proposed that 5-HT released during wakefulness promotes wakefulness *per se*, and triggers subsequent sleep via sleep-inducing factor(s) (Jouvet *et al*., 1983; Jouvet, 1999).

The IL-1 and serotonergic systems influence each other and exhibit a wide range of overlapping biological activities (Imeri & De Simoni, 1999). IL-1 stimulates 5-HT release in the hypothalamus (Gemma *et al*., 1997) and other brain areas (Imeri & De Simoni, 1999). An intact brain serotonergic system is necessary for the full manifestation of IL-1 effects on NREM sleep. Depletion of brain 5-HT (Imeri *et al*., 1997) or blockade of 5-HT2 receptors (Imeri *et al*., 1999) antagonizes part of IL-1-induced enhancement of NREM sleep. Collectively, these data suggest that IL-1 effects on NREM sleep are mediated, in part, by the serotonergic system (Imeri & De Simoni, 1999).

As IL-1 microinjected into several brain regions (extending from the brain stem to the diencephalon) does not enhance NREM sleep (Walter *et al*., 1989), the site(s) in brain where IL-1 acts to enhance NREM sleep remain to be identified. Observations that IL-1 increases NREM sleep in part through the serotonergic system suggest a neurochemical

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substrate that might mediate IL-1 effects on NREM sleep. Because the dorsal raphe nucleus (DRN) is the origin of the major ascending serotonergic pathways to the forebrain (Jacobs & Azmitia, 1992) and it contains IL-1 receptors (Cunningham & De Souza, 1993; Schöbitz *et al*., 1994), the same observations also suggest that the DRN might represent one brain region that mediates IL-1 effects on NREM sleep.

The present study tested two distinct, yet related hypotheses. First, to test the hypothesis that the DRN represents one brain region where IL-1 acts to enhance NREM sleep, IL-1β was microinjected into the DRN of freely behaving rats and subsequent sleep–wake activity was quantified. Second, to test the hypothesis that the activity of DRN serotonergic neurons is modified by IL-1β, intracellular recordings were obtained from physiologically and pharmacologically identified DRN serotonergic neurons using a guinea-pig brain stem slice preparation and changes induced by IL-1β in firing rate and membrane potential were determined.

Materials and methods

Substances

IL-1β (human recombinant IL-1β expressed in *Escherichia coli*) was purchased from R & D System (Minneapolis, MN, USA). Lyophilized IL-1β was dissolved in pyrogen-free saline containing 0.1% bovine serum albumin, aliquoted, frozen and stored at −80 °C until used. Serotonin and noradrenaline (NA; Sigma, St Louis, MO, USA) used in the *in vitro* experiments were dissolved just prior to use in artificial cerebrospinal fluid (ACSF) of the following composition (mM): NaCl 120, KCl 2.5, KH_2PO_4 1.25, NaH_2PO_4 1.25, $NaHCO_3$ 22, $MgSO_4$ 2, $CaCl_2$ 2, glucose 10 (final pH 7.35) and applied via bath perfusion.

In vivo *experiments*

Animals

Male Sprague–Dawley rats (225–275 g at the time of surgery, Charles River, Calco CO, Italy) were anaesthetized (pentobarbital sodium 40 mg/kg + chloral hydrate 180 mg/kg, intraperitoneally), positioned in a stereotaxic apparatus and surgically prepared for chronic polygraphic recordings. Stainless steel screws served as electroencephalographic (EEG) and ground electrodes after placement over frontal, parietal and occipital cortices. Teflon-coated silver wires were inserted in the neck muscles to record electromyographic activity. A calibrated 30-kΩ thermistor (Omega Engineering, Stamford, CT, USA) was implanted between the dura mater and the skull over the parietal cortex to monitor cortical brain temperature (T_{cort}). A stainless steel guide cannula (length, 1.5 cm; outer diameter, 0.5 mm) was stereotaxically placed 3 mm above the DRN. In order to avoid damage to the midline sinus or to the Sylvius aqueduct, cannulae were implanted at a 30° angle to the sagittal plane. The following stereotaxic coordinates (adapted from Paxinos & Watson, 1986) were used: anterioposterior, +0.7 mm from interaural line; lateral, 3.3 mm; dorsoventral, 3.6 mm below the dura mater (tooth bar, −3.3 mm). Insulated leads were routed from the screws, the electromyographic electrodes, and the thermistor to a Teflon pedestal (Plastics One Inc., Roanoke, VA, USA) that was cemented in place with dental acrylic (Isocryl; Lang Dental Supply, Wheeling, IL, USA). The incision was treated topically with polysporin (polymixin B sulphate – bacitracin zinc) and the animals were placed under heat lamps and monitored until recovery from anaesthesia. On the third postsurgical day, the rats were connected to the recording apparatus (see later) via a flexible tether (connected to the Teflon pedestal) that allowed relatively unrestricted movement within the cage. For the next 5 days the animals were allowed to adapt to the experimental procedures before the experiments began. Rats were always housed individually in environmentally controlled chambers, maintained at 22 ± 1 °C with a 12 : 12 h light–dark cycle. Food and water were available *ad libitum*. Rats were fed a standard rat maintenance diet (Mucedola SRL, Settimo Milanese MI, Italy). All procedures involving animals and their care were conducted in conformity with institutional guidelines that are in compliance with European Union (EEC Council Directive 86/609, OJ L 358,1; 12 December 1987) and Italian (D.L. n.116, G.U. suppl. 40, 18 February 1992) laws and policies, as well as with the United States Department of Agriculture Animal Welfare Act and the United States Public Health Service Policy on Humane Care and Use of Laboratory Animals.

