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CHOLINERGIC NEURONS OF THE BASAL FOREBRAIN MEDIATE BIOCHEMICAL AND ELECTROPHYSIOLOGICAL MECHANISMS UNDERLYING SLEEP HOMEOSTASIS

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Abstract

The tight coordination of biochemical and electrophysiological mechanisms underlies the homeostatic sleep pressure (HSP) produced by sleep deprivation (SD). We have reported that during SD the levels of inducible nitric oxide synthase (iNOS), extracellular nitric oxide (NO), adenosine [AD]_{ex}, lactate [Lac]_{ex} and pyruvate [Pyr]_{ex} increase in the basal forebrain (BF). However, it is not clear whether all of them contribute to HSP leading to increased electroencephalogram (EEG) delta activity during non-rapid eye movement (NREM) recovery sleep (RS) following SD. Previously, we showed that NREM delta increase evident during RS depends on the presence of BF cholinergic (ChBF) neurons. Here, we investigated the role of ChBF cells in coordination of biochemical and EEG changes seen during SD and RS in the rat. Increases in low theta power (5–7Hz), but not high theta (7–9Hz), during SD correlated with the increase in NREM delta power during RS, and with the changes in nitrate/nitrite [NO_x]_{ex} and [AD]_{ex}. Lesions of ChBF cells using IgG 192-saporin prevented increases in [NO_x]_{ex}, [AD]_{ex} and low theta activity, during SD, but did not prevent increases in [Lac]_{ex} and [Pyr]_{ex}. Infusion of NO donor DETA NONOate into the saporin-treated BF failed to increase NREM RS and delta power, suggesting ChBF cells are important for mediating NO homeostatic effects. Finally, SD-induced iNOS was mostly expressed in ChBF cells, and the intensity of iNOS induction correlated with the increase in low theta activity. Together, our data indicate ChBF cells are important in regulating the biochemical and EEG mechanisms that contribute to HSP.

Keywords

adenosine; rat; nitric oxide; inducible nitric oxide synthase

INTRODUCTION

Investigation of the biochemical neural changes triggered during SD, and their correlation with electrophysiological changes, is key towards the understanding the mechanisms

underlying sleep homeostasis. The two-process model of sleep regulation accurately predicts changes in electroencephalogram (EEG) parameters during spontaneous sleep-wake cycles, as well as during sleep deprivation (SD) and recovery sleep (RS) (Borbely, 1982; Daan *et al.*, 1984). However, the biochemical events which underlie these EEG changes are less understood. Recent reports from our group and others have shown that the cholinergic basal forebrain (BF) is an important site involved in homeostatic sleep control (Porkka-Heiskanen *et al.*, 1997; Kalinchuk *et al.*, 2003; Thakkar *et al.*, 2003; Basheer *et al.*, 2004; Murillo-Rodriguez *et al.*, 2004; Methippara *et al.*, 2005; McCarley, 2007). A biochemical cascade crucial for RS response is initiated within the BF during SD (Kalinchuk *et al.*, 2006a; Kalinchuk *et al.*, 2006b). The initial part of this cascade is comprised of rapid (< 1h) induction of inducible nitric oxide synthase (iNOS) in wake-active neurons, which is followed by a release of NO and an increase of extracellular adenosine [AD]_{ex} (Kalinchuk *et al.*, 2006a; Kalinchuk *et al.*, 2006b; Kalinchuk *et al.*, 2010). A parallel increase is observed in the levels of extracellular lactate [Lac]_{ex} and pyruvate [Pyr]_{ex} (Kalinchuk *et al.*, 2003; Wigren *et al.*, 2007) which indicates neuronal activation (Magistretti *et al.*, 1999). However, it is still not clear whether all these biochemical changes correlate with EEG indicators of homeostatic sleep pressure (HSP) and serve as biomarkers of sleep homeostasis.

BF cholinergic (ChBF) neurons are the primary source of cholinergic innervation of the cerebral cortex, and play an essential role in cortical activity and promotion of behavioral states (Jones, 2004). Depletion of ChBF cells using the immunotoxin 192 IgG-saporin (saporin) blocked the increase of [AD]_{ex} (Kalinchuk *et al.*, 2008) and in non-rapid eye movement (NREM) RS (Kalinchuk *et al.*, 2008; Kaur *et al.*, 2008). We therefore hypothesize that ChBF cells are a crucial component of the neural circuitry responsible for HSP, and serve as a link between the biochemical and electrophysiological mechanisms that contribute to HSP.

To test this hypothesis we performed within-animal comparisons of changes in biochemical factors, correlated with EEG indicators of HSP during SD and RS, before and after BF cholinergic lesions. Our data indicate that low frequency (5–7Hz), but not high frequency (7–9Hz), theta power during SD, strongly correlates with NREM delta power during subsequent RS, and serves as a reliable indicator of HSP during SD as previously proposed (Vyazovskiy & Tobler, 2005). Increased levels of extracellular nitrate/nitrite [NO_x]_{ex} and [AD]_{ex}, but not [Lac]_{ex} and [Pyr]_{ex}, strongly correlated with increased low frequency theta. Finally, after cell-specific lesions of the ChBF cells, SD failed to produce increased biochemical and EEG markers typical of HSP, suggesting a homeostatic role of ChBF neurons.

METHODS

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 under 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. The effect of saporin lesions on biochemical and electrophysiological correlates of HSP—We showed previously that the levels of two important biochemical sleep factors, $[\text{NO}_x]_{\text{ex}}$ and $[\text{AD}]_{\text{ex}}$, increased during SD in the rat BF (Basheer *et al.*, 1999; Kalinchuk *et al.*, 2006a). Similarly, extracellular lactate $[\text{Lac}]_{\text{ex}}$ and pyruvate $[\text{Pyr}]_{\text{ex}}$, known markers of neuronal activation, also increase in BF during SD (Kalinchuk *et al.*, 2003; Wigren *et al.*, 2007). We also demonstrated that the increase in $[\text{AD}]_{\text{ex}}$ during 6h SD is prevented following the lesion of the ChBF cells, using local injections of saporin which produced a decreased intensity of NREM delta during RS (Kalinchuk *et al.*, 2008).

