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The Role of Cholinergic Basal Forebrain Neurons in Adenosine-Mediated Homeostatic Control of Sleep: Lessons from 192 IgG-Saporin Lesions

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Abstract

A topic of high current interest and controversy is the basis of the homeostatic sleep response, the increase in non-rapid-eye-movement (NREM) sleep and NREM-delta activity following sleep deprivation (SD). Adenosine, which accumulates in the cholinergic basal forebrain (BF) during SD, has been proposed as one of the important homeostatic sleep factors. It is suggested that sleep-inducing effects of adenosine are mediated by inhibiting the wake-active neurons of the BF, including cholinergic neurons. Here we examined the association between SD-induced adenosine release, the homeostatic sleep response and the survival of cholinergic neurons in the BF after injections of the immunotoxin 192 IgG-saporin (saporin) in rodents. We correlated SD-induced adenosine level in the BF and the homeostatic sleep response with the cholinergic cell loss 2 weeks after local saporin injections into the BF, as well as 2 and 3 weeks after intracerebroventricular (ICV) saporin injections.

Two weeks after local saporin injection there was an 88% cholinergic cell loss, coupled with nearly complete abolition of the SD-induced adenosine increase in the BF, the homeostatic sleep response, and the sleep-inducing effects of BF adenosine infusion.

Two weeks after ICV saporin injection there was a 59% cholinergic cell loss, correlated with significant increase in SD-induced adenosine level in the BF and an intact sleep response. Three weeks after ICV saporin injection there was an 87% cholinergic cell loss, nearly complete abolition of the SD-induced adenosine increase in the BF and the homeostatic response, implying that the time course of ICV saporin lesions is a key variable in interpreting experimental results.

Taken together, these results strongly suggest that cholinergic neurons in the BF are important for the SD-induced increase in adenosine as well as for its sleep-inducing effects and play a major, although not exclusive, role in sleep homeostasis.

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Keywords

sleep deprivation; cholinergic lesion; delta activity; stereology; rat; immunotoxin

INTRODUCTION

The cholinergic basal forebrain (BF), adenosine and their relevance to sleep is a topic of high current interest. The BF contains cortically projecting wake-active neurons (Szymusiak et al., 2000; Semba, 2000; Jones, 2004) and, as referred in this paper, is comprised of the horizontal diagonal band (HDB) substantia innominata (SI) and magnocellular preoptic nucleus (MCPO). Together with the medial septum (MS), vertical limb of the diagonal band (VDB), and the nucleus basalis magnocellularis (NBM), which also contain cholinergic neurons, these nuclei form a continuous cholinergic column in the BF.

In the BF, the somnogenic effects of the inhibitory neuromodulator, adenosine, have been suggested to be mediated via the A1 adenosine receptors (see Strecker et al., 2000, Basheer et al., 2004, McCarley 2007). In vivo, the BF extracellular adenosine was shown to increase gradually during sleep deprivation (SD), while the increase in homeostatic sleep response following SD (recovery sleep) was mimicked by increasing extracellular adenosine level with a transporter blocker, S-(p-nitrobenzyl)-6-thioinosine (NBTI) (Porkka-Heiskanen et al., 1997) and by adenosine infusion (Portas et al., 1997; Basheer et al., 1999). Adenosine, acting postsynaptically at the A1 receptor, inhibited BF cholinergic and some non-cholinergic neurons *in vitro* (Rainnie et al., 1994; Arrigoni et al., 2006) and inhibited BF wake-active neurons *in vivo* (Alam et al., 1999; Thakkar et al., 2003a), while antisense against the A1 receptor in the BF blocked the SD-induced increase in non-rapid-eye-movement (NREM) sleep and the increase in delta activity (Thakkar et al., 2003b). Taken together, these observations led to the hypothesis that BF adenosine accumulation during SD plays an important role in sleep homeostasis, promoting sleep by inhibiting BF wake-active neurons.

The BF contains several neurotransmitter phenotypes, including cortically projecting cholinergic, GABAergic and glutamatergic neurons (Manns et al., 2003; Steriade and McCarley, 2005). Cholinergic neurons were initially thought to be the major BF component promoting cortical activation/arousal since cortical acetylcholine release increased during cortical activation states of waking and REM sleep (Szerb 1967; Marrosu et al., 1995) and blocking cholinergic receptors produced diminished cortical activation (Longo 1966). These data led us to hypothesize that cholinergic neurons play an important but non-exclusive role in the BF adenosine actions, including sleep homeostasis.

However, the precise role of cholinergic neurons in adenosine-mediated homeostatic sleep control remained untested, and hence the interest in the use of the immunotoxin 192 IgG-saporin (saporin), a conjugate of a ribosomal inactivating enzyme, and the monoclonal antibody 192 IgG, which specifically binds to the p75 nerve growth factor-receptor located on BF cholinergic neurons and destroys them (Book et al. 1992; Heckers et al., 1994). Several studies which employed intracerebroventricular (ICV) saporin injections have failed to detect stable significant changes in the sleep-wake cycle and the homeostatic sleep response when measured within 14–16 days post-injection (Bassant et al., 1995; Kapas et al., 1996; Gerashchenko et al., 2001; Blanco-Centurion et al., 2006). However, there are reports on the differential effect on the extent of cholinergic cell loss between rostral parts of BF (including MS and VDB) and the caudal nuclei of BF (including HDB, MCPO, SI and NBM) (Wrenn et al., 1999; Traissard et al., 2007; Moreau et al., 2008) after ICV saporin injections. An almost complete loss of cholinergic cells located in the rostral areas was

contrasted with only up to 60% of cholinergic cell loss in the caudal BF in these studies suggesting a slower time course for larger lesion development in caudal areas. This effect might be attributed to better diffusion of the toxin through the parenchyma to the rostral BF than to the caudal BF, which is more distant from the lateral ventricles (Moreau et al., 2008). Also bearing on measurements of the time course of effects, SD-induced adenosine levels in the HDB/SI/MCPO was not increased when measured 3 week after ICV saporin injection, but this data was not correlated with measurements of recovery sleep at the same time point (Blanco-Centurion et al., 2006). In contrast to ICV injections, which destroy cholinergic cells throughout the BF, the method of local saporin injections induces regionally precise destruction of cholinergic neurons (Pizzo et al., 1999). Studies using this method found minor changes in spontaneous sleep-waking cycles (Berntson et al., 2002; Kaur et al., 2008) but dramatic changes in the homeostatic sleep response (Kaur et al., 2008). However, to our knowledge, the effect of local injections on adenosine accumulation during SD as well as adenosine-induced sleep have not been studied.

The present study addressed the role of the BF cholinergic cells in adenosine-mediated homeostatic sleep control by using and comparing two different methods of saporin immunotoxin delivery, ICV and local injection into HDB/SI/MCPO. We compared SD-induced adenosine increase in the BF, recovery sleep response after SD and adenosine-induced sleep in the same animals before and 2 weeks after local saporin injections. In order to examine whether the effects of ICV saporin injections follow slower time course, we measured SD-induced adenosine increase in the BF and recovery sleep response in the same animals both 2 and 3 weeks after ICV saporin injections. We correlate our findings with the extent of cholinergic cell loss in HDB/SI/MCPO. Parts of these studies have appeared as abstracts (Kalinchuk et al., 2005, 2007).

EXPERIMENTAL PROCEDURES

This section first presents the experimental design and rationale, followed by description of methods and specific experimental details. All surgical and experimental protocols were approved by the Ethical Committee for Animal Experiments at the University of Helsinki and the provincial government of Uusimaa (Finland), were in accordance with the laws of Finland and the European Union and the Association for Assessment and Accreditation of Laboratory Animal Care and Use Committee at Boston VA Healthcare system, Harvard University and U.S. National Institute of Health. Every effort was made to minimize animal suffering and to reduce the number of animals used.