Apparatus and recording

Signals from the EEG and electromyographic electrodes as well as from the thermistor were fed into a Grass (Quincy, MA, USA) polygraph in the adjacent room. The EEG was amplified (factor of 3000) and analog bandpass filtered between 0.1 and 40 Hz (frequency response, ±3 dB; filter frequency roll off, 12 dB/octave). These conditioned signals were digitized with 12-bit precision at a sampling rate of 128 Hz (AT-MIO-64F5; National Instruments, Austin, TX, USA). The digitized EEG waveform, the T_{cort} samples, and integrated values for EMG activity were stored as binary computer files until subsequent analyses. Post-acquisition determination of vigilance state was done by visual scoring of 12-s epochs using custom-written software. The animal's behaviour was classified as wakefulness, NREM or REM sleep. EEG power density values were obtained for each artefact-free 12-s scoring epoch for the frequency range of 0.5–20 Hz. Values in the 0.5–4.0 Hz (delta) frequency range were collapsed and integrated for 12-s epochs, and used as measures of slow wave activity (SWA). These values for SWA were then matched to corresponding 12-s epochs that had already been scored for behavioural state to obtain SWA during NREM sleep.

Experimental protocol

Each animal was injected with both vehicle (pyrogen-free saline containing 0.1% bovine serum albumin) and at least one dose of IL-1β, so each rat served as its own control. No animal received more than three microinjections, one vehicle and two IL-1β. Animals also were studied in two further conditions: after simple handling for the time required by the microinjection procedure and after sham injection, during which an injection needle was inserted, but no microinjection made. Experiments were scheduled randomly with an interval of at least 3 days between microinjections. IL-1β and vehicle were administered 15 min prior to dark onset in a constant volume of 100 nL, using a stainless steel needle (length, 1.8 cm; outer diameter, 0.3 mm), connected via polyethylene tubing to a Hamilton microsyringe and inserted through the guide cannula. The needle extended 3 mm past the tip of the guide cannula, reaching the DRN. Microinjections were performed over a 1-min period. After the microinjection the needle was left in place for 3 min. The highest IL-1β dose microinjected (0.5 ng, 29 fmol) corresponds to one-fifth of the lowest IL-1 dose that effectively enhances NREM sleep when administered intracerebroventricularly into rats (Opp *et al*., 1991; Lancel *et al*., 1996; Imeri *et al*., 1997). IL-1β and vehicle were dissolved, aliquoted and delivered using sterile procedures. Polygraphic recordings began immediately after the microinjections and continued for 12 h.

At the end of the experiments an overdose of chloral hydrate was administered and dye was microinjected (100 nL) into the microinjection site; the brains were then removed and fixed. The position of the dye spot was determined from 40-μm-thick, frozen, neutral-red-stained sections. In seven of 20 animals used in this study the histological examination of the site of injection revealed that the

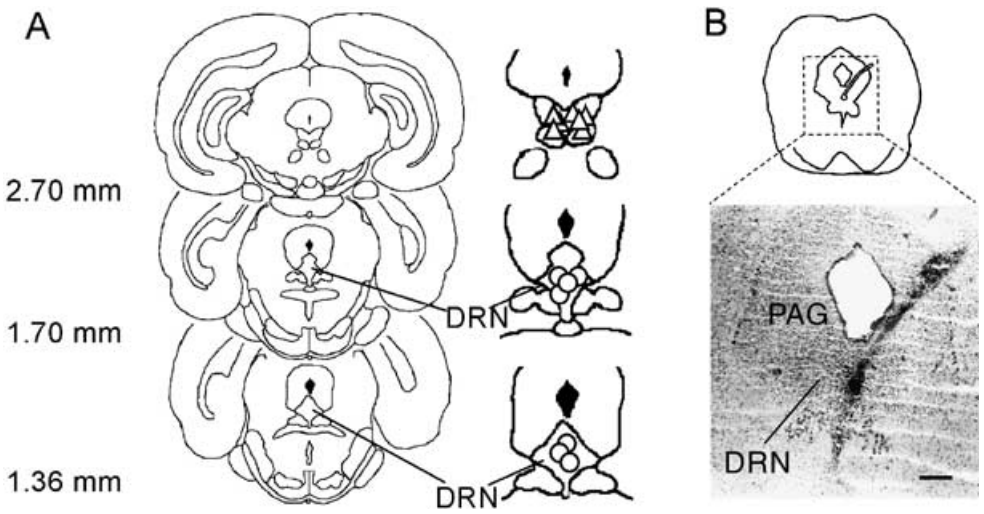

FIG. 1. (A) Schematic representation of location of microinjection sites described in this study. Coronal sections (left) and corresponding and enlarged details of the areas of interest (right) (from Paxinos & Watson, 1986, with modifications). Distances (mm) are from the interaural line. Microinjection sites within the dorsal raphe nucleus (DRN) ($n = 7$) are represented by open circles. Microinjection sites outside and rostral to the DRN ($n = 7$), lying in the region of the oculomotor nucleus, are represented by open triangles. For the sake of clarity of the illustration, injection sites outside the DRN between coronal planes at 1.7 mm and 1.36 mm from the interaural line ($n = 6$) are not represented. (B) Microphotograph and schematic camera lucida drawing of a histological section showing track and site of microinjection into the DRN. Section shown corresponds approximately to the section at 1.7 mm from the interaural line shown in A. PAG, periaqueductal grey matter. Scale bar = 500 μm.

injecting needle was in the DRN (Fig. 1A and B). In the remaining 13 animals the injection sites were located outside the DRN (Fig. 1A).

Statistical analysis

One-way analysis of variance (ANOVA) was used to determine if the experimental variables measured in this study were significantly different between the three control conditions tested (vehicle microinjection vs. needle insertion vs. handling). Dependent variables were: (i) amount of time spent in the different phases of the sleep–wake cycle, (ii) EEG SWA during NREM sleep, (iii) sleep architecture parameters and (iv) T_{cort} values. 'Manipulation' was the main (fixed) effect. An α level of $P < 0.05$ was taken to indicate a statistically significant difference between conditions.