Here we performed simultaneous measurements of four biochemical markers, including $[\text{NO}_x]_{\text{ex}}$, $[\text{AD}]_{\text{ex}}$, $[\text{Lac}]_{\text{ex}}$ and $[\text{Pyr}]_{\text{ex}}$ during SD, and correlated these biochemical changes with changes in EEG theta activity measured during SD, proposed to be an electrophysiological marker of HSP during wakefulness (Vyazovskiy & Tobler, 2005). All measurements were performed in the same animals before (pre-injection **control** condition) and 2 weeks after saporin treatment (**saporin** condition, **saporin group**, $N=6$). Another group of rats (**saline group**, $N=6$) injected with saline served as treatment (injection) controls. The experiment included two experimental days. On Day 1 (baseline (**BL**) day), EEG/electromyogram (EMG) was recorded during the spontaneous sleep-waking cycle for 24h starting at 7AM (Lights ON). Artificial cerebrospinal fluid (aCSF) was infused into BF and baseline collection of microdialysis samples was performed between 7AM–3PM. On Day 2 (**SD** day), EEG was again recorded for 24h starting at 7AM, and microdialysis samples were collected during 2h pre-SD baseline (**pre-SD BL**, 7–9AM), 3h SD (9AM–12PM), and 3h RS (12–3PM). To demonstrate the effect of SD on $[\text{NO}_x]_{\text{ex}}$, $[\text{AD}]_{\text{ex}}$, $[\text{Lac}]_{\text{ex}}$ and $[\text{Pyr}]_{\text{ex}}$, we normalized values from samples collected during 3h SD to values evaluated on the same day during 2h pre-SD BL (=100%). Theta power during SD and delta power during recovery NREM sleep were determined by averaging values obtained during 3h SD or first 3h of recovery NREM sleep, normalized to the corresponding time-of-day values collected during BL EEG (=100%).

Experiment 2. The effect of saporin lesions on sleep induced by the infusion of NO donor, DETA NONOate—We previously demonstrated that unilateral administration of the NO donor DETA NONOate into the BF for 3h mimics the effects of SD, producing increased levels of $[\text{NO}_x]_{\text{ex}}$, $[\text{AD}]_{\text{ex}}$, $[\text{Lac}]_{\text{ex}}$, $[\text{Pyr}]_{\text{ex}}$, as well as electrophysiological measures of HSP including increased subsequent NREM sleep and NREM sleep delta power (Kalinchuk *et al.*, 2006a). We also previously reported that saporin lesions of ChBF cells prevented the induction of sleep produced by microdialysis infusion of AD into the BF (Kalinchuk *et al.*, 2008). However, the effect of saporin lesions on NO donor-induced sleep has not been studied. Therefore, to determine if ChBF neurons play a role in mediating the somnogenic effects of NO, we infused DETA NONOate for 3 hours into the BF by reverse microdialysis ipsilateral to the site of BF saporin injection, both

before (pre-injection **control** condition) and 2 weeks after local saporin injection (**saporin** condition). We also recorded corresponding EEG/EMG, to report measured NREM sleep and delta power values (**saporin group**, N=6). Microdialysis samples collection was not performed in this experiment, for our previous experience showed that DETA NONOate significantly interferes with the measurements of other metabolites (Kalinchuk *et al.*, 2006a).

For each of the two evaluated time points (control vs. saporin conditions), each experimental day lasted for 29h. On baseline (BL) day, EEG/EMG was recorded during the spontaneous sleep-waking cycle starting at 7AM. aCSF was infused into BF between 7AM–12PM. On DETA NONOate infusion day, EEG/EMG was again recorded starting at 7AM, and animals were infused with aCSF between 7–9AM, followed by DETA NONOate infusion between 9AM–12PM. In this experiment, we evaluated the long-term changes in NREM sleep and delta power within 24h after DETA NONOate infusion, starting at 12PM. Post-treatment recordings were divided into 6h bins and changes in NREM sleep/NREM delta power were calculated as the percentage of the correspondent time bin of BL Day 1 EEG (=100%).

Experiment 3. Investigation of the neurotransmitter identity of the BF cells expressing iNOS and their contribution to the generation of HSP—

We previously showed that during SD iNOS expression occurred in BF wake-active cells (Kalinchuk *et al.*, 2010). However, the neurotransmitter identity of activated cells was not determined. Here we used immunohistochemistry (IHC) for iNOS and post-hoc double-labeling of one of two major neurotransmitter types in BF wake-active neurons: acetylcholine (choline acetyl transferase, ChAT) and parvalbumin (Parv). We compared the number of iNOS+, ChAT+/iNOS+ and ChAT–/iNOS+ neurons in three groups of animals: sleep deprived for 6h (9AM–15PM, **SD group**, N=6), 6h sleeping control (9AM–15PM, **SC group**, N=4) and 6h waking control (9PM–15AM, **WC group**, N=4). Also, we monitored the EEG in all groups of animals, and correlated the intensity of iNOS+ staining in ChAT+ cells with the intensity of EEG theta power. To detect changes in waking theta power during SD, SC or WC we normalized the value obtained during every hour of SD, SC or WC period to the first two hours of the experiment, considered as a pre-treatment baseline (7AM–9AM for SD and SC groups; 7PM–9PM for WC group). For the correlative analysis with the increase in iNOS intensity, we used values obtained during the last hour of the experiment before animals were killed.

Description of methods

Animals and surgery—The total of 30 Male Wistar rats (250–300g) were used in this study, kept in constant temperature (23.5–24°C) and 12-h light-dark cycle (lights ON at 7AM). Water and food were provided *ad libitum*. Starting 5 days before surgery, animals were habituated to experimenters by daily 10min training sessions that included handling and removal of animals from the cages by the experimenter. During surgery, under general anesthesia (i.m. ketamine 7.5mg/100g body weight, xylazine 0.38mg/100g, acepromazine 0.075mg/100g), rats were implanted with EEG and EMG electrodes. EEG electrodes (stainless steel screws) were implanted epidurally over the frontal (primary motor, AP=+2.0; ML=2.0) and parietal (retrosplenial agranular, AP=–4.0; ML=1.0) cortices. EMG recording

electrodes (silver wires covered with Teflon) were implanted into neck muscles. In **Experiments 1 and 2**, for the collection of microdialysis samples, infusion of DETA NONOate, and saporin injection, unilateral guide cannulae (CMA/11 Guide, CMA/Microdialysis, Stockholm, Sweden) were implanted targeting the BF nuclei, including horizontal limb of diagonal band (HDB), substantia innominata (SI) and magnocellular preoptic area (MCPO) (AP=−0.3; ML=2.0; V=6.5) (Paxinos & Watson, 1998).

Recovery and adaptation—After surgery, rats were housed in individual cages and allowed 1 week of recovery from surgery before procedures. Beginning 3 days after surgery, daily 10min training sessions were resumed. Habituation was complete when there was no fear reaction when the researcher approached the cage and touched the rat.