Experimental design and rationale

Experiment 1. Investigation of the effects induced by local saporin administration on SD-induced adenosine accumulation and sleep homeostasis

The method of local, intraparenchymal injections of saporin allows to perform targeted lesion of cholinergic cells in the area of interest and avoid the extra-BF lesions (suprachiasmatic and cerebellar neurons) caused by ICV saporin injection (Pizzo et al., 1999). Kaur et al. (2008) reported that bilateral local injections into the caudal part of BF (NBM/SI) resulted an attenuation of the homeostatic sleep response, but these authors did not measure changes in adenosine (Kaur et al., 2008). The present study used unilateral injections to determine if this minimal BF cholinergic lesion would be able to alter both adenosine accumulation during SD and the homeostatic sleep response. We reasoned that unilateral injections, if successful in altering sleep homeostasis, would be preferable to bilateral injection in that they would cause less non-specific damage, and be in agreement with the general principle that the minimal lesion producing behavioral effects should be used. Although we were prepared to use bilateral injections if necessary, we thought there

was a high likelihood that unilateral injections would be successful, since our previous studies had shown that unilateral pharmacological manipulations in the BF (HDB/SI/MCPO) such as adenosine infusion (Portas et al., 1997; Basheer et al., 1999), nucleoside transport blocker NBTI infusion (Porkka-Heiskanen et al., 1997), dinitrophenol application (Kalinchuk et al., 2003), and nitric oxide donor application (Kalinchuk et al., 2006a) were sufficient to cause marked changes in sleep. We studied the effects of local saporin injections on adenosine increase during 6h SD by comparing adenosine values before and during SD, and simultaneously we studied the changes in recovery sleep following 6h SD by measuring NREM sleep, NREM delta activity, and REM sleep. All measurements were performed in the same animals before and 2 weeks after saporin treatment (group **L-SD-saporin**, N=6). Another group of rats (group **L-SD-saline**, N=5) injected with saline served as treatment (injection) controls.

Experiment 2. Investigation of the effects induced by local saporin administrations on adenosine-induced sleep

—Previously we have shown that unilateral administration of adenosine into BF induces sleep in the rat (Basheer et al., 1999). To determine the role of cholinergic neurons, if any, in mediating the somnogenic effects of adenosine, we infused adenosine into the BF by reverse microdialysis into side ipsilateral to the saporin injection, in the same animals both before and 2 weeks after local saporin injection and measured changes in NREM sleep, NREM delta activity, and REM sleep (group **L-AD-saporin**, n=6).

Experiment 3. Investigation of the time course of ICV saporin injection's effects on SD-induced adenosine accumulation and sleep homeostasis

—To determine the time course of ICV-saporin lesions, in the same animals we measured the effects of 6 hr SD on adenosine increase and the homeostatic sleep response 2 and 3 weeks post-ICV injection (group **ICV-SD-saporin**, N=7). To correlate these changes with cell loss in HDB/SI/MCPO, 4 additional animals were injected with saporin and sacrificed 2 weeks post-injection (see experiment 4). A group of saline-treated animals (group **ICV-SD-saline**, N=5) served as treatment (injection) controls.

Experiment 4. Comparison of the histological effects induced by local and ICV-saporin injections

—To examine the extent of cholinergic loss 2 weeks after local saporin injections, and to follow the time course and compare the extent of cholinergic loss between 2 and 3 weeks following ICV-saporin injections, we counted the cholineacetyltransferase (ChAT)-positive neurons in the BF using stereological counting, methodology more precise than non-stereological counting (Gundersen 1986; West 1993). Counting was done in four groups of animals sacrificed at these times: 2 weeks after local injection (N=4, randomly chosen rats from experiment 1 (**L-SD-saporin**) were used); 2 weeks after ICV saporin injection (N=4); 3 weeks after ICV saporin injection (N=4, randomly chosen rats from experiment 3 (**ICV-SD-saporin**) were used); and 3 weeks after ICV-saline injection (N=4, randomly chosen rats from experiment 3 (**ICV-SD-saline**) were used). In the same groups of animals we also examined the acetylcholinesterase (AChE)-positive fibers in the cortex. To rule out the possibility of non-specific lesions in the BF induced by local injections, we performed control staining for glutamic acid decarboxylase (GAD), followed by non-stereological counting of GAD-positive cells.

Animals and surgery: general procedures

The total of 33 male Wistar rats (300–400g) used in this study were kept in a room with constant temperature (23.5–24°C) and 12-h light-dark cycle (lights on at 8.00 AM). Water and food were provided *ad libitum*. Under general anesthesia (i.m. ketamine 7.5mg/100g body weight, xylazine 0.38mg/100g, acepromazine 0.075mg/100g) rats were implanted with

electroencephalogram (EEG) and electromyogram (EMG) electrodes. EEG electrodes (stainless steel screws) were implanted epidurally over the frontal (primary motor, AP=+2.0; ML=2.0) and parietal (retrosplenial, AP=-4.0; ML=1.0) cortices. EMG recording electrodes (silver wires covered with Teflon) were implanted into neck muscles. For collection of microdialysis samples, and also for local injections of saporin, unilateral guide cannula (CMA/11 Guide, CMA/Microdialysis, Stockholm, Sweden) were implanted in such a way that the tip was located 2mm above target area HDB/SI/MCPO (AP=-0.3; ML=2.0; V=6.5) (Paxinos & Watson, 1998).

Administration of the immunotoxin 192 IgG -Saporin

In experiments 1 and 2, saporin (Chemicon International, Inc; batch #0703054253) was injected locally through microdialysis probes which were modified by removing the microdialysis membrane from the tip after completion of control measurements. For local injections, 1µl of saporin at a concentration of 0.23µg/µl was injected at the flow rate of 0.1µl/min into the HDB/SI/MCPO at the following coordinates: AP=-0.3; ML=2.0; V=8.5 (Paxinos & Watson, 1998). According to the literature, this dose should provide selective lesion of the BF cholinergic cells without any loss of parvalbumin- and GAD-immunopositive neurons which are intermingled with the cholinergic neurons in the BF (Pizzo et al., 1999, Kaur et al., 2008). In experiment 3, ICV injections of saporin were performed under stereotaxic control during the surgery for EEG electrode implantation. For the ICV injection, 6µl of saporin (1µg/µl) was unilaterally injected into the lateral ventricle at coordinates: AP=+1.0; ML=0.3; V=5.9 (Paxinos and Watson, 1998). This dose for ICV injections was the same as in a recently published study by Blanco-Centurion et al. (2006). Treatment control group rats were injected with 0.9% saline either into lateral ventricle (6µl) or into the CBF (1µl). In all cases saporin was injected using a microdialysis pump at the flow rate of 0.1µl/min. After all injections the probe was kept in place for an additional 3–4 min and then was slowly removed.

Recovery, adaptation and sleep deprivation: general procedures

After surgery the rats were housed in individual cages and were allowed 1 week of recovery from surgery before procedures. Beginning 3 days after surgery, animals were socially habituated to experimental conditions by daily 10 min training sessions that included handling and removal from the cages to play with the experimenter. Habituation was regarded as complete when there was no fear reaction (manifested as startling, excessive urinating, etc.) when the researcher approached the cage and touched the rat.

After 1 week of recovery period, rats were connected to EEG/EMG recording leads for adaptation which lasted 4 days. Before microdialysis probe insertion, rats were connected to the recording cables at 08.00AM and EEG/EMG was continuously recorded for at least 24h to monitor the stabilization of EEG and sleep-waking cycles. If after 72 h of recording there was no stabilization, the animal was not used in the experiments.

SD was done by gentle handling (Franken et al. 1991) which included presentation of new objects into the cages or gentle touching by a brush or hands when rats became sleepy. SD started at 10.30AM and ended at 4.30PM. EEG/EMG recordings were continuously performed during SD and continued during recovery sleep for 24h after SD.

EEG recording and analysis

The EEG/EMG signals were amplified and sampled at 104Hz. EEG recordings were scored using the Spike 2 program (Version 5.11, Cambridge Electronic Devices, Cambridge, UK) in 30-sec bins semi-automatically for NREM sleep and manually for REM sleep and wakefulness as described previously (Kalinchuk et al., 2006a & b). The scoring of NREM

sleep was validated by comparing the semi-automatic scoring with manual scoring for 17 records of 30h each; the average match was $94.1 \pm 3.1\%$ (mean $\pm$ SEM). Recordings were divided into 6 h bins; the amounts of NREM sleep, REM sleep and slow-wave EEG power in delta range (0.4–4.5Hz) during NREM episodes in each bin during the experimental day were compared with the corresponding time bin on the baseline day (see below) and percentage differences were calculated. In all experiments a total of 18 hours after the treatments (1.30 PM – 7.30 AM for adenosine infusion or 4.30PM – 10.30AM for 6h SD) was used for these quantitative analyses. Additionally, to compare EEG power density before and after saporin injection, we examined the spectra during recovery NREM sleep after SD, vigilance states were manually scored for 4s epochs during the first 3h after SD and EEG power spectra were calculated within the frequency range of 0–15Hz with a resolution of 0.4Hz.