One-way ANOVA was also used to determine if values obtained after administration of IL-1 deviated statistically from values obtained after vehicle microinjection. Experimental variables (see above) were the dependent variables and 'manipulation' (vehicle vs. IL-1 doses) the main (fixed) effect. On the basis of visual inspection of the results, statistical analyses were conducted across 2-h time blocks. An α level of $P < 0.05$ was used to indicate a statistically significant difference between vehicle and IL-1. If ANOVA indicated statistically significant drug main effect, *post hoc* multiple comparisons were made using Fisher's least significant difference.

In vitro *experiments*

Preparation of brain stem slices

Guinea-pig brain slices were chosen for this study because they have been extensively used in the electrophysiological investigation of neurons involved in the regulation of sleep–wake activity and thermoregulatory mechanisms (see, for instance, Ono *et al*., 1987; Shibata & Blatteis, 1991; Xin & Blatteis, 1992; Bal & McCormick, 1993; Leonard & Llinas, 1994; Alonso *et al*., 1996; Khateb *et al*., 1998). Guinea-Pigs (Hartley strain, 150–175 g at time of experiment; Charles River, Italy) were anaesthetized with ether and decapitated. The brain then was removed rapidly and placed in ice-cold (4 °C) ACSF, continuously bubbled with an O_2–CO_2 mixture (95 : 5%). Coronal sections (400 μm thick) were then cut with a Vibratome® from a block of tissue containing the DRN in ice-cold carbogenated ACSF. Two slices were taken from each animal for subsequent *in vitro* recording. The slices were then incubated at room temperature in carbogenated ACSF and were left to recover for at least 1 h. Then a single slice was transferred to a submersion-type slice recording chamber, through which carbogenated ACSF prewarmed to 34 °C was continuously superfused at a rate of 2.5 mL/min.

The DRN was located in the midline of the slice, between the medial longitudinal fasciculi extending dorsally towards the aqueduct. Only sections containing the midline decussating fibres of the superior cerebellar peduncle were selected for use. Intracellular recordings were made throughout the midline and paramidline DRN.

Recording methods and electrophysiological characterization of serotonergic neurons

Neurons were impaled with glass microelectrodes pulled from 1.5-mm filament-containing tubing filled with 2 M potassium-acetate (impedance, 60–80 MΩ). Conventional intracellular recordings were made from DRN neurons using the bridge balance mode on an Axoclamp 2B amplifier. The bridge was frequently checked to ensure that it was balanced throughout experiments. Serotonergic DRN neurons were identified by their distinctive discharge and membrane properties (see *in vitro* results). Only spontaneously active neurons (about 80% of the impaled neurons) were included in the study. Neurons that did not display electrophysiological properties of serotonergic neurons (<10%) were discarded and not further analysed. Because most of the serotonergic DRN neurons display a steady discharge, without sustained pauses or bursts in firing, their mean membrane potential was defined as the membrane potential value measured in the middle of each interspike interval. All neurons that met the electrophysiological criteria described above were further characterized pharmacologically (see *in vitro* results).

In vitro *drug application*

Test substances, dissolved in warmed carbogenated ACSF just prior to use and applied via bath perfusion, as described above, arrived in the bath 2 min after application. Serotonin and NA were applied for 1 min.

IL-1 was applied for 2 min. The dose of IL-1β (25 ng/mL; 1.5 nM) was chosen on the basis of previous *in vitro* electrophysiological studies (Miller *et al*., 1991; Shibata & Blatteis, 1991; Pringle *et al*., 1996; D'Arcangelo *et al*., 1997). The effects of the different drugs were evaluated during intracellular recordings by comparing the mean neuronal discharge frequency and the membrane potential before any treatment (baseline record) and 3 min after their addition to the superfusing ACSF. All data are from neurons that showed recovery upon washout of applied substances.

Data acquisition and analysis

Signals were conditioned through an Axoclamp 2B amplifier (Axon, Foster City, CA, USA), followed online on a paper chart recorder, and continuously acquired onto a VHS tape recorder by means of an Instrutech VR 100B analog–digital converter (Instrutech Corp., Great Neck, NY, USA). The off-line analysis of data was performed with a Macintosh computer equipped with a direct memory access board (Instrutech ITC 16, Instrutech Corp.) and Axograph 4.6 software (Axon). One-way analysis of variance and Student's *t*-test, when appropriate, were used to determine if the electrophysiological variables investigated differed significantly between the three conditions tested (i.e. before, during and following substance application). Statistical differences were considered significant at $P < 0.05$.

Results

In vivo *results*

IL-1β enhanced NREM sleep when microinjected into the DRN

NREM sleep was significantly increased for 2 h after microinjection of 0.5 ng IL-1β into the DRN ($n = 7$) from 33.5 ± 3.7% of recording time (after vehicle) to 42.9 ± 3.0% ($P < 0.05$; Fig. 2, Table 1). This increase in the amount of NREM sleep was due to a significant increase in the number of NREM sleep bouts, from 17.1 ± 1.8 to 29.9 ± 3.8 ($P < 0.05$). EEG SWA during NREM sleep was not modified at any time after microinjection of 0.5 ng IL-1β.