All efforts were made to achieve maximal adaptation of animals to the experimental conditions and minimize possible stress related to novelty of manipulations during SD experiments (Kalinchuk et al., 2010). After 1 week of post-surgery recovery, rats were connected to EEG/EMG recording leads for adaptation, which lasted 4 days. Before the beginning of experiments, 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 of sleep/wake, the animal was not used in the experiments.

Sleep deprivation paradigm—SD was done by gentle handling (Franken *et al.*, 1991) which included presentation of new objects into the cages or tactile stimulation, including gentle touching by a brush. In **Experiment 1**, EEG/EMG recording continued for 19h after SD. In **Experiment 3**, animals were killed immediately after the end of SD with their matching controls and RS was not recorded.

EEG recording, analysis of vigilance states and power spectral analysis—EEG/EMG signals were amplified and sampled at 256Hz (filter settings for EEG: 0.3 Hz high pass and 40 Hz low pass, for EMG: 30 Hz high pass and 100 Hz low pass). For vigilance state and NREM delta band analyses in **Experiment 2**, EEG recordings were scored semi-automatically in 30-sec epochs using Spike 2 (Version 5.11, Cambridge Electronic Devices, Cambridge, UK) for NREM sleep and manually for rapid eye movement (REM) sleep. The number of waking epochs was calculated from these scores as described previously (Kalinchuk *et al.*, 2006a; Kalinchuk *et al.*, 2006b). Recordings used for power spectral analysis (**Experiment 1**, 3h SD and 3 first hours of recovery sleep on SD day and correspondent time period 9.00AM – 3.00PM during BL day) were scored manually in 5-sec epochs using Spike 2 sleep scoring script Sleepscore, version 1.01 (CED). Vigilance states were scored according to standard criteria: NREM sleep was recognized by high-amplitude EEG associated with low-voltage EMG and slow waves in the delta range (0.4–4.5 Hz); REM sleep was recognized by low-amplitude, high-frequency EEG associated with the absence of EMG, as well as evident EEG theta activity (5–9 Hz); wakefulness was recognized as low-amplitude, high-frequency EEG with high-voltage EMG. Epochs with artifacts were excluded from the analysis.

In **Experiment 1**, on Day 1, EEG/EMG recording was performed for 24h for BL, and on Day 2, recorded during 2h pre-SD BL, 3h SD and 19h RS. All recordings were divided into

1h bins. EEG power spectra were generated in Spike 2 for consecutive 5-sec wakefulness or NREM sleep epochs (fast Fourier transform routine, Hanning window, 0.4 Hz resolution, frequency range 0.4–25Hz) and averaged over the periods investigated (3h of spontaneous wakefulness on EEG BL day or 3h SD on SD day, 9PM–12AM; 3h of spontaneous NREM sleep on EEG BL day and 3h NREM RS on SD day, 12AM–3PM). The EEG power in the low theta band (5–7Hz), high theta band (7–9Hz) during 3h of SD and delta band (0.4–4.5Hz) during the first 3 hours of NREM RS was normalized to the corresponding time bins of the EEG BL (=100%), and percentage differences were calculated.

In **Experiment 2**, each experimental day lasted for 29h. On 1st experimental day, EEG/EMG recording was performed for 29h for BL. On 2nd experimental day, animals were infused with DETA NONOate between 9AM–12PM. To evaluate the long-term changes in NREM sleep and delta power during NREM sleep, we continued EEG/EMG recording within 24h after DETA NONOate infusion (29h total). All post-treatment recordings were divided into 6h bins and changes for NREM sleep and NREM delta power in each bin were calculated as the percentage of the corresponding time bin of EEG BL (=100%).

In **Experiment 3**, EEG/EMG was recorded within 2h pre-treatment period (pre-treatment BL) and 6h of SD, SC or WC (7AM–15PM for SD and SC groups; 7PM–15AM for WC group). To define changes in wake theta power during SD, SC or WC, we normalized values obtained during every hour of SD, SC or WC period (9AM–15PM for SD and SC groups; 9PM–15AM for WC group) to the 2h pre-treatment BL (7AM–9AM for SD and SC groups; 7PM–9PM for WC group). The episodes of wakefulness accompanied by low EMG activity were included into analysis (Vyazovskiy & Tobler, 2005).

In vivo microdialysis—For **Experiments 1 and 2**, microdialysis probes (CMA11; 1mm membrane length, CMA/Microdialysis) were inserted 16h before the beginning of experiments. In all microdialysis experiments, aCSF (Harvard Apparatus, Holliston, MA) or DETA NONOate (Diethylenetriamine NONOate, Cayman Chemical, Ann Arbor, MI, 1mM) were perfused with a flow rate 1µm/min. In **Experiment 1**, microdialysis samples were collected every 30min. For each rat, microdialysis experiments were performed on 2 days (see Experimental design and rationale). Day 1 (BL) involved aCSF infusion in combination with EEG/EMG recording. On Day 2, SD or DETA NONOate infusion was performed between 9AM–12PM. In **Experiment 1**, for the measurements of SD-induced changes in $[NO_x]_{ex}$, $[AD]_{ex}$, $[Lac]_{ex}$ and $[Pyr]_{ex}$, collection of samples continued through pre-SD BL, SD, and the first 3h of RS.

Administration of the saporin—Immunotoxin 192 IgG-saporin is a ribosomal inactivating enzyme conjugated to the monoclonal antibody 192 IgG. It binds to the p75 nerve growth factor-receptor on ChBF neurons and destroys them (Heckers *et al.*, 1994). In **Experiments 1 and 2**, saporin (Chemicon International, Temecula, CA) was injected intracerebrally through microdialysis probes modified by removing the membrane from the probe tip after completion of control measurements, as described previously (Kalinchuk *et al.*, 2008). 1µl of saporin at a concentration of 0.23µg/µl was injected into the HDB/SI/MCPO region of the BF at the following coordinates: AP=−0.3; ML=2.0; V=8.5 (Paxinos & Watson, 1998). According to our previous experience and the literature, saporin selectively

depletes ChBF cells, with no loss of parvalbumin and GABAergic (Glutamate Decarboxylase 67-labeled) neurons intermingled with ChBF neurons (Pizzo *et al.*, 1999; Kalinchuk *et al.*, 2008; Kaur *et al.*, 2008). Treatment control group rats (**saline** group) were injected with 0.9% saline into BF (1µl). In all cases, saporin or saline were injected using a microdialysis pump at a flow rate of 0.1µl/min. After all injections, the microdialysis probe was kept in place for an additional 3–4 min, and then slowly removed. Lesion location and effectiveness was validated histologically after the end of experiments.