Adenosine measurements during SD using *in vivo* microdialysis

To measure the SD-induced changes in the extracellular adenosine levels in BF, microdialysis probes (CMA/11, membrane length and diameter 2mm and 0.24mm, respectively; CMA/Microdialysis, Stockholm, Sweden) were inserted into the HDB/SI/MCPO (AP=−0.3; ML=2.0; V=8.5) (Paxinos and Watson, 1998) at least 20 h before the start of the experiments, as described by Porkka-Heiskanen et al. (1997). For experiments, animals were connected to the combined leads (EEG/EMG recording cable and microdialysis tubing) after lights on at 08.00 AM – 08.20 AM. Artificial cerebrospinal fluid (ACSF, NaCl 147mM; KCl 3mM; CaCl₂ 1.2mM; MgCl₂ 1.0mM) was pumped through the microdialysis probe at 1μl/min. On each experimental day microdialysis samples were collected at 30 min intervals during the course of the experiment. For each rat, microdialysis experiments were performed on 2 consecutive days (see experimental schedule for 6h SD, Table 1A). The first day was always ACSF infusion, on the second day SD was performed. For measurements of SD-induced changes in adenosine, collection of samples started at 8.30AM and continued through the SD and first two hours of recovery sleep. At the end of microdialysis experiment the combined microdialysis and EEG/EMG leads were disconnected and replaced by ordinary EEG/EMG recording leads and recordings continued till the end of 24h of recovery sleep.

Adenosine infusion using *in vivo* microdialysis

We tested the effects of infusions of adenosine (300μM) (Basheer et al., 1999) for 3h on sleep before and 2 weeks after local injections of saporin using reverse microdialysis. Microdialysis probes were inserted into the HDB/SI/MCPO as described in the previous paragraph. According to the manufacturer's description of CMA probes and our previous measurements (Portas et al., 1997), the efficiency of probe recovery was ~10%, which allowed an estimate of effective BF concentrations of adenosine as 30μM. Table 1B describes the protocol. Animals were connected to combined microdialysis and EEG/EMG recording leads at 08.00–08.20AM. Adenosine infusions started at 10.30AM and ended at 1.30 PM. ACSF was infused 2 h before and 2 h after adenosine infusion. EEG/EMG recording was continuously performed during microdialysis period and continued further for 24 h post-infusion period.

Baselines for SD experiments and for adenosine microdialysis measurements

1) Baseline for sleep/delta power measurements during recovery sleep after SD—The baseline recording of EEG/EMG (hereafter referred to as **baseline**) was performed on the day preceding SD (hereafter referred to as **baseline day**) and was combined with ACSF infusion. To provide a control for handling, during baseline recording rats were gently handled for 2–4min during episodes of spontaneous waking at the same

circadian time as the SD. Microdialysis samples were collected during ACSF infusion to confirm that adenosine levels were stable during the period corresponding to the circadian time of SD experiment performed on the following day (8.30AM-6.30PM).

2) Baseline for sleep/delta power measurements after adenosine infusion—

The baseline recording of EEG/EMG (also hereafter referred to as **baseline**) was performed on the day preceding adenosine infusion (hereafter referred to as **baseline day**) and was combined with ACSF infusion.

3) Baseline for adenosine measurements during SD—Our measurements from baseline day revealed that adenosine level was not fluctuating during the light period of the day when normally SD is performed (data not shown). Thus, to study the effects of SD on adenosine level, we compared the samples collected on the same day before and during SD. The average of 2 samples collected during 2 hours before SD (hereafter referred to as **pre-SD baseline**) served as the baseline for adenosine measurements during SD.

HPLC analysis

Adenosine was measured using high performance liquid chromatography coupled to a UV detector (Waters 486). Details of the adenosine assays have been published previously (Porkka-Heiskanen et al., 1997). The detection limit of the assay was 0.8 nM (signal to noise ratio 2:1). Mean concentrations of the samples (n=5) collected during 6h SD (average of every other 30-min samples, collected during the hours 2–6) were normalized to the mean concentration of samples (n=2) collected during 2h pre-SD period (=100%).

Histological verification of the cells lesion and probe locations

After the experiments, rats were given a lethal dose of pentobarbital and perfused transcardially with 50–100 ml 0.9% saline followed by 150–200ml 4% paraformaldehyde in 0.1M phosphate buffer (PBS, pH 7.4). The brains were removed, postfixed in the 4% paraformaldehyde in 0.1M PBS overnight and immersed in a 30% sucrose solution at 4°C for 4–5 days for cryoprotection. After brains sank, they were frozen and stored at –80°C. Coronal 50-µm-thick sections were cut through the entire brain (except olfactory bulbs and cerebellum) using a cryostat and placed into 0.1M PBS. Sections from the BF region (between AP=0.0 and AP=–0.8 (Paxinos and Watson, 1998); approximately 11/12 sections per brain with 250 µm intervals (every fifth) were taken for immunohistochemical staining for ChAT. For local saporin injections, staining for GAD was also performed in the BF sections. Histological verification of probe locations was performed in parallel. For AChE staining, every tenth section including regions rostral and caudal to the BF was taken. Density of AChE-positive fibers was examined using light microscopy in cortical areas which receive projections from the BF, including prefrontal and frontal areas (Gaykema et al., 1990).

Immunohistochemistry for ChAT, GAD and AChE

After washing in PBS, the sections were incubated in 0.02% Triton-X for 2h, then incubated in 3% donkey serum for 1h at room temperature, and finally placed in primary antibody solution. ChAT-immunoreactivity (ChAT-ir) was detected using the polyclonal rabbit anti-ChAT antibody at 1:400 dilution and GAD-immunoreactivity by using polyclonal rabbit anti-GAD antibody at 1:500 dilution (Chemicon International, Temecula, CA). For ChAT staining, sections were incubated for 24h at 4°C, and for GAD – for 48h at 4°C. Sections were then treated with the secondary antibody (donkey anti-rabbit IgG, biotin conjugated, Chemicon International, Temecula, CA) at 1:200 dilution for 1h followed by ABC solution for 1h (Vectarin ABC kit, Vector Laboratories Inc., Burlingame, CA). For visualization of

the ChAT- and GAD-immunoreactivity, diaminobenzidine (DAB; peroxidase kit, Vector laboratories Inc., Burlingame, CA) was used. AChE staining was performed using AChE Rapid Staining Kit (MBL, Japan) according to manufacturer's protocol.

Stereological Counting of ChAT-ir neurons in the BF

The stereological cell counting was performed using Stereoinvestigator software version 7 (Microbrightfield, Williston, VT). The integrated set-up for cell counting consisted of an Olympus BX51 microscope fitted with a motorized stage (3-axis computer controlled stepping motor system comprised of 4"×3" XY stepping stage with 0.1 μm resolution, and Z-axis motor with 0.1 μm resolution), connected to a workstation via a Digital Video (DV-20) color camera. Counting of the ChAT-ir neurons was performed in the HDB/SI/MCPO. The outlines of the area were drawn at low magnification (4× Uplan Fluorite objective, NA 0.13, WD 17mm) based on the Paxinos and Watson rat brain atlas at three levels (−0.26, −0.4, −0.8) and matched with sections for counting. Three sections/rat, at an interval of 250 μm (1 in 5 series), were used for counting by applying systematic unbiased sampling using optical fractionator probe of Stereoinvestigator. ChAT-ir cells were counted on one side of the brain using a fractionator area of 50 × 50 μm (2500 μm^2) within a randomly laid grid of 200 × 200 μm (40,000 μm^2).

The thickness of the stained sections was measured at multiple sites within the counting frame at the time of counting using Uplan S-APO 100× oil immersion objective (NA 1.40, WD 0.12). The average thickness of the stained sections was calculated to be ~ 40 μm . The plane for the top of the counting frame in z-dimension was set to 5 μm from the surface of the section (guard zone), allowing a 30 μm probe height and a 5 μm guard zone set at the bottom of the section. The cell counting was done using the 100× oil immersion objective using optical fractionator method (Gundersen 1986; West 1993). The counting included 20% of the BF sections and 80% of the height of each section. An average of 125 sampling sites was employed for an average volume of the CBF of ~ 1.1 mm^3 . The inter-animal variability in cell counts within each group showed an average Gundersen coefficient of error (CE) of 0.08.

Statistics

Data are expressed as mean±SEM.