In the first 2 h post-injection, 0.5 ng IL-1β induced a significant decrease in the amount of REM sleep from 9.9 ± 1.5% of recording time to 2.9 ± 1.2% ($P < 0.05$; Fig. 2, Table 1). Amount of REM sleep was also reduced 3–4 h post-injection, from 8.2 ± 1.7% of recording time (control condition) to 1.0 ± 0.6% (Table 1). Owing to these changes in the amount of both NREM and REM sleep, the amount of NREM sleep/total sleep per cent ratio significantly increased from 77.3 ± 3.2 to 93.4 ± 2.5 ($P < 0.01$). In the first 2 h post-injection, 0.5 ng IL-1β also induced a fragmentation of the sleep–wake cycle, with a statistically significant increase in the number of transitions (from one state to another) from 79.6 ± 8.8 to 139.1 ± 15 ($P < 0.01$). Over the same time interval, 0.5 ng IL-1β significantly increased T_{cort} from 37.0 ± 0.1 °C (control condition) to 37.6 ± 0.1 °C (Fig. 2, Table 1). At 3–4 h post-injections, 0.5 ng IL-1β significantly increased T_{cort} from 37.3 ± 0.1 °C (control condition) to 37.9 ± 0.1 °C (Table 2). At the lower dose tested in this study (0.25 ng) IL-1β did not significantly affect sleep–wake activity (Fig. 2, Table 1), EEG SWA during NREM sleep and T_{cort} (Fig. 2, Table 1).

When microinjections missed the DRN (Fig. 1), IL-1β did not alter the amount of NREM sleep (Fig. 2, Table 1) or EEG SWA during NREM sleep irrespective of dose and time. However, 0.5 ng IL-1β microinjected outside the DRN significantly reduced REM sleep 1–2 h post-injection (from 11.2 ± 1.6% after vehicle to 5.6 ± 1.5% of recording time, $P < 0.05$; Fig. 2, Table 1) and 3–4 h post-injection (from 7.9 ± 1.1 to 3.7 ± 1.2%, $P < 0.05$; Table 1). The reduction in amoutn of REM sleep was due to a significant decrease in mean duration of REM bouts (from 1.7 ± 0.2 min to 1.0 ± 0.4 min; $P < 0.05$). T_{cort} was not altered by IL-1β during any of the post-injection time blocks (Fig. 2, Table 1). The lower IL-1β dose tested did not induce any significant change in sleep–wake activity (Fig. 2, Table 1), EEG SWA during NREM sleep and T_{cort} (Fig. 2, Table 1).

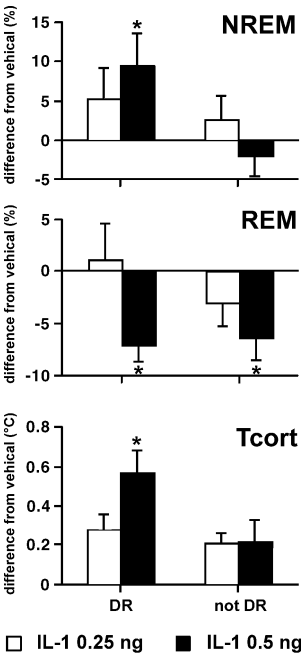

FIG. 2. Interleukin-1 (IL-1) microinjected into the dorsal raphe nucleus (DRN) increased non-rapid eye movements (NREM) sleep. Changes induced in NREM, rapid eye movements (REM) sleep, and brain cortical temperature (T_{cort}) by IL-1 microinjection into the DRN and outside this area (not DRN) during the first 2 h post-injection. REM sleep was inhibited when IL-1 was microinjected both into and outside the DRN. Values are means ± SEM and are expressed as differences from vehicle. $^*P < 0.05$ vs. vehicle.

Sham injection or vehicle microinjection into and outside the DRN did not alter sleep–wake behaviour and T_{cort}

Values for experimental variables did not differ between the three control conditions tested (see above, Materials and methods – Experimental protocol), irrespective of whether the microinjection sites were located inside or outside the DRN (Table 2). The amount of time the rats spent in different vigilance states is consistent with values previously reported during control conditions for Sprague–Dawley rats during the dark phase of the light–dark cycle (Opp, 1997).

In vitro *results*

Serotonergic neurons in the DRN were inhibited by 5-HT and excited by NA

Thirty-five spontaneously active neurons were electrophysiologically characterized as serotonergic on the basis of their distinctive discharge and membrane properties (Williams *et al*., 1988): slow and regular firing rate (range, 0.5–4 Hz; Fig. 3A), large after-hyperpolarization (9–20 mV; Fig. 3A and B), high input resistance (150–400 MΩ), long spike duration (1.5–2.5 ms; Fig. 3B). Because serotonergic neurons have been shown to be inhibited by 5-HT and stimulated by NA (Baraban *et al*., 1978; Vandermaelen & Aghajanian, 1983; Yoshimura & Higashi, 1985; Williams *et al*., 1988), electrophysiologically identified neurons were further characterized pharmacologically. Following 5-HT perfusion, 32 of the 35 neurons either ceased firing (Fig. 3C) or their firing rate was significantly reduced. This reduction in firing rates from 1.4 ± 0.3 Hz before 5-HT perfusion to 0.2 ± 0.1 Hz during

TABLE 1. Effects of interleukin-1β (IL-1β) microinjection into and outside the dorsal raphe nucleus (DRN) on the percentage of recording time spent in vigilance states and on average brain cortical temperature (T_{cort})