Measurements of $[\text{NO}_x]_{\text{ex}}$ —As no endogenous source other than NO is known for nitrate/nitrite $[\text{NO}_2^-/\text{NO}_3^-]$, collectively $[\text{NO}_x]$, this metabolite has been taken as indicative of NO production (Mackenzie *et al.*, 1996). The dietary intake of nitrate can considerably affect the plasma nitrate concentrations, but as these anions do not penetrate the blood–brain barrier, their effect on brain concentrations is not significant (Clark *et al.* 1996). $[\text{NO}_x]_{\text{ex}}$ concentrations were measured using a Nitrate/Nitrite Fluorometric Assay kit (Cayman Chemical Company, Ann Arbor, MI, USA). The detection limit of the assay was 0.06 µM in the final reaction mixture. The results were calculated as $[\text{NO}_x]_{\text{ex}}$. Values from the samples collected during 3h SD (N=6 samples) were averaged and normalized to the mean concentration of samples (N=4 samples) collected during the 2h pre-SD period (=100%).

Measurements of $[\text{AD}]_{\text{ex}}$, $[\text{Lac}]_{\text{ex}}$ and $[\text{Pyr}]_{\text{ex}}$ —Details of these assays have been published previously (Kalinchuk *et al.*, 2003; Kalinchuk *et al.*, 2008). $[\text{AD}]_{\text{ex}}$ was measured using high performance liquid chromatography (HPLC) coupled to a fluorescent detector (Waters 2475). $[\text{Lac}]_{\text{ex}}$ and $[\text{Pyr}]_{\text{ex}}$ were measured using HPLC coupled to a UV detector (Waters 486) as previously described (Kalinchuk *et al.*, 2011). The detection limit of the assay was 0.8 nM for AD (signal to noise ratio 2:1), 0.6 µM for Pyr (3:1) and 10 µM for Lac (3:1). Concentrations of samples collected during SD (average of 6 samples for 3h SD) were normalized to the mean concentration of samples (n=4) collected during the 2h pre-SD period (=100%).

Immunohistochemistry for iNOS, ChAT and Parv—After the end of microdialysis 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 4% paraformaldehyde in 0.1M PBS overnight, and then immersed in a 30% sucrose solution at 4°C for 4–5 days. Coronal 50-µm-thick sections were cut through ChBF (between AP=0.0 and AP=−0.8 (Paxinos and Watson, 1998)) using a cryostat and placed into 0.1M PBS.

In **Experiments 1 and 2**, for staining of ChAT+ cells, after washing in PBS, tissue was incubated in 3% Triton-X for 1h at RT, then blocked in 0.3% donkey serum for 1h at RT, and incubated overnight at 4°C with primary antibody for ChAT (1:500, rabbit polyclonal, Chemicon, Temecula, CA). Next day, sections were treated with secondary antibody (donkey anti-rabbit IgG, biotin conjugated, Chemicon, 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-immunoreactivity, diaminobenzidine (DAB; peroxidase kit, Vector laboratories Inc., Burlingame, CA) was used.

In **Experiment 3**, sections were first stained for iNOS and then labeled for neurotransmitter phenotype (ChAT or Parv). After the incubation in 3% Triton-X and 0.3% donkey serum, sections were incubated overnight with primary antibody for iNOS (mouse monoclonal, 1:15000, Sigma). Next day, sections were treated for 1h with anti-mouse fluorescent secondary antibodies with green fluorescence (1:500, Alexa Fluor 488, Invitrogen Molecular Probes, Eugene, OR). For double-labeling, sections were blocked in 0.3% mouse serum and incubated overnight at 4°C with different primary antibody: anti-ChAT (1:500, rabbit polyclonal, Chemicon) or anti-Parv (1:100; rabbit polyclonal, Thermo Scientific, Rockford, IL), followed by incubation with a secondary anti-rabbit antibody of red color (1:500, Alexa Fluor 568, Invitrogen Molecular Probes). Further, sections were mounted with Fluoromount-G (SouthernBiotech, Birmingham, AL). The specificity of iNOS antibody was previously tested using iNOS KO mice (Kalinchuk *et al.*, 2010).

Quantitative analysis of iNOS+ fluorescence intensity and cell counting—In **Experiment 3**, to quantify the SD-induced changes in iNOS production in the ChBF cells, we compared the iNOS fluorescence intensity of individual ChAT+/iNOS+ cells in the SD, SC, and WC groups. Also, we compared numbers of iNOS+, ChAT+, ChAT+/iNOS+ and ChAT–/iNOS+ in three groups. Fluorescence microscopy was performed using the Olympus BX51 microscope and quantitative analysis performed using Metamorf Software (version 6.1; Universal Imaging Corp., Downingtown, PA). Images from the BF area in each animal were collected at 20× magnification using blue and/or red filters. 5 comparable coronal sections from different rats were matched using anatomical landmarks and Bregma coordinates (AP levels +0.48; +0.2; –0.26; –0.4; –0.8; Paxinos & Watson, 1998) and HDB/Sl/MCPo area was outlined in each section.

The details of the counting procedure were described previously (Kalinchuk *et al.*, 2010). Briefly, to count ChAT+, iNOS+, ChAT+/iNOS+ and ChAT–/iNOS+ cells, 2 images of each of 5 sections per rat stained for both iNOS and ChAT were taken. For counting of double-labeled cells, 2 images were merged using Metamorf Software. Only cells that had intensity at least 1.5 times greater than background were counted. Counts from 5 sections were summarized to yield a total number of cells for each rat.

For the analysis of iNOS fluorescence intensity of ChAT+/iNOS+ cells, each cell was outlined and its fluorescence intensity was defined. The average fluorescence intensity of all cells in each section was normalized against the background by moving the outline of a cell to the neighboring area on tissue where no fluorescent cell body was detected. These values from 5 sections were averaged to form the grand average for each rat.

Histological verification of the cholinergic lesions and probe locations—In **Experiments 1 and 2**, 12 sections per brain with 250 µm intervals (1 of 5 series) were taken for IHC labeling of ChAT, as well as verification of the location and quality of saporin lesions. The details of counting procedure were described in Kalinchuk *et al.* (2008). In all experiments, histological verification of probe locations was performed in parallel.

Statistics

Data are expressed as mean $\pm$ SEM. Statistical analysis was performed using SigmaPlot 11.0 Statistical software (Systat Software Inc., San Jose, CA, USA).