Statistical analysis was performed using SigmaStat 3.0 Statistical software (SPSS Inc., Chicago, IL, USA). To evaluate the statistical significance of the effects of SD and adenosine infusion on the time spent in NREM sleep, REM sleep and on NREM delta activity over 18h after the treatment vs. baseline we used a t-test. To compare the SD- or adenosine-induced changes in the same animals before vs. 2 weeks after local saporin injection (Experiment 1 and 2), or 2 weeks vs 3 weeks after ICV saporin injection (Experiment 3) we used Repeated Measures Analysis of Variance (RM-ANOVA). The 18h recovery sleep was further divided into three 6h bins and, if the 18h values were statistically different, pair-wise comparisons of individual bins were done using a t-test. A RM-ANOVA comparison of EEG power spectrum density was followed by Holm-Sidak post hoc tests for pair-wise comparisons. The effect of SD on concentrations of adenosine vs. pre-SD baseline was determined using a paired t-test, and to compare SD-induced changes before vs. 2 weeks after local saporin injection (Experiment 1 and 2), or 2 weeks vs 3 weeks after ICV saporin injection (Experiment 3) we used RM-ANOVA. For cholinergic cell counts, One Way ANOVA was done followed by Holm-Sidak post-hoc tests. In the case when data were non-normally distributed nonparametric Mann-Whitney Rank Sum Test was applied.

RESULTS

1. Effects of local saporin administration 2 weeks post-injection on adenosine accumulation during SD and recovery sleep response

In this experiment, we measured changes in SD-induced increase in the adenosine levels in the HDB/SI/MCPO and correlated them with changes in homeostatic sleep response before and 2 weeks after local injection.

1.1. Adenosine release during sleep deprivation following local saporin injection—First, we examined the SD-induced changes in adenosine in rats sleep-deprived for 6h before the local saporin injection (**pre-injection control condition**) and 2 weeks after the saporin injection (**post-injection condition**) (**L-SD-saporin**, N=6). Microdialysates were collected 2h before SD (**pre-SD baseline**), during SD and 2 h after the end of SD in conjunction with simultaneous EEG recording (Table 1A). Similar measurements were performed in rats that received local saline injection (**L-SD-saline**, N=5).

In saporin injected group (**L-SD-saporin**) in pre-injection control conditions adenosine level in the HDB/SI/MCPO (Figure 1A, B) during 6h SD (average of 5 every other 30min samples) was increased by $245.7 \pm 71.8\%$ as compared to its 2h pre-SD baseline (average of 2 every other samples collected on the same day before SD) (paired t-test, $t=2.274$, $P<0.05$). In contrast, in the same rats 2 weeks after saporin injection adenosine concentration was not increased ($-3.0 \pm 10.0\%$ compared to pre-SD baseline). Adenosine level during the 6h SD in pre-injection control conditions was significantly higher as compared to post-injection conditions (RM ANOVA, $F(1,5)=11.388$, $P=0.02$) (Figure 1C).

In saline injected group (**L-SD-saline**) injection of saline into HDB/SI/MCPO region did not affect the SD-induced increases in adenosine level which was significantly increased as compared with pre-SD level (data not shown). Comparison of SD-induced adenosine levels between the **L-SD-saporin** and the **L-SD-saline** groups showed no significant differences at pre-injection control conditions (t-test, $t=-0.042$ (9), $p=0.967$), whereas 2 weeks post-injection, the SD-induced adenosine increase in saline-treated rats was significantly higher than in saporin-injected rats (t-test, $t=-2.78$ (9), $p=0.02$).

1.2. Homeostatic sleep response after sleep deprivation following local saporin injection—Changes in the homeostatic sleep response were evaluated by comparing the time spent in recovery NREM sleep, REM sleep and the change in delta power during recovery NREM sleep before the saporin injection (**pre-injection control condition**) and 2 weeks after the local saporin injection (**post-injection condition**) in the same rats (group **L-SD-saporin**, N=6). We first evaluated the changes during the entire 18h of recovery sleep after 6h SD by comparing them to the respective baseline (recording performed on pre-SD day) both in pre-injection and post-injection conditions. Then we evaluated the difference between changes in the pre-injection control and post-injection conditions. Similar measures were performed in animals receiving saline (group **L-SD-saline**, N=5).

In saporin injected group (**L-SD-saporin**) in pre-injection control condition, the total time spent in NREM sleep over 18h period after 6h SD was increased by $29.0 \pm 3.7\%$ as compared to its baseline (t-test, $t=4.671$, $P<0.05$). After saporin injection, however, there was only non-significant increase by $9.1 \pm 8.1\%$ (t-test, $t=0.957$, $P>0.05$). A repeated measures ANOVA (RM ANOVA) comparison revealed a significant difference between amount of recovery NREM sleep over 18h period in pre-injection control vs. post-injection conditions (RM ANOVA, $F(1,5)=7.316$, $P=0.043$) (Figure 2A). A post-hoc t-test applied to each of the 6h bins in the 18h period showed that the increase in NREM sleep was

significantly higher in the first 6h bin in the pre-injection control vs. the post-injection conditions ($t=7.162$, $P<0.001$) (see Figure 2B). With respect to REM sleep, the total time spent in REM sleep within 18h after 6h SD was increased by $91.8\pm15.0\%$ in the pre-injection control condition compared to its baseline (t -test, $t=8.660$, $P<0.001$), and by $58.3\pm22.4\%$ after saporin injection (t -test, $t=3.701$, $P<0.05$). The percentage REM sleep changes were not significantly different during 18h of recovery sleep in pre-injection vs. post-injection conditions (RM ANOVA, $F(1,5)=1.220$, $P=0.32$) (Figure 2C).

The NREM delta power during 18h of recovery NREM sleep following 6h SD was increased by $40.5\pm8.2\%$ in the pre-injection control condition compared with its baseline (t -test, $t=2.251$, $P<0.05$). However, after saporin injection the increase was minimal and statistically non-significant ($5.2\pm8.7\%$; t -test, $t=0.03$, $P>0.05$). The percentage increases in the pre-injection control and post-injection conditions were significantly different (RM ANOVA $F(1,5)=6.617$, $p=0.048$) (Figure 3A). Post-hoc comparisons showed that NREM delta power was significantly higher in the first 6h bin of recovery in the pre-injection control vs. the post-injection conditions (t -test, $t=2.236$, $P<0.05$) (Figure 3B). To define whether the blunted decrease in NREM delta power in saporin injected animals encompassed the full range of frequencies, we analyzed the EEG power spectrum density in the range 0–15Hz with resolution of 0.4 Hz (between 0.4 to 14.8Hz) in NREM sleep during the first 6 h after 6h SD. Of all frequencies measured, delta activity in pre-injection control conditions was significantly higher as compared to post-injection conditions only in the low frequency delta range (0.4–2.4Hz) (RM ANOVA $F(5,59)=3.148$, $P<0.001$; Holm-Sidak post hoc test, $2.315 \leq t \leq 5.615$, all P 's <0.05) (Figure 3C).

In saline injected group (**L-SD-saline**), injection of saline into the HDB/SI/MCPO area did not induce any significant differences in pre-injection control vs. post-injection conditions over the post-SD 18h period in measures of NREM recovery sleep (RM ANOVA $F(1,4)=0.047$, $p=0.839$), NREM delta power (RM ANOVA $F(1,4)=0.033$, $p=0.866$), nor in REM recovery sleep (RM ANOVA $F(1,4)=1.013$, $p=0.371$).

A between group comparison of NREM sleep and NREM delta power after 6h SD in saporin injected (**L-SD-saporin**) and saline injected (**L-SD-saline**) animals revealed no difference in pre-injection control condition (NREM, t -test, $t=0.279$, $p=0.787$ and NREM delta, $t=0.756$, $p=0.469$). However, after injection there was a significant difference in NREM sleep after 6h SD, with saline injected rats showing a significantly greater increase than saporin injected rats (t -test, $t=2.323$, $p=0.045$). The NREM delta also showed significantly greater increases in the saline injected group compared with the saporin injected group (t -test, $t=2.329$, $p=0.045$).

2. Effects of local saporin administration 2 weeks post-injection on adenosine-induced sleep

Changes in the sleep response after the infusion of adenosine ($300\mu\text{M}$) for 3h (see experimental schedule in Table 1B) into BF were evaluated by comparing the time spent in NREM sleep, REM sleep and change in NREM delta power during the 18h post-infusion period (group **L-AD-saporin**, $N=6$) (Figure 4A) before saporin injection (**pre-injection control condition**) and 2 weeks after the saporin injection (**post-injection condition**).