	Microinjection into DRN			Microinjection outside DRN		
	NREM	REM	T_{cort}	NREM	REM	T_{cort}
Hours 1–2						
Vehicle	33.5 ± 3.7	9.9 ± 1.5	37.0 ± 0.1	33.2 ± 2.3	11.2 ± 1.6	37.1 ± 0.1
IL-1β 0.25 ng	36.3 ± 3.1	10.7 ± 2.5	37.4 ± 0.1	37.0 ± 2.9	9.7 ± 1.6	37.2 ± 0.1
IL-1β 0.5 ng	42.9 ± 3.0*	2.9 ± 1.2*	37.6 ± 0.1*	30.7 ± 2.2	5.6 ± 1.5*	37.3 ± 0.1
Hours 3–4						
Vehicle	38.4 ± 4.5	8.2 ± 1.7	37.3 ± 0.1	37.1 ± 3.3	7.9 ± 1.1	37.5 ± 0.1
IL-1β 0.25 ng	34.0 ± 2.5	2.6 ± 1.2	37.8 ± 0.1	40.6 ± 3.2	4.3 ± 1.1	37.6 ± 0.1
IL-1β 0.5 ng	40.8 ± 1.9	1.0 ± 0.6*	37.9 ± 0.1*	40.4 ± 3.1	3.7 ± 1.2*	37.7 ± 0.1
Hours 5–6						
Vehicle	34.3 ± 4.9	4.5 ± 1.3	37.4 ± 0.1	40.4 ± 2.8	10.9 ± 1.5	37.4 ± 0.1
IL-1β 0.25 ng	36.6 ± 3.8	6.2 ± 1.6	37.7 ± 0.1	38.1 ± 3.5	5.8 ± 1.3	37.6 ± 0.1
IL-1β 0.5 ng	34.7 ± 4.0	6.6 ± 1.9	37.6 ± 0.1	38.9 ± 3.0	7.5 ± 1.2	37.6 ± 0.1
Hours 7–8						
Vehicle	31.8 ± 5.6	7.2 ± 1.7	37.3 ± 0.1	27.9 ± 2.8	6.4 ± 1.2	37.4 ± 0.1
IL-1β 0.25 ng	24.6 ± 4.1	8.5 ± 2.7	37.4 ± 0.1	34.2 ± 4.2	6.4 ± 1.6	37.4 ± 0.1
IL-1β 0.5 ng	32.9 ± 5.0	10.6 ± 2.4	37.2 ± 0.1	30.1 ± 3.4	9.3 ± 1.4	37.3 ± 0.1
Hours 9–10						
Vehicle	27.1 ± 4.0	3.3 ± 0.8	37.2 ± 0.1	17.5 ± 2.2	2.5 ± 0.7	37.3 ± 0.1
IL-1β 0.25 ng	22.6 ± 4.1	3.8 ± 1.4	37.2 ± 0.1	33.5 ± 3.8	5.5 ± 1.3	37.1 ± 0.1
IL-1β 0.5 ng	28.8 ± 5.2	5.8 ± 1.5	37.0 ± 0.1	20.6 ± 3.1	4.5 ± 1.0	37.2 ± 0.1
Hours 11–12						
Vehicle	25.5 ± 5.4	3.4 ± 1.3	37.1 ± 0.1	15.4 ± 3.5	2.2 ± 0.8	37.2 ± 0.1
IL-1β 0.25 ng	17.8 ± 4.1	3.8 ± 1.3	37.1 ± 0.1	23.0 ± 2.7	2.3 ± 0.7	37.0 ± 0.1
IL-1β 0.5 ng	28.3 ± 5.2	7.8 ± 1.7	36.8 ± 0.1	21.4 ± 4.3	4.9 ± 1.5	37.1 ± 0.1

Values are the mean ± SEM percentage of recording time within consecutive 2-h time blocks after administration of vehicle or two different doses of IL-1. *$P < 0.05$ vs. vehicle.

5-HT perfusion ($P < 0.001$) was associated with membrane hyperpolarizations ranging from −2 to −14 mV, with a mean value of −6.4 ± 1 mV (from −54.2 ± 1.9 mV before 5-HT perfusion to −60.6 ± 2.3 mV during 5-HT perfusion, $P < 0.001$). These effects were fully reversible: within 5 min after cessation of tissue exposure to 5-HT, firing rate and membrane potential returned to 1.2 ± 0.2 Hz and 54.9 ± 2.1 mV, respectively. The three neurons that were not inhibited by 5-HT were not further analysed.

In 13 neurons, following IL-1β perfusion and full wash-out, perfusion with NA 30 μM increased the firing rate from a baseline value of 1.5 ± 0.3 Hz to 3.6 ± 0.5 Hz ($P < 0.001$; Fig. 3D). This effect was associated with a mean depolarization of 1.9 ± 0.6 mV (from a resting membrane potential value of −54.9 ± 2.1 mV before NA perfusion to −53.0 ± 2 mV during NA perfusion; $P < 0.05$). NA effects were fully reversible. The electrophysiological and neuropharmacological criteria used for the identification of DRN serotonergic neurons, first developed in rats, proved to be valid and reliable when used in guinea-pigs, for *in vivo* and *in vitro* extra- and intracellular elctrophysiological recordings (Craven *et al*., 1994, 2001; Mundey *et al*., 1994; Conley *et al*., 2002).

IL-1β inhibited serotonergic DRN neurons

Twenty-six (81%) of 32 presumed serotonergic DRN neurons were inhibited by bath application of IL-1 (25 ng/mL, Fig. 4). IL-1β perfusion induced in these 26 neurons a significant decrease in spontaneous firing rate, from 1.6 ± 0.2 Hz in control conditions to 0.8 ± 0.2 Hz

TABLE 2. Effects of handling, sham injection and vehicle administration into and outside the dorsal raphe nucleus (DRN) on the percentage of recording time spent in vigilance states and on average brain cortical temperature (T_{cort})