In **Experiment 1**, the effects of SD on concentrations of [NO_x]_{ex}, [AD]_{ex}, [Lac]_{ex} and [Pyr]_{ex} were determined using a paired t-test. To evaluate the statistical significance of the effects of saporin lesion on the levels of [NO_x]_{ex}, [AD]_{ex}, [Lac]_{ex}, [Pyr]_{ex}, EEG theta and delta activity, we used repeated measures ANOVA (RM-ANOVA) followed by post hoc Bonferroni t-test to reveal the difference between groups. A repeated measures comparison of EEG power spectra was followed by Holm-Sidak post hoc tests for pair-wise comparisons. To evaluate the correlation between groups we used Pearson's correlation analysis; for the comparison of correlation coefficients we used Fisher Z-transformation.

In **Experiment 2**, to evaluate the effects of saporin lesion on DETA NONOate-induced NREM sleep and NREM delta power, we used RM-ANOVA followed by post hoc Bonferroni t-test (as presented in the text). The 24h post-infusion period was further divided into four 6h bins and, if the 24h values were statistically different, pair-wise comparisons of individual bins were done using a t-test.

In **Experiment 3**, to evaluate the statistical significance of the effects of SD on iNOS intensity, EEG theta power, and to compare numbers of iNOS+, ChAT+, ChAT+/iNOS+ and ChAT-/iNOS+ cells in three groups of rats (SD, WC and SC), we used one-way ANOVA, followed by Student-Newman-Keuls post hoc test for pair-wise comparison. To evaluate the correlation between groups we used Pearson's correlation analysis.

RESULTS

1. Saporin lesions of ChBF neurons prevented the SD-induced increase in [NO_x]_{ex}

In the saporin group, in control conditions, the [NO_x]_{ex} level in the BF during 3h SD (average from 6 samples) was increased by 71.7 $\pm$ 14%, compared to its 2h pre-SD baseline (average from 4 samples) (paired t-test, $t(5)=-3.790$, $P=0.013$). In contrast, in the same rats 2 weeks after the saporin injection the [NO_x]_{ex} level was not increased (1.0 $\pm$ 5% compared to pre-SD baseline). [NO_x]_{ex} levels during 3h SD in pre-injection control conditions were significantly higher compared to post-injection conditions (RM ANOVA, $F(2)=13.389$, $P=0.001$; post-hoc Bonferroni t-test, $t=4.449$, $P=0.004$) (Figure 1A). In the saline group, 2 weeks after injection the SD-induced increases in [NO_x]_{ex} level remained significantly elevated as compared with pre-SD baseline level (data not shown). Similar results were obtained when SD was extended to 6h: in pre-injection control condition, [NO_x]_{ex} was increased by 93.5 $\pm$ 14% compared to pre-SD baseline (paired t-test, $t(5)=-3.517$, $P=0.017$), while in the post-injection condition this increase was eliminated (increase by 9.5 $\pm$ 4% as compared to pre-SD baseline). RM ANOVA revealed significant difference between pre- and post-injection conditions ($F(2)=10.960$, $P=0.003$; post-hoc Bonferroni t-test, $t=3.821$, $P=0.01$). These data indicate that lesion of ChBF neurons prevents NO production during SD.

2. SD-induced [AD]_{ex} increase, but not [Lac]_{ex} and [Pyr]_{ex}, was prevented after saporin lesions of ChBF neurons

The increase in the level of [AD]_{ex} observed during SD in the pre-injection control condition (by $135.7 \pm 14\%$ as compared to pre-SD baseline; paired t-test, $t(5) = -4.823$, $P = 0.005$) was completely eliminated after lesions of ChBF neurons (increase by $24.2 \pm 18\%$ as compared to pre-SD baseline; paired t-test, $t(5) = -0.807$, $P = 0.456$) (Figure 1B). RM-ANOVA revealed the difference between pre- and post-injection conditions ($F(2) = 10.341$, $P = 0.004$; post-hoc Bonferroni t-test, $t = 3.503$, $P = 0.017$). In saline group, the injection did not affect the SD-induced increases: the [AD]_{ex} level remained significantly increased as compared with pre-SD baseline level (data not shown).

In agreement with our previous data (Kalinchuk *et al.*, 2003), the levels of [Lac]_{ex} and [Pyr]_{ex} were significantly increased during SD before saporin lesions (by $31.3 \pm 4\%$ and $34.2 \pm 7\%$ as compared to pre-SD baseline; paired t-test, $t(5) = -10.114$, $P < 0.001$ and $t(5) = -3.03$, $P = 0.029$, respectively). In contrast to [NO_x]_{ex} and [AD]_{ex}, saporin lesions could not prevent these increases. After saporin lesion, [Lac]_{ex} was increased by $24.8 \pm 3\%$ (paired t-test, $t(5) = -4.992$, $P = 0.004$). RM ANOVA revealed overall significant difference in [Lac]_{ex} between pre-SD baseline and SD ($F(2) = 26.811$, $P < 0.001$), however, there was no difference between pre- and post-injection conditions (post-hoc Bonferroni t-test, $t = 1.458$, $P = 0.527$) (Figure 1C). After saporin injection, [Pyr]_{ex} was increased by $33.4 \pm 5\%$ (paired t-test, $t(5) = -3.714$, $P = 0.014$). There was overall a significant difference in [Pyr]_{ex} between pre-SD baseline and SD (RM ANOVA, $F(2) = 6.792$, $P = 0.014$), however, there was no difference between pre- and post-injection conditions (post-hoc Bonferroni t-test, $t = 0.077$, $P = 1.0$) (Figure 1D). These data indicate that lesion of ChBF neurons prevents an increase in [AD]_{ex}, but does not affect increases in [Lac]_{ex} and [Pyr]_{ex}.

3. Saporin lesions of ChBF neurons blocked increasing HSP during SD

First, we evaluated changes in EEG power (0.4–25Hz) during spontaneous wakefulness and NREM sleep during EEG BL days before and after saporin lesion. Power spectra were calculated for the periods corresponding to SD (9AM–12PM) and first 3h of NREM RS (12PM–3PM) of SD days. During waking, saporin injection led to significant increase in delta power (1.6–2.8Hz) and significant decrease in theta power (6–9.2Hz) (Figure 2A). During NREM sleep, after saporin lesion we also observed a significant increase in delta power (1.2–2.8Hz) and moderate but significant decrease in theta power (6–8Hz) (Figure 2B).