In pre-injection control conditions, adenosine infusion resulted in an overall 18h increase in NREM sleep of $21.0\pm6.4\%$ as compared with its baseline (t -test, $t=2.673$, $P<0.05$); in contrast there was only non-significant increase by $4.0\pm7.2\%$ 2 weeks after saporin injection (t -test, $t=0.236$, $P>0.05$). These percentage increases were significantly different (RM ANOVA, $F(1,5)=5.139$, $P<0.02$). Post-hoc t -tests revealed that the increase in NREM sleep

in post-injection vs. pre-injection control conditions was significantly lower over both the first 6h ($t=2.330$, $P<0.05$) and second 6h ($t=2.502$, $P<0.05$) bins after infusion (Figure 4B).

Similarly, NREM delta power within the 18h after adenosine infusion in pre-injection control conditions was increased by $22.0\pm2.4\%$ as compared to respective baseline, but in post-injection conditions – only by $5.4\pm4.7\%$ (t -test, $t=0.999$, $P>0.05$). This difference between percentage increases in pre-injection control vs. post-injection conditions was significant (RM ANOVA $F(1,5)=13.153$, $P=0.015$). Pair wise t -test comparisons revealed significant differences between these two conditions in the first 6h after infusion ($t=2.480$, $P<0.05$) (Figure 4C).

In contrast, REM sleep was almost equally increased during the 18h after adenosine infusion as compared with its baseline in the pre-injection control condition ($+27.5\pm6.5\%$) and after saporin treatment ($+16.2\pm5.2\%$). Percentage increases in pre- and post-injection conditions were not significantly different (RM ANOVA, $F(1,5)=0.911$, $P=0.384$) (Figure 4D).

3. Effects of ICV saporin administration 2 and 3 weeks post-injection on adenosine accumulation during SD and recovery sleep response

Previous study by Blanco-Centurion et al. (2006) revealed no change in homeostatic sleep response 2 weeks after ICV saporin injection. However, measurements of adenosine concentration during SD in the BF, performed one week later, showed a dramatic decrease. Here we tested the hypothesis that effect of ICV-saporin administration can be time-dependent and correlated the BF (HDB/SI/MCPO) adenosine concentration during 6h SD with the homeostatic sleep responses following 6h SD in the same rats 2 and 3 weeks post-ICV saporin using the dose ($6\mu\text{g}$) described by Blanco-Centurion et al. (2006).

3.1. Adenosine release during sleep deprivation following ICV saporin injection—We examined the SD-induced changes in adenosine in rats sleep-deprived for 6h which received ICV injection of saporin (**ICV-SD-saporin**, $N=7$) 2 weeks after injection (**2 weeks post-injection condition**) and 3 weeks after injection (**3 weeks post-injection**). Microdialysates were collected 2 h before (**pre-SD baseline**), during SD and 2 h after the end of SD in conjunction with simultaneous EEG recording (Table 1A). As a treatment control, the same measurements were performed after 2 and 3 weeks in the group of rats injected with saline (group **ICV-saline**, $N=5$).

In saporin injected group (**ICV-SD-saporin**) 2 weeks post-injection, adenosine levels in HDB/SI/MCPO (see Figure 5A for probe tips locations) during 6h SD (average of 5 every other 30min samples) was increased by $141.7\pm23.0\%$ as compared to 2h pre-SD baseline (average of 2 every other samples, paired t -test, $t=4.102$, $P<0.05$). In contrast, 3 weeks after saporin injection in the same rats adenosine concentrations were increased only by $9.9\pm7.3\%$ compared to the 2h pre-SD baseline (paired t -test, $t=0.459$, $P>0.05$). These differences in adenosine increases at 2 and 3 weeks post-injection were statistically significant (RM ANOVA, $F(1,6)=30.152$, $P=0.002$) (Figure 5B).

In contrast, in the saline-treated rats (**ICV-SD-saline**), increase in adenosine levels observed 3 weeks post-injection did not differ from that at 2 weeks post-injection (data not shown). Comparison of SD-induced adenosine level between **ICV-SD-saline** and **ICV-SD-saporin** groups showed no difference 2 weeks post-injection (t -test, $t=0.148$ (10), $p=0.88$), whereas 3 weeks post-injection the SD-induced increase in the saline-treated rats was significantly higher (t -test, $t=-2.531$ (10), $p=0.03$) when compared to the saporin-injected group.

3.2 Homeostatic sleep response after sleep deprivation following ICV saporin injection—The changes in the homeostatic sleep response were determined by comparing

the time spent in NREM sleep, REM sleep and delta power during NREM sleep over 18h of recovery sleep after 6h SD in the same rats (group **ICV-SD-saporin**, N=7) 2 weeks after ICV saporin injection (**2 weeks post-injection condition**) and 3 weeks after ICV saporin injection (**3 weeks post-injection**). Similar measurements were performed in the group of rats injected with saline (group **ICV-SD-saline**, N=5) which served as a treatment control.

In saporin-injected group (**ICV-SD-saporin**) 2 weeks post-injection recovery NREM sleep over the 18h period following 6h SD was significantly increased by $46.5 \pm 10.2\%$ compared with its baseline (t-test, $t=3.493$, $P<0.05$). In contrast, 3 weeks after injection there was no statistically significant increase in NREM sleep after SD ($+1.7 \pm 5.7\%$, t-test, $t=0.882$, $P>0.05$). These differences in NREM percentage increases between 2 and 3 weeks post-injection conditions were statistically significant (RM ANOVA, $F(1,6)=14.181$, $P=0.009$) (Figure 6A). Post-hoc t-test pair-wise comparisons showed significant differences between 2 weeks and 3 weeks post-injection groups for the first 6h ($t=2.828$, $P<0.05$, Figure 6B) and second 6h ($t=3.815$, $P<0.05$) bins of recovery NREM sleep. REM sleep was significantly increased both 2 weeks ($+73.1 \pm 11.8\%$, t-test, $t=5.287$, $P<0.001$) and 3 weeks ($+93.4 \pm 15.0\%$, t-test, $t=2.936$, $P<0.05$) post-injection as compared with their respective baselines, without a significant overall difference between changes observed 2 and 3 weeks after injection (RM ANOVA, $F(1,6)=1.286$, $P=0.3$) (Figure 6C).

Two weeks post-injection, delta power during the 18h recovery NREM sleep period increased by $81.2 \pm 17.8\%$ as compared with its baseline (t-test, $t=2.298$, $P<0.05$), but 3 weeks post-injection the increase was only $14.5 \pm 6.6\%$ (t-test, $t=2.269$, $P<0.05$) (Figure 7A). These differences in delta activity increase at 2 and 3 weeks post-injection were highly significant (RM ANOVA, $F(1,6)=14.607$, $P=0.009$). Post hoc t-tests showed that delta activity for the 2 weeks post-injection condition was significantly higher than for 3 weeks post-injection condition in the first 6h ($t=3.915$, $P<0.05$) (Figure 7B) and second 6h ($t=3.150$, $P<0.05$) bins.

In saline injected group (**ICV-SD-saline**) there was no significant difference in the 18h period after SD in NREM sleep (RM ANOVA, $F(1,4)=0.529$, $P=0.507$), NREM delta power (RM ANOVA, $F(1,4)=0.028$, $P=0.875$) and REM sleep (RM ANOVA, $F(1,4)=0.018$, $P=0.9$) between 2 and 3 weeks post-injection conditions.

A between group comparison of ICV saporin injected group (**ICV-SD-saporin**) and ICV saline injected group (**ICV-SD-saline**) showed that the increases in NREM sleep after 6h SD were similar when measured 2 weeks after injections (no statistical difference (t-test, $t=1.643$, $p=0.131$)). However, the increase in recovery NREM sleep after SD was significantly greater after 3 weeks in saline injected group as compared with saporin injected group (t-test, $t=-2.474$, $p=0.03$). Similarly, there was no significant difference in the NREM sleep delta after SD 2 weeks after saporin and saline injections (Mann-Whitney Rank Sum Test, $T=33.5$, $p=0.876$), whereas after 3 weeks, NREM delta was significantly higher increased in the saline injected group when compared to the saporin injected group (Mann-Whitney Rank Sum Test, $T=45.5$, $p=0.03$).