	Microinjection into DRN			Microinjection outside DRN		
	NREM	REM	T_{cort}	NREM	REM	T_{cort}
Hours 1–2						
Handling	32.7 ± 7.9	12.2 ± 3.3	37.1 ± 0.1	31.3 ± 3.6	10.7 ± 2.2	36.8 ± 0.1
Sham injection	32.3 ± 4.7	9.7 ± 2.5	36.9 ± 0.1	31.2 ± 4.4	10.5 ± 1.6	37.0 ± 0.1
Vehicle	33.5 ± 3.7	9.9 ± 1.5	37.0 ± 0.1	33.2 ± 2.3	11.2 ± 1.6	37.1 ± 0.1
Hours 1–12						
Handling	28.8 ± 2.7	8.5 ± 1.1	37.2 ± 0.1	28.7 ± 2.4	8.7 ± 1.3	37.1 ± 0.1
Sham injection	32.1 ± 2.2	7.3 ± 0.8	37.1 ± 0.1	31.7 ± 1.8	7.4 ± 0.7	37.3 ± 0.1
Vehicle	31.8 ± 1.9	6.1 ± 0.6	37.2 ± 0.1	28.6 ± 1.4	6.9 ± 0.6	37.3 ± 0.1

Values are the mean ± SEM percentage of recording time for the first 2 h post-injection and for the whole recording period for the condition indicated.

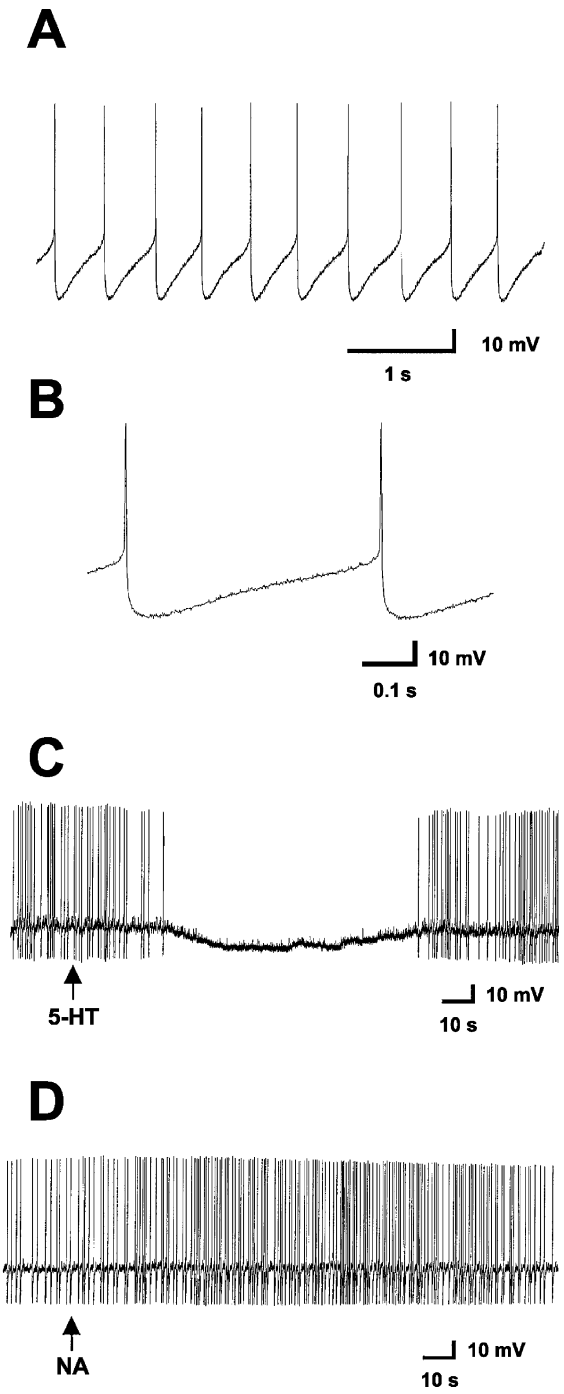

FIG. 3. Electrophysiological and pharmacological identification of serotonergic dorsal raphe nucleus (DRN) neurons. Representative voltage recordings from DRN serotonergic neurons obtained under current clamp in brain slice. No direct current current was injected. (A) Slow and regular firing rate of serotonergic DRN neurons in spontaneous conditions. (B) Detail of the recording in A, showing typical serotonergic action potentials, characterized by an initiating ramp of depolarization and by a large after-hyperpolarization. (C) Intracellular recording showing the effect of 1-min bath perfusion with 5-HT 40 μM (starting at arrow) on a serotonergic DRN neuron. In this case 5-HT induced a complete cessation of firing and a hyperpolarization of about 10 mV. (D) In the same neuron depicted in C, 1-min bath perfusion (starting at arrow) with NA 30 μM induced a reversible increase in the firing rate. The effects in C and D were rapidly reverted after wash out.

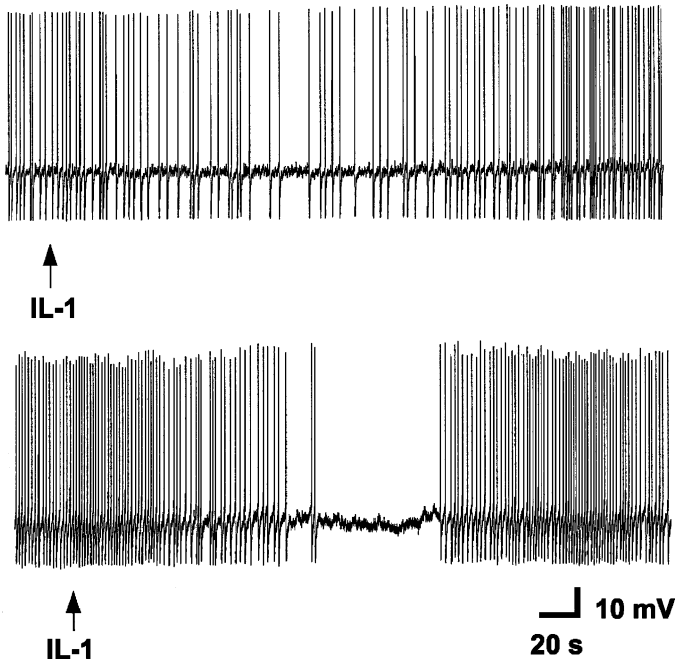

FIG. 4. Interleukin-1 (IL-1) inhibited serotonergic dorsal raphe nucleus (DRN) neurons intracellularly recorded *in vitro*. Intracellular recordings showing the effects of IL-1 (25 ng/mL) addition to bath perfusate for 2 min on two different representative serotonergic DRN neurons. Upper panel: IL-1 perfusion (starting at arrow) induced a decrease in spontaneous firing rate. Lower panel: in this neuron during perfusion with IL-1 the spontaneous activity first decreased and then ceased. In both neurons the effects were rapidly and completely reverted by washout.