Further, we evaluated the effects of SD on EEG power. To evaluate SD-induced changes in EEG theta or delta power, we compared them to the time-matched periods of spontaneous wakefulness or NREM sleep on the EEG BL days (=100%). First, we looked at changes in waking EEG during 3h SD. We found that in control condition before saporin lesion 3h SD lead to a significant increase in theta power (4.8–9.2Hz), decrease in delta power (0.4–4.4Hz) and increase in sigma (12–14Hz) power (Figure 3A). After saporin injection, we detected a significant decrease in the delta range (0.4–4.4Hz) and increase in high theta range (6.8–9.2Hz) (Figure 3B). Next, we evaluated changes during 3h of NREM RS. We

found that in control conditions, SD led to a significant increase in both delta and theta (0.4–9.2Hz) power (Figure 3C), eliminated after saporin lesions (Figure 3D).

Statistical analysis revealed that in control condition waking EEG theta power during SD increased by $35.9 \pm 4\%$, compared to baseline day (RM ANOVA, $F(2)=34.950$, $P<0.001$; post-hoc Bonferroni t-test, $t=8.338$, $P<0.001$) (Figure 4A). After saporin lesions, the increase in EEG theta power was still significant ($15.6 \pm 5\%$) compared to baseline day (post-hoc Bonferroni t-test, $t=3.642$, $P=0.014$), but significantly smaller as compared to control (post-hoc Bonferroni t-test, $t=4.697$, $P=0.003$) (Figure 4A). Further dissection of the increase in theta activity revealed that in the control condition SD led to increases in both high theta power (7–9Hz) (by $43.3 \pm 6\%$ as compared to baseline, RM ANOVA, $F(2)=28.962$, $P<0.001$; post-hoc Bonferroni t-test, $t=7.597$, $P<0.001$) and low theta power (4.8–7Hz) (by $27.1 \pm 4\%$ as compared to baseline, RM ANOVA, $F(2)=18.495$, $P<0.001$; post-hoc Bonferroni t-test, $t=5.775$, $P<0.001$). After saporin injection, the increase in high theta power still was significant ($24.0 \pm 4\%$ as compared to baseline day, post-hoc Bonferroni t-test, $t=4.199$, $P=0.005$), although it was significantly smaller compared to control (post-hoc Bonferroni t-test, $t=3.397$, $P=0.02$). However, the increase in low theta was completely eliminated (increase by $5.8 \pm 6\%$ as compared to baseline day, post-hoc Bonferroni t-test, $t=1.237$, $P=0.733$) (Figure 4A).

Statistical analysis of changes in NREM delta power during RS revealed that in control conditions NREM delta was increased by $71.8 \pm 3.4\%$, compared to baseline (RM ANOVA, $F(2)=121.059$, $P<0.001$; post-hoc Bonferroni t-test, $t=13.018$, $P<0.001$). After saporin lesions, this increase was eliminated (as compared to control condition, post-hoc Bonferroni t-test, $t=0.873$, $P=1.000$) (Figure 4B).

We correlated increases in EEG theta power during SD with EEG delta power during NREM RS. In control condition, while there was a strong positive correlation between low theta power and delta power ($R=0.949$; $P=0.004$; Figure 4C, middle panel). There was no correlation between total theta power and delta power (Pearson correlation coefficient, $R=0.732$; $P=0.09$; Figure 4C, left panel) and between high theta and delta power ($R=0.431$; $P=0.394$; Figure 4C, right panel). There was a statistically significant difference between correlation coefficients calculated for delta power and low theta versus delta power and high theta (Fisher Z-transformation, $Z=1.67$; $P=0.047$). After saporin injection, the correlation between low theta and delta was eliminated ($R=0.277$, $P=0.595$, respectively; Figure 4D, middle panels). There was a statistically significant difference between correlation coefficients calculated for low theta and delta power before and after the saporin lesions ($Z=1.88$; $P=0.03$). These data indicate that low theta power (5–7Hz) is an accurate electrophysiological marker of HSP present in waking EEG during SD, and this EEG component depends on the presence of ChBF cells.

4. Biochemical and electrophysiological markers of HSP

We performed correlative analyses between increases in the levels of $[\text{NO}_x]_{\text{ex}}$, $[\text{AD}]_{\text{ex}}$, $[\text{Lac}]_{\text{ex}}$ and $[\text{Pyr}]_{\text{ex}}$ during SD with EEG theta power during SD. We found a positive correlation between total theta and $[\text{Lac}]_{\text{ex}}$ (Pearson correlation coefficient, $R=0.870$; $P=0.024$), but not between total theta and $[\text{NO}_x]_{\text{ex}}$ ($R=0.692$; $P=0.128$), theta and $[\text{AD}]_{\text{ex}}$

($R=0.743$; $P=0.09$), and theta and $[\text{Pyr}]_{\text{ex}}$ ($R=0.771$; $P=0.072$) (Figure 5A). Further, we found that both $[\text{NO}_x]_{\text{ex}}$ and $[\text{AD}]_{\text{ex}}$ were positively correlated with low theta power ($R=0.964$; $P=0.002$ and $R=0.984$; $P=0.001$, respectively; Figure 5B). However, increases in $[\text{Lac}]_{\text{ex}}$ or $[\text{Pyr}]_{\text{ex}}$ did not correlate with the increase in low theta ($R=0.486$; $P=0.328$ and $R=0.089$; $P=0.868$, respectively; Figure 5B). There was no correlation between increases in $[\text{NO}_x]_{\text{ex}}$ or $[\text{AD}]_{\text{ex}}$ and high theta ($R=0.386$; $P=0.450$ and $R=0.441$; $P=0.382$, respectively;) but a significant and positive correlation was observed between increases in $[\text{Lac}]_{\text{ex}}$ or $[\text{Pyr}]_{\text{ex}}$ and high theta power ($R=0.851$; $P=0.031$ and $R=0.927$; $P=0.008$, respectively; Figure 5C). There was a statistically significant difference between correlation coefficients calculated for $[\text{NO}_x]_{\text{ex}}$ and low theta versus $[\text{NO}_x]_{\text{ex}}$ and high theta (Fisher Z-transformation, $Z=1.95$; $P=0.026$), and for $[\text{AD}]_{\text{ex}}$ and low theta versus $[\text{AD}]_{\text{ex}}$ and high theta ($Z=2.37$; $P=0.009$).