4. Comparison of the histological effects induced by local and ICV 192 IgG-saporin injections: Stereological counting of cholinergic cells

An average section thickness of $\sim 40 \mu\text{m}$ was measured for all the sections consistent with 20% shrinkage during the process of ChAT immunostaining. The darkly stained ChAT-positive cells were evenly distributed through the entire thickness of the sections. The ChAT-positive cells were of medium ($20 \mu\text{m}$) to large ($35 \mu\text{m}$) size, mostly fusiform in shape with a few interspersed polygonal cells. Cell counting in the locally lesioned animals were done ipsilateral to the injection and the numbers presented are for this side. In saporin

injected animals darkly stained cells (as seen in saline treated rats) were counted. Some lightly stained cells with a 'ghost-like' outline were observed in lesioned rats, but not seen in saline rats; these were considered to be 'dying cholinergic cells' and not counted. There was an overall significance determined by One Way ANOVA on the changes in the cholinergic cell count ($F=38.041$; $p<0.001$) between the groups of: (i) Saline injected; (ii) 2 weeks after local injection; (iii) 2 weeks after ICV injection; and (iv) 3 weeks after ICV injection. Post hoc pair-wise comparisons revealed significant reduction in cholinergic neurons in all three saporin conditions when compared to saline treated rats. The total number obtained in control (saline injected) animals using the Optical Fractionator method of Stereoinvestigator was 8619 ± 846.9 on one side (BF volume $1.18 \pm 0.07 \text{ mm}^3$) (see Table 2, Figure 8). Rats with local injections had 1070.82 ± 174.4 (12.4% of saline, $p<0.004$, CBF volume $1.04 \pm 0.03 \text{ mm}^3$). The ICV injected rats showed a time dependent decrease in the surviving cholinergic cells. The rats killed 2 weeks post-injection had an average of 3567.16 ± 392.6 ChAT- positive cells (41% of saline count, $p<0.001$, BF volume $1.14 \pm 0.14 \text{ mm}^3$), whereas rats killed 3 weeks post-injection had significantly reduced ChAT-positive mean cell counts of 1129.67 ± 168.2 (13.1% of saline, $p<0.001$, BF volume $1.08 \pm 0.05 \text{ mm}^3$) (Table 2, Figure 8).

The effect of cholinergic cell loss was also observed at the level of cortex where staining of cholinergic processes using an AChE antibody showed the highest density in the saline treated rats and a clearly visible reduction in rats that were locally injected with saporin (Figure 9). The rats that were killed 2 weeks post ICV injection had less dense AChE stained processes when compared to the saline controls but more than the density of rats killed 3 weeks post ICV injection, as well as rats with local injections (Figure 9).

In addition to the stereological counting just described, non-stereological counting of GAD-positive cells in locally saporin-injected rat was performed simultaneously with probe localization verification at the end of experiments. Counting of GAD-positive cells (Figure 8G) indicated that saporin did not affect this neuronal population (94% survived, data not shown).

DISCUSSION

The results of this study, summarized in table 3, have two major findings. **First**, local injection of saporin into the HDB/SI/MCPPO at 2 weeks post-injection is highly effective in producing: i) a marked reduction in SD-induced adenosine increase; ii) a decrease in the homeostatic sleep response, as measured by changes in the percentage of recovery NREM sleep and delta power during recovery NREM sleep; iii) attenuation of the sleep-inducing effects of adenosine infusion into BF; and iv) an 88% cholinergic cell loss. **Second**, ICV injections of saporin have a longer time course for cholinergic cell degeneration than local injections, which delays changes in adenosine release and sleep. At **2 weeks post-injection** we observed: i) intact adenosine release during SD; ii) intact homeostatic sleep response; iii) only 59% cholinergic cell loss. However, at **3 weeks post-injection** we observed: i) dramatic decrease in SD-induced adenosine release; ii) significant decrease in homeostatic sleep response; iii) an 87% cell cholinergic cell loss.

Taken together, these results provide, in our opinion, compelling evidence for the importance of cholinergic neurons in: 1) causing the increase in SD-induced extracellular adenosine accumulation in the BF; 2) mediating the effects of adenosine in the BF on sleep, as evinced by decreased NREM sleep and delta activity after adenosine infusion.

Circadian variations in the levels of adenosine in different brain areas have been reported by several investigators, strongly supporting its role in sleep-wake regulation (Chagoya de

Sanchez et al., 1993; Huston et al., 1996). Pharmacological studies of the effects of an A1 receptor agonist together with glycogen measurements led to the suggestion that adenosine plays a role of biochemical substrate for energy restoration during sleep (Bennigton and Heller, 1995). Measurements of extracellular adenosine levels using *in vivo* microdialysis from our and other laboratories showed a progressive and selective increase in adenosine during prolonged wakefulness in both cats (Porkka-Heiskanen et al., 1997, 2000) and rats (Basheer et al., 1999; McKenna et al., 2003; Kalinchuk et al., 2003; Murillo-Rodriguez et al., 2004), and a slow decline during recovery sleep. Measured over 6h of SD, the increase in extracellular adenosine was most marked in the BF, where increases were 140% of baseline, and increases were present to a lesser extent in the cortex; SD-related increases were not seen in the other five sleep-wake related brain regions including thalamus, preoptic area of hypothalamus, dorsal raphe nucleus and pedunculopontine tegmental nucleus (Porkka-Heiskanen et al., 2000).

The current saporin lesion data indicate that cholinergic neurons play a major, although not exclusive, role in the phenomenon of adenosine increase with SD. A decrease in extracellular adenosine levels was also reported by Blanco-Centurion et al., (2006) following **3 weeks** after ICV saporin injection. They also reported no reduction in the NREM sleep and delta power or homeostatic sleep response when measured **2 weeks** after ICV saporin injection, consistent with our data (Table 3). However the present data make it clear that the adenosine and the NREM sleep/delta response to SD were decreased **3 weeks** after ICV saporin injection, a time point when these variables were not measured simultaneously in the Blanco-Centurion et al., (2006) study. Table 3 illustrates the temporal dynamics of changes in SD-induced adenosine and in homeostatic sleep response after ICV saporin injection and emphasizes the need for simultaneous measurements of all studied parameters.

In addition to the impaired homeostatic sleep response after SD, our data also show that in cholinergic-lesioned rats exogenous adenosine infusion into the HDB/SI/MCPO fails to produce somnogenic effects, implying an important role for cholinergic neurons. Our finding that loss of cholinergic cells alone results in decreased NREM and delta during NREM sleep following SD is in agreement with a recent report by Kaur et al. (2008) of the effects of bilateral local injections of saporin and ibotenic acid into the caudal part of the BF (NBM/SI). These authors found that both ibotenic and saporin lesions were effective in reducing rebound NREM sleep (32 and 77% less respectively) and NREM delta power (78% and 53% less), and concluded that both cholinergic and non-cholinergic BF neurons play important roles in recovery NREM sleep and increased delta power after SD. The present data indicate a 60–75% reduction in NREM delta rebound following local and ICV saporin, somewhat higher than Kaur et al. (2008), perhaps due to the fact that in our study injections were more rostral and affected mostly HDB/SI/MCPO region. The present data on adenosine effects, a measure not done by Kaur et al. (2008) strongly indicate that lesions of cholinergic neurons reduce NREM delta response to infused adenosine, further supporting a major role of cholinergic neurons in producing delta in NREM rebound sleep. *In vitro* studies have shown that both cholinergic and noncholinergic neurons in the BF are hyperpolarized by adenosine (Arrigoni et al., 2006) and *in vivo* studies have shown that BF wake-active neurons are inhibited by adenosine (Alam et al., 1999; Thakkar et al., 2003a) while cholinergic neurons have been shown to be wake-active (Lee et al., 2005). These studies and the present data thus support the hypothesis that adenosine may inhibit both cholinergic and non-cholinergic neurons to produce the sleep-inducing effects.