during IL-1β perfusion. After wash-out, the firing rate returned to 1.6 ± 0.3 Hz. The IL-1β-induced reduction in firing rate was associated with a non-significant membrane hyperpolarization. Three neurons were not responsive to IL-1β. Three neurons were inhibited by IL-1β, but data from these neurons were not analysed as the effect was not washable. None of the 32 neurons studied was depolarized or its spontaneous firing was increased by IL-1β perfusion.

Discussion

The results of the present study show that IL-1β, when microinjected into the DRN (but not into adjacent brain stem areas) of freely behaving rats enhances NREM sleep. Furthermore, IL-1β inhibits the firing rates of physiologically and pharmacologically identified DRN serotonergic neurons in brain slice preparations. Collectively, these results suggest that IL-1β may increase NREM sleep by acting at the level of the DRN, perhaps due to the inhibition of DRN serotonergic neurons.

IL-1β can enhance NREM sleep by acting at the level of the DRN

Specific brain areas at which IL-1 can act to enhance NREM sleep remain to be fully identified, although there have been some studies to address this question. A purified IL-1 preparation obtained from stimulated human monocytes failed to enhance NREM sleep when microinjected into several areas of the rabbit brain, extending from the brain stem to the diencephalon and including areas in the rostral medulla, pons and midbrain, the hypothalamic preoptic area and basal forebrain, the posterior hypothalamus (Walter *et al.*, 1989). Recent evidence suggests that IL-1 may alter NREM sleep by actions on the preoptic/anterior hypothalamic area; IL-1 increases firing rates of

neurons active during NREM sleep and reduces firing rates of neurons active during waking (Alam *et al*., 2001). Consistent with these results are recent observations that IL-1 increases cytosolic Ca^{2+} in cultured hypothalamic neurons (De *et al*., 2002). IL-1, when microinjected into the rat locus coeruleus, potentiates EEG frequencies in the 3–9 Hz range and enhances sleep [as defined on the basis of visual inspection of the animal behaviour (De Sarro *et al*., 1997)]. By contrast, IL-1 induces wakefulness when microinjected into the rat hypothalamic paraventricular nucleus (Slisli & de Beaurepaire, 1999). As IL-1 stimulates the release of corticotropin-releasing hormone (Barbanel *et al*., 1990), this latter effect is likely to be due to the increased levels of this peptide, which enhances wakefulness (Chang & Opp, 2001).

The involvement of both IL-1 and 5-HT in sleep regulation, as well as growing evidence showing that IL-1-induced enhancement of NREM sleep is mediated, in part, by the serotonergic system, are rewieved in the Introduction. The DRN is the origin of the major ascending serotonergic pathways to the forebrain (Jacobs & Azmitia, 1992) and it contains IL-1 receptors (Cunningham & De Souza, 1993; Schöbitz *et al*., 1994). Therefore, the results of the present study, by demonstrating for the first time that IL-1β enhances polygraphically defined NREM sleep when microinjected into the DRN of conscious animals, indicate that the DRN might represent one brain region where IL-1 can affect the serotonergic system and, moreover, one brain region that mediates IL-1 effects on NREM sleep.

IL-1β inhibits DRN serotonergic neurons

Results in the present paper are the first to provide electrophysiological evidence that in brain slices IL-1β inhibits physiologically and pharmacologically identified DRN serotonergic neurons. As 5-HT is a wake-promoting agent (Jouvet, 1999; Pace-Schott & Hobson, 2002), data in the present paper suggest that the increase in NREM sleep induced by IL-1β microinjection into the DRN can result, in part, from the inhibition of DRN serotonergic neurons.

Serotonin, which promotes wakefulness *per se*, also has been proposed to induce the synthesis and/or release of factor(s) that subsequently enhance sleep (Jouvet *et al*., 1983; Jouvet, 1999). Recent data directly support a dual role for 5-HT in sleep regulation. Increasing serotonergic activity by injecting rats with the 5-HT precursor 5-hydroxytryptophan (5-HTP) first enhances wakefulness and later increases NREM sleep (Imeri *et al*., 2000).

The hypothalamic preoptic area and basal forebrain play a crucial role in the regulation of NREM sleep (reviewed in Jones, 2000; McGinty & Szymusiak, 2000; Saper *et al*., 2001). IL-1 releases 5-HT from axon terminals in the hypothalamus (Shintani *et al*., 1993), suggesting that IL-1 could promote NREM sleep not only by inhibiting wake-promoting DRN serotonergic neurons, but also by enhancing axonal 5-HT release in specific brain areas. The anterior hypothalamus is the only brain area where stimulation of serotonergic activity can restore physiological sleep in cats that have been made insomniac by brain 5-HT depletion (Denoyer *et al*., 1989). Serotonin hyperpolarizes basal forebrain cholinergic neurons responsible for cortical activation (Khateb *et al*., 1993). Furthermore, because 5-HT induces IL-1 both *in vitro* (Silverman *et al*., 1989) and *in vivo* (Gemma *et al*., 2003), it could amplify the effects of exogenously administered IL-1. These findings would explain previous results showing that 5-HT is necessary for the full manifestation of IL-1 effects on NREM sleep (reviewed in Imeri & De Simoni, 1999). In summary, IL-1-induced NREM sleep enhancement could result from both the inhibition of the serotonergic cell bodies and the enhancement of 5-HT release from axon terminals in specific brain regions involved in NREM sleep generation. These effects may occur at different time points after IL-1 is administered or released in physiological or pathological conditions. They may also depend on sites in brain accessed by IL-1.