Further, we investigated the correlation between changes in $[\text{NO}_x]_{\text{ex}}$, $[\text{AD}]_{\text{ex}}$, $[\text{Lac}]_{\text{ex}}$, $[\text{Pyr}]_{\text{ex}}$ with theta power during SD after saporin lesions. We found that positive correlations between $[\text{NO}_x]_{\text{ex}}$ and $[\text{AD}]_{\text{ex}}$ with low theta power were eliminated ($R=0.390$; $P=0.445$ and $R=0.060$; $P=0.910$, respectively) (data not shown). There was a statistically significant difference between correlation coefficients calculated for $[\text{NO}_x]_{\text{ex}}$ or $[\text{AD}]_{\text{ex}}$ and low theta power before and after the saporin lesions (Fisher Z-transformation, $Z=1.94$; $P=0.026$ and $Z=2.88$; $P=0.002$). However, the positive correlations between $[\text{Lac}]_{\text{ex}}$ and total theta ($R=0.886$; $P=0.018$), and both $[\text{Lac}]_{\text{ex}}$ and $[\text{Pyr}]_{\text{ex}}$ and high theta were preserved ($R=0.905$; $P=0.013$ and $R=0.855$; $P=0.030$, respectively). These data demonstrate a strong correlation between biochemical and electrophysiological markers of HSP, such as $[\text{NO}_x]_{\text{ex}}$ and $[\text{AD}]_{\text{ex}}$, with low theta during SD, and ChBF neurons are crucial for regulation of both biochemical and electrophysiological processes.

5. Saporin lesions of ChBF cells prevented the increase of NREM sleep induced by infusion of the NO donor, DETA NONOate, into BF

We infused NO donor, DETA NONOate (1mM), for 3h into ChBF of the same animals ($N=6$) before (pre-injection control condition) and after saporin lesions (post-injection saporin condition), and measured changes in sleep and delta power during NREM sleep after infusion. We found that in the control condition DETA NONOate infusion induced an increase of NREM sleep over the period of 18h after infusion (by $22.3\pm 4\%$ as compared to baseline day, RM ANOVA, $F(2)=22.138$, $P<0.001$; post-hoc Bonferroni t-test, $t=5.178$, $P=0.001$). This increase was completely eliminated after saporin injection ($-4.5\pm 4\%$ as compared to baseline day; post-hoc Bonferroni t-test, $t=1.040$, $P=0.968$); there was a statistically significant difference between pre- and post-injection conditions (post-hoc Bonferroni t-test, $t=6.218$, $P<0.001$) (Figure 6A).

Infusion of DETA NONOate in control condition induced the increase in NREM delta power for 18h after the infusion (by $34.0\pm 7\%$ as compared to baseline day, RM ANOVA, $F(2)=13.877$, $P=0.001$; post-hoc Bonferroni t-test, $t=3.929$, $P=0.008$). After saporin injection, DETA NONOate did not induce an increase in NREM delta power (post-hoc Bonferroni t-test, $t=1.074$, $P=0.924$); this effect statistically differed from that observed before the lesion (post-hoc Bonferroni t-test, $t=5.004$, $P=0.002$) (Figure 6B). These data

indicate that infusion of NO donor to the BF mimics effects of SD by increasing NREM sleep and delta power during NREM sleep, and this effect is mediated via ChBF neurons.

6. SD-induced iNOS expression occurred preferentially in ChBF neurons

Our data suggest that ChBF neurons are important for NO production during SD. However, it is uncertain if they are the source of iNOS expression during SD. We previously showed that, during SD, iNOS expression occurs in BF wake-active cells (Kalinchuk *et al.*, 2010). Their neurotransmitter identity, though, was not clarified. Here we used IHC for iNOS and post-hoc double-labeling for different wake-active neurons: ChAT+ and Parv+. First, we compared the numbers of ChAT+, iNOS+ and ChAT+/iNOS+ in ChBF of three groups of rats (SC; WC and SD; n=6 or 4/group). The numbers of ChAT+ cells were not different between the three treatment groups (Figure 7A). However, the number of iNOS+ cells were significantly higher in SD, as compared to SC and WC (by 273% and 230%, respectively; one-way ANOVA, $F=36.879$, $P<0.001$; post-hoc Student-Newman-Keuls test, $q=10.247$, $p<0.001$) (Figure 7A). Also, we found that the majority of iNOS+ cells (74%) after SD were ChAT+, and only 26% cells were ChAT-/iNOS+ (Figure 7B). The number of ChAT+/iNOS+ cells was significantly higher after SD, compared to SC and WC (by 643% and 415%, respectively; one-way ANOVA, $F=110.047$, $P<0.001$; post-hoc Student-Newman-Keuls test, $q=17.488$, $p<0.001$) (Figure 7B). The number of ChAT-/iNOS+ cells did not change after SD compared to SC and WC. Double labeling with markers for non-cholinergic neurons revealed that some ChAT-/iNOS+ cells after SD were Parv+ (Figure 7C). In summary, our data indicate that the ChBF neuronal population is the primary source of iNOS-dependent NO production during SD.

7. The intensity of SD-induced iNOS expression in ChBF cells correlated with the increase in low theta activity

We correlated the fluorescence luminance intensity of iNOS expression in ChAT+ cells with the EEG power in the low theta band in animals after 6h SD and in their controls, SC and WC (N=6 or 4/group). In this experiment, to calculate the increase of EEG theta power, we normalized the theta power values during last 3h of SD or spontaneous sleeping/waking period to the theta power values within the two hour of pre-SD or pre-sleeping/waking period (baseline, =100%, 7AM–9AM for SD and SC groups; 7PM–9PM for WC). Animals were immediately killed at the end of SD or spontaneous sleep/wake. In SD group we found that low theta power was increased by 36% as compared to pre-SD baseline (one-way ANOVA, $F=12.695$, $P<0.001$; post-hoc Student-Newman-Keuls test, $q=5.849$, $p=0.001$). We found no increase in EEG theta produced by SC or WC (post-hoc Student-Newman-Keuls test, $q=1.499$, $p=0.553$) (Figure 8A). Also, in SD group the iNOS fluorescence intensity was significantly higher as compared to WC and SC groups, which is in agreement with our previous report (Kalinchuk *et al.*, 2010) (Kruskal-Wallis one-way ANOVA on Ranks, $H=11.024$, $P<0.001$; post-hoc Student-Newman-Keuls test, $q=10.293$, $p<0.001$). Finally, in SD group, there was a very strong positive correlation between iNOS fluorescence intensity with EEG theta power during SD (Pearson correlation coefficient, $R=0.879$; $P=0.02$; Figure 8B). These data indicate that iNOS-dependent NO production in ChBF neurons is a biochemical marker of HSP, manifested as an increase in low theta power during SD.