Our data strongly demonstrate that lesion of cholinergic neurons in HDB/SI/MCPO region follows slower time course as compared to local injections showing greater (87%) cell loss 3 weeks post-injection as compared to 2 weeks post-injection (59%). This observation

supports other reports showing differences in the extent of cholinergic cell loss in the different parts of the BF after ICV saporin injections when measured at the same post-injection time. For example, the loss of cholinergic neurons is much lower in caudal BF as compared to MS/VDB after ICV injection of 192 IgG-saporin in rats (44–68% vs. 75–87%, Wrenn et al., 1999; 65% vs. 90%, Traissard et al., 2007) and its analogue mu p75-saporin in mice (55–43% vs. 82%, Moreau et al., 2008). Also, there are indications that, with increase in the dose of injected ICV saporin, the percentage of cholinergic lesion in the caudal BF is higher (Wrenn et al., 1999) and that behavioral changes, such as myoclonic and/or tonic convulsions, develop faster (Moreau et al., 2008). Our study, to our knowledge, is the first to demonstrate a time dependent increase in cholinergic lesion after ICV saporin injections that is well corroborated with the SD-induced adenosine increase and homeostatic sleep response. The delayed effect may be due to, i) slower diffusion of saporin from ventricles through parenchyma to HDB/SI/MCPO when compared to rostral areas, and/or ii) the decrease in the effective concentration of saporin during the course of this diffusion. In support of this suggestion, we found that when saporin was administered locally into the HDB/SI/MCPO, the lesion of cholinergic cells in this area was developing faster. It is important to mention that in our study we used the same batch of immunotoxin: it is known that its potency might vary from batch to batch, and thus might potentially underlie the difference in results reported by different laboratories.

Another important outcome of this study is the recognition of the threshold effect on behavior for the number of cholinergic neurons. All three parameters, SD-induced adenosine, NREM recovery sleep and NREM delta remained unchanged with a cell loss of 59% but showed dramatic reduction with 87% cell loss. Thus, the critical level of the BF cholinergic cell loss to affect the homeostatic sleep response is appears to be more than 60%. In agreement with this observation, in study by Kaur et al. (2008) the cell loss of 69% was able to induce impairment in sleep homeostasis. Other studies have also shown a critical threshold level of lesion needed to impact behavior. For example, in measurement of effects of local saporin injections, there was no behavioral effect in a non-match to sample paradigm one week after injection (73–82% cholinergic cell loss) but marked deficits at one month (88–95% loss; Paban et al., 2005; Chambon et al., 2007). Also, there is an indication that after ICV saporin administration impaired passive avoidance performance is observed only when >80% of NBM cholinergic cells is lesioned (Wrenn and Whiley, 1998). The presence of a critical threshold level for behavioral effects to occur is present for dopamine where ~60% dopamine cell loss in the substantia nigra is required to produce Parkinsonism symptoms (Lang, 2007). Interestingly, it seems that SD-induced adenosine release and recovery sleep were affected only when lesion in the caudal areas of the BF reached its threshold level. In contrast, the level of cholinergic cells lesion in the rostral areas (MS/VDB) did not have any impact: the fully evolved effects of ICV injection, which destroys both rostral and caudal areas at 3 weeks post-injection, and local saporin injection into caudal regions, which do not affect the MS/VDB (Kaur et al., 2008; our own observations) were similar. This allows us to suggest that MS/VDB cholinergic cells are not important in sleep homeostasis parameters measured here.

To our knowledge, this study is the first using stereological techniques for ChAT positive cell counting after saporin-induced lesions. The BF cholinergic cell count in non-lesioned animals (saline injections) described in our study, 8620 ± 847 unilaterally and hence a bilateral estimate of 17240, very closely matches two previous studies reporting totals of 16,715 (Miettinen et al., 2002) and 16,075 (Gritti et al., 2006), studies that used similar methods of unbiased cell counting with optical fractionator/Stereoinvestigator. The congruence of values supports the accuracy of our stereological counts. Differences in cell loss counts 3 weeks following ICV saporin injection in our study (~87%) and in a non-stereologic report (98%) (Blanco-Centurion et al., 2006) may be due to the 3-dimensional

stereological counting method we used, which has been suggested to yield an unbiased/more accurate estimate of cell numbers than non-stereologic techniques (Coggeshall and Lekan, 1996). Non-stereologic count, used in our other study, indicated 95% loss at 2 weeks after local saporin injection, which was higher than our stereologic counts and more congruent with a 98% loss reported by Blanco-Centurion et al., (2006), and 55% cell loss at 2 weeks after ICV saporin injection which was not far from our stereologic count of 59% loss (A.V. Kalinchuk, unpublished observations).

Taken together, these results provide important evidence for a major but not exclusive role of BF cholinergic neurons in sleep homeostasis, and for the role of extracellular adenosine as a mediator of this response. The effect of cholinergic lesions was restricted to NREM recovery sleep while REM recovery remained unchanged, suggesting different regulatory mechanisms. Although BF cholinergic neurons have been shown to be maximally active during REM sleep (Lee et al., 2005), absence of any effects on REM rebound suggests non-cholinergic mechanisms also importantly underlie the homeostatic REM response. Previously we have found a similar differential effect of NO donor on NREM and REM sleep (Kalinchuk et al., 2006b).

What features of cholinergic neurons might make the effects of lesions so dramatic? BF cholinergic neurons uniquely respond to adenosine by increasing intracellular calcium release in vitro and nuclear translocation of the transcription factor NF- κ B in vivo, also seen after 3h of SD (Basheer et al., 2002; Ramesh et al., 2007). Blocking nuclear translocation of NF- κ B during SD significantly reduced the recovery NREM delta activity as well as the upregulation of A1 receptor protein that follows sleep deprivation (Ramesh et al., 2007; Basheer et al., 2007). SD-increased delta activity, especially low frequency delta, is one of the hallmarks of the homeostatic response (Tobler and Borbely, 1990; Franken et al., 1991). In the present study we found that lesion of the BF cholinergic neurons dramatically affected low frequency delta power (0.4–2.4Hz). In a recent report of a hypofunctioning BF cholinergic system in an animal model of Alzheimer's disease showed decreased low frequency delta activity in NREM, thus also suggesting a role of the cholinergic system in sleep homeostasis (Wisor et al., 2005).

In summary, this study has shown that the effects of BF adenosine on homeostatic sleep are importantly mediated by cholinergic neurons and that the time course of saporin cholinergic lesions must be taken into account in assaying effects.

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Abbreviations

ABC	avidin-biotin complex
AC	anterior commissure
AChE	acetylcholinesterase
ACSF	artificial cerebrospinal fluid

ANOVA	analysis of variance
BF	basal forebrain
CE	coefficient of error
Cg	cingulate cortex
ChAT	choline acetyltransferase
ChAT-ir	cholinacetyltransferase immunoreactivity
DAB	diaminobenzidine
EEG	electroencephalogram
EMG	electromyogram
GABA	gamma-aminobutyric acid
GAD	glutamic acid decarboxylase
HDB	horizontal diagonal band
ICV	intracerebroventricular
IgG	immunoglobulin G
M2	secondary motor cortex
MCPO	magnocellular preoptic area
MS	medial septum
NBTI	S-(p-nitrobenzyl)-6-thioinosine
NF-κB	nuclear factor- κ B
NREM sleep	non-rapid-eye movement sleep
OX	optic chiasm
PBS	phosphate buffer
REM sleep	rapid-eye movement sleep
RM-ANOVA	repeated measures analysis of variance
SCN	suprachiasmatic nucleus
SD	sleep deprivation
SI	substantia innominata
UV	ultraviolet
VDB	vertical diagonal band

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Local BF saporin

Figure 1.

The effects of local saporin injection into the BF on adenosine release during 6h SD at 2 weeks post-injection. Adenosine was sampled on the side ipsilateral to injection. (A) Example of histology of probe tip localization in HDB (horizontal diagonal band) where saporin was injected and adenosine samples were collected. AP=-0.26mm. Scale bar: 2mm. (B) Localization of the microdialysis probe tips in 1) HDB; 2) MCPO (magnocellular preoptic region); and 3) SI (substantia innominata) (group L-SD-saporin, N=6). Arrow indicates site shown in (A). (C) Average changes in adenosine concentrations during SD before (Control, black bar) and after saporin injection (Saporin, open bar). For both pre- and post-injection conditions, 5 measurements from 6h SD period were averaged and expressed as percentage of the respective pre-SD baseline (2 measurements averaged). Note increases in adenosine levels during the 6h SD in control conditions were significantly higher than those after the saporin lesion (#, $P<0.05$). * indicates that in the pre-injection control condition, SD values vs. pre-SD baseline were significantly different ($P<0.05$).

Local BF saporin

Figure 2.

The effects of local saporin injection into BF on recovery NREM sleep and REM sleep after 6h SD (group L-SD-saporin, N=6) 2 weeks post-injection. (A) After saporin injection (open circles) recovery NREM sleep was significantly decreased as compared to pre-injection control conditions (closed circles). Values are grand mean parentages of baseline day values for each of 6h bins calculated for each rat. (B) Data for individual animals for first 6h of recovery sleep are shown as percentage change relative to baseline before and after saporin injection. (C) REM sleep was not affected. The shaded area indicates the first 18h period after SD, as used for percentage difference calculations presented in the text. **= $p < 0.001$, significant difference between saporin and control conditions. The X-axis in A and C shows lights on period (open horizontal bars), SD during the light period (hatched horizontal bar) and lights off period (black horizontal bar). Error bars in this and subsequent figures are $\pm$ SEM.