IL-1β-induced inhibition of REM sleep is not mediated by DRN serotonergic neurons

The firing rate of DRN serotonergic neurons and 5-HT release from axon terminals are state-dependent: they are maximal during wakefulness, reduced during NREM sleep and suppressed (or minimal) during REM sleep (McGinty & Harper, 1976; Trulson & Jacobs, 1979; Cespuglio *et al*., 1981, 1990; Lydic *et al*., 1987; Wilkinson *et al*., 1991; Imeri *et al*., 1994; Portas & McCarley, 1994). The observation that inhibition of the serotonergic system results in the enhancement of REM sleep (Adrien, 1995) suggests that suppressed serotonergic activity is permissive for REM sleep generation (Steriade & McCarley, 1990; Pace-Schott & Hobson, 2002). *In vitro* results of the present study indicate that IL-1β significantly reduces, but does not suppress, the firing rate of DRN neurons. *In vivo* results indicate that IL-1β microinjection into the DRN and adjacent brain stem areas does not increase, but rather inhibits, REM sleep. Taken together, these observations suggest that a reduced serotonergic activity is not sufficient to permit REM sleep, which could be enhanced only when serotonergic activity is abolished. Moreover, REM sleep is inhibited also by the noradrenergic neurons of the locus coeruleus (Pace-Schott & Hobson, 2002), which were not targeted in this study.

Observations that blockade of 5-HT2 receptors (Imeri *et al*., 1999) does not modify IL-1-induced inhibition of REM sleep suggests that IL-1 inhibitory effects on REM sleep are not mediated by the serotonergic system (or at least not through this receptor subtype), but by other neurochemical systems. The possible spreading of IL-1 into adjacent cholinergic structures of the pontine tegmentum from microinjection sites within and outside the DRN cannot be ruled out. As the cholinergic system is involved in the generation of REM sleep (Steriade & McCarley, 1990; Semba, 1999; Baghdoyan, 1997) and IL-1 inhibits ACh release *in vivo* in the hippocampus (Rada *et al*., 1991), as well as IL-1 synthesis in cultured pituitary cells (Carmeliet *et al*., 1989), IL-1 action on the cholinergic structures of the pontine tegmentum might account for the inhibition of REM sleep described in the present paper.

IL-1β-induced inhibition of DRN serotonergic neurons can increase brain cortical temperature

IL-1β administration into the DRN nucleus induces a transient and moderate increase in T_{cort}. The typical response to IL-1 administration through several routes includes an increase in NREM sleep and a febrile response [reviewed in Terao *et al*., 1998; Opp & Imeri, 1999; Krueger & Fang, 2000). Although it is well established that, under certain circumstances, increases in body and brain temperature promote NREM sleep (reviewed in McGinty & Szymusiak, 1990), an extensive literature shows that NREM sleep enhancement induced by IL-1 is a specific effect and it is not merely a byproduct of fever, because it occurs also when febrile response is blocked (Krueger *et al*., 1984; Opp *et al*., 1989).

Observations that IL-1β increases T_{cort} when microinjected into the DRN, whereas no changes in T_{cort} are observed when IL-1 is microinjected into brain stem areas surrounding the DRN, suggest that this IL-1β effect is due to specific actions on the DRN. Whereas the firing rate of serotonergic DRN neurons is not affected by the peripheral administration of synthetic muramyl dipeptide, an analogue of Gram-positive bacterial cell wall component (Fornal *et al*., 1987), the present data show that DRN serotonergic neurons are inhibited by direct IL-1β administration.

Although the role of 5-HT in thermoregulation is complex, there is evidence that under certain conditions 5-HT may lower body/brain

temperature, as 5-HT excites hypothalamic warm-sensitive neurons, inhibits cold-sensitive neurons and stimulates the release of pro-opiomelanocortin-derived peptides, including α-melanocyte-stimulating hormone, whose hypothermic effect is well established (reviewed in Imeri *et al*., 2000). Observation that serotonergic activation induced by the administration of the 5-HT precursor 5-HTP decreases brain temperature (Imeri *et al*., 2000) supports such a role for 5-HT. The present results, showing that IL-1 inhibits DRN serotonergic neurons in brain slices and increases T_{cort} when microinjected into the DRN of freely behaving animals, are in agreement with the hypothesis that 5-HT lowers body/brain temperature and might explain the increase in T_{cort} observed following IL-1 microinjection into the DRN.

In conclusion, the results presented here demonstrate for the first time that IL-1β enhances NREM sleep when microinjected into the DRN of conscious animals and inhibits the firing rates of DRN serotonergic neurons in a slice preparation. These data suggest that the DRN may be one specific brain region where IL-1β inhibits the wake-promoting serotonergic system, with subsequent enhancement of NREM sleep.

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Abbreviations

5-HT, 5-hydroxytryptamine, serotonin; ACSF, artificial cerebrospinal fluid; DRN, dorsal raphe nucleus; EEG, elctroencephalogram/electroencephalographic; IL-1, interleukin-1; NA, noradrenaline; NREM, non-rapid eye movements; REM, rapid eye movements; SWA, slow wave activity; T_{cort}, cortical brain temperature.

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