DISCUSSION

In summary, changes in four biochemical markers studied here, as well as EEG indicators of HSP, evaluated before and after selective lesions of ChBF neurons, suggest that cholinergic neurons are key to the generation of HSP, since: (1) Under control conditions increases in low theta power (5–7Hz), but not high theta power (7–9Hz), during SD had a significant correlation with the increased NREM delta power during RS, and thus served as a suitable marker of HSP during waking; however such increases were not seen following ChBF saporin lesions; (2) While the levels of $[NO]_{ex}$, $[AD]_{ex}$, $[Lac]_{ex}$, $[Pyr]_{ex}$ and both low and high theta increased during SD, significant correlations with low theta activity were observed only with $[NO]_{ex}$ and $[AD]_{ex}$. After ChBF saporin lesions, there were no increases in $[NO]_{ex}$, $[AD]_{ex}$, low theta during SD and delta power during NREM RS in contrast with significant increases in $[Lac]_{ex}$ or $[Pyr]_{ex}$; (3) Infusion of NO donor increased NREM sleep/ NREM delta power under control conditions, but not after ChBF saporin lesions, suggesting ChBF neurons are important in mediating the sleep-inducing effects of NO; (4) SD-induced expression of iNOS is selective to cholinergic neurons in BF, and the levels of expression of iNOS showed a positive correlation with the increases in low theta power during SD.

Low theta activity as an electrophysiological marker of HSP

It has been suggested that, in rodents, EEG theta activity in low-frequency theta band (5–7Hz) is associated with quiet waking and sleepiness, while high-frequency theta (7–9Hz) is associated with active wakefulness and related cortical arousal (Vinogradova, 1995; Maloney *et al.*, 1997; Kahana *et al.*, 2001; Vyazovskiy & Tobler, 2005; Wigren *et al.*, 2009; Young & McNaughton, 2009). It has been previously shown that an increase in low theta (5–7Hz) during quiet waking during SD predicts the subsequent enhancement of delta power during recovery NREM sleep (Vyazovskiy & Tobler, 2005). Enhancing high theta (7–9Hz) by histamine infusion to the BF failed to increase delta power (Zant *et al.*, 2012). Our data agree with these findings and indicate a different functional role for the low and high EEG theta activity in that the increase in low theta during 3h SD had a strong correlation with the increase in subsequent delta during NREM RS, while total theta and high theta did not show any correlation with delta during NREM RS.

iNOS, NO and AD as biological markers of HSP

Work from our laboratory and others has demonstrated that a time dependent increase first in $[NO]_{ex}$, followed by $[AD]_{ex}$ in BF, is essential for HSP (Basheer *et al.*, 2004; Porkka-Heiskanen & Kalinchuk, 2011; Brown *et al.*, 2012). While increased $[AD]_{ex}$ depends on NO, the latter depends on induction of iNOS in ChBF neurons (Kalinchuk *et al.*, 2006a; Kalinchuk *et al.*, 2006b). There is ample evidence that the biochemical cascade $iNOS \rightarrow NO \rightarrow AD$ triggered in the BF during SD plays an important role in HSP. However, the exact mechanism explaining how NO and $[AD]_{ex}$ contribute to HSP, and particularly, what is their relationship with the EEG changes taking place in homeostatic regulation of sleep, was unclear. Our current data indicate that both $[AD]_{ex}$ and $iNOS/[NO]_{ex}$ contribute to the generation of HSP during SD and correlate with the increase of an electrophysiological marker of HSP, low theta power.

The selective induction of iNOS during SD can be attributed to a presumed increase in glutamatergic tone in BF during SD. The BF receives a robust glutamatergic input from several known wake-promoting areas including the brainstem, cerebral cortex, lateral hypothalamus and amygdala (Carnes *et al.*, 1990; Holmes & Jones, 1994; Jolkkonen *et al.*, 2001). Glutamatergic activation of ChBF neurons promotes cortical arousal (Hawryluk *et al.*, 2012). Glutamate induces iNOS expression in neurons in culture (Cardenas *et al.*, 2000), which may potentially produce iNOS induction in BF.

Lac, Pyr and high theta as indicators of neuronal activation and arousal

There is extensive evidence in the literature indicating that increase in Lac and Pyr indicate neuronal activation. Lac is an important cerebral oxidative energy substrate (Pellerin & Magistretti, 1994; Schurr & Payne, 2007). Activity-dependent [Lac]_{ex} release can be detected in the activated brain areas during sensory or cognitive stimulation in humans (Prichard *et al.*, 1991; Urrila *et al.*, 2003) and during behavioral or experimental activation in animals (Kuhr & Korf, 1988; Caesar *et al.*, 2008). The level of [Lac]_{ex} increases in the cortex during spontaneous wakefulness as compared to spontaneous NREM sleep and REM sleep, and even more so during active wake (Shram *et al.*, 2002). Glutamatergic stimulation of the BF increases [Lac]_{ex} levels in parallel with the increases in active wake and high-frequency theta power (7–9Hz), indicating that [Lac]_{ex} increase might result from the increased neuronal activity (Wigren *et al.*, 2009). Recent data demonstrate a robust temporal relationship between lactate concentration and slow-wave activity in the cerebral cortex: when EEG is desynchronized (during waking or REM sleep), lactate concentration builds; when EEG is synchronized during NREM sleep or during induction of cortical 1-Hz oscillations, but not 10-Hz oscillations, by optogenetic stimulation, lactate concentration declines (Wisor *et al.*, 2013).

The SD-induced increase in [Lac]_{ex} and [Pyr]_{ex} levels, described previously by us and others (Kalinchuk *et al.*, 2003; Wigren *et al.*, 2007), may reflect an increased energy demand (Dworak *et al.*, 2010) in the BF, possibly produced by the increased glutamatergic tone. A previous study showed that the increase in [Lac]_{ex} seen during SD correlates with an increase in high theta (Wigren *et al.*, 2009). In agreement, our data indicate that during SD the increase in both [Lac]_{ex} and [Pyr]_{ex} correlates with an increase in high theta, indicating arousal, but not with low theta, which is a measure of HSP. Further, lesion of ChBF neurons failed to prevent the increases in BF levels of [Lac]_{ex} and [Pyr]_{ex} during SD, and also partially spared the increase in high theta, while completely eliminating increases in [NO_x]_{ex}, [AD]_{ex}, and low theta activity.

Cholinergic modulation of cortical activity during spontaneous sleep-waking cycles

Acetylcholine release is increased with cortical activation during attentive wakefulness and REM sleep (Celesia & Jasper, 1966; Marrosu *et al.*, 1995; Himmelheber *et al.*, 2000), and can stimulate fast cortical activity by depolarizing rhythmic-bursting pyramidal neurons via muscarinic receptors (Metherate *et al.*, 1992). ChBF cells have intrinsic capacities to fire in rhythmic bursts in low theta range through low-threshold Ca²⁺ spikes (Khateb *et al.*, 1992). ChBF cells discharge in bursts at maximal rates during