Local BF saporin

Figure 3.

The effects of local saporin injection into the BF on delta power during recovery NREM sleep after 6h SD 2 weeks post-injection. (A) Overall delta power (0.4–4.5Hz) during NREM recovery sleep was significantly decreased after saporin treatment (open circles) as compared to control conditions (closed circles). Values are grand mean percentages of respective baseline day values for each of 6h bins calculated for each rat. Shaded area indicates the first 18h period after SD, used for calculation of percentage difference presented in the text. Effect was maximal during first 6h of recovery sleep. $^* = P < 0.05$. (B) Percentage change after SD relative to baseline for each animal for the first 6h of recovery sleep. (C) Spectral analysis of 6h period after SD. $^* = P < 0.05$, $^{**} = P < 0.001$. Note EEG delta power was significantly decreased in the range 0.4–2.4Hz.

Local BF saporin

Figure 4.

The effects of local saporin injection on adenosine infusion-induced NREM sleep, NREM delta power and REM sleep at 2 weeks post-injection. Values are grand mean percentages of respective baseline day values for each of 6 hour bins for each rat. (A) Localization of microdialysis probe tips in HDB/Sl/MCPO (as identified in Fig. 1) where adenosine was infused (group L-AD-saporin, N=6). (B) After local saporin injection (open circles) recovery NREM sleep was significantly decreased as compared to pre-saporin control conditions (closed circles). $^* = P < .05$ here and in following two panels. (B) Overall delta power during NREM recovery sleep was significantly decreased after saporin treatment as compared to pre-saporin control. (C) REM sleep was not different at pre- and post-injection conditions. Shaded area indicates the first 18h period after SD, used for percentage difference calculations described in text.

ICV saporin

Figure 5.

The effect of ICV saporin injection on adenosine release during SD measured 2 and 3 weeks post-injection in the same animals. (A) Localization of the microdialysis probe tips for adenosine measurements in HDB/SI/MCPO (as identified in Fig.1) (group ICV-SD-saporin, N=7, closed circles). (B) Changes in average SD-induced adenosine concentrations 2 weeks (black bars) and 3 weeks (open bars) after ICV saporin injection. For both conditions, 5 measurements from 6h SD period were averaged and expressed as percentage of the respective pre-SD baseline (2 measurements averaged). Note that 2 weeks post-injection, the level of adenosine was significantly increased, while 3 weeks post-injection it remained unchanged and was significantly different from that 2 weeks post-injection. *= $P < 0.05$, SD vs. pre-SD baseline.

ICV saporin

Figure 6.

The effects of ICV saporin injection on recovery NREM sleep and REM sleep after 6h SD, measured 2 and 3 weeks post-injection in the same animals (group ICV-SD-saporin, N=7). (A) NREM sleep is significantly decreased over 18h period at 3 weeks (open circles) as compared with 2 weeks (closed circles). Values are expressed as percentage of respective baseline day values for each of the 6 hour bins calculated for each rat. $*=P<.05$. (B) Data for individual animals for first 6h of recovery sleep are shown as percentage change to respective baseline 2 and 3 weeks after saporin injection. (C) REM sleep did not differ between 2 and 3 weeks post-injection over 18h period. Shaded area indicates the first 18h period after SD, used for percentage difference calculations presented in the text.

ICV saporin

Figure 7.

The effects of ICV saporin injection on delta power during recovery NREM sleep after 6 h SD measured 2 weeks and 3 weeks post-injection in the same animals (group ICV-SD-saporin, N=7). (A) NREM sleep delta power is significantly decreased over 18h period at 3 weeks (open circles) as compared with 2 weeks (closed circles). Values are expressed as percentages of respective baseline day values for each of 6 hour bins calculated for each rat. *=P<.05. Shaded area indicates the first 18h period after SD, used for percentage difference calculations presented in the text. (B) Normalized data to respective baselines for individual animals for the first 6h of recovery sleep, when maximal changes were observed.

Figure 8.

ChAT-positive and GAD-positive neurons in the MCPO/SI/HDB after local and ICV saporin injection. (A) Coronal schematic from Paxinos and Watson (1998) indicating regions magnified in B–G. (B and C) ChAT-positive cells after saline control injection, 4X (large square in A schematic) and 20X (small square in A). Square in B shows area which magnified in C–G. D) ChAT-positive cells 2 weeks after ICV saporin injection. E) 3 weeks after ICV saporin injection. F) ChAT-positive cells 2 weeks after local saporin injection. G) GAD-positive cells 2 weeks after local saporin injection. Note that the decreases in ChAT-positive neurons relative to control at 2 weeks after local saporin and 3 weeks after ICV saporin injection were approximately equivalent, in contrast to 2 weeks after ICV injection

when many more ChAT-positive cells remain. Scale bar: 100 μ m. Abbreviations. AC=anterior commissure. OX=optic chiasm.

Figure 9.

AChE-positive fibers in cortex after saporin injected locally in the BF and ICV. (A) Schematic from Paxinos and Watson (1998) showing cortical area magnified in B–F. (B and C) After saline control injection, at 4X (B) and 20X(C). Square in B shows area which was magnified in C–F. (D) 2 weeks after local saporin injection. (E) 2 weeks after ICV saporin injection. (F) 3 weeks after ICV saporin injection. Note that the decreases in AChE-positive fibers were similar when measured 2 weeks after local and 3 weeks after ICV saporin injection, in contrast to 2 weeks after ICV injection when many more AChE-positive fibers remain. Scale bar: 100µm. Abbreviations: M2, secondary motor cortex; Cg, cingulate cortex.

Table 1

Protocol for microdialysis experiments. (A) Timing of 6h sleep deprivation (SD) experiments with simultaneous sample collection for adenosine (experiments 1 and 3). White background = periods when microdialysis leads were attached and microdialysis samples for adenosine were collected; hatched background = periods when microdialysis leads were connected/disconnected. (B) Schedule of adenosine infusion experiment (experiment 2). White background indicates perfusion with ACSF or adenosine. Hatched background as in (A). Grey colour indicates periods when only EEG/EMG recording leads were connected. Light on: light off were at 8.00AM:8.00PM.

	8AM	9AM	10AM	11AM	12PM	1PM	2PM	3PM	4PM	5PM	6PM	7PM
A	<div><div></div><div>ACSF spontaneous sleep-wake</div><div></div><div>ACSF SD</div><div></div><div>ACSF recovery sleep</div><div></div></div>											
B	<div><div></div><div>ACSF spontaneous sleep-wake</div><div>Adenosine infusion spontaneous sleep-wake</div><div>ACSF spontaneous sleep-wake</div><div></div><div></div><div></div></div>											

Cholinergic cell counting using StereoInvestigator in HDB/SI/MCPO region (Bregma, 0 to -0.8).

Table 2

Treatment	Sections	Sites sampled	Cells counted	Sampled volume (mm ³)	Cell number	% of saline	p values
Saline control (n=4)	1 in 5, 3 sampled	130.25±8.2	71.5±10.4	1.18±0.07	8619.75±846.9	100	
local (after 2 weeks) (n=4)	1 in 5, 3 sampled	114.0±3.0	10.5±1.7	1.04±0.03	1070.82±174.4	12.4	p<0.004 *
ICV (after 2 weeks) (n=4)	1 in 5, 3 sampled	124.0±15.89	35.25±3.9	1.14±0.14	3567.16±392.6	41	p<0.001 *, p<0.01 **
ICV (after 3 weeks) (n=4)	1 in 5, 3 sampled	118.0±6.0	10.5±1.3	1.08±0.05	1129.67±168.2	13.1	p<0.001

* saline versus 2 weeks;

** 2 weeks versus 3 weeks post-injection, see text for sites sampled and statistics description.

Table 3

Summary of results.

Measure	Local injection 2 weeks post-injection	ICV injection 2 weeks post-injection	ICV injection 3 weeks post-injection
Adenosine release during SD	Decreased	Not affected	Decreased
NREM recovery sleep	Decreased	Not affected	Decreased
NREM delta power	Decreased	Not affected	Decreased
Adenosine-induced sleep	Decreased		
% loss of cholinergic cells	88%	59%	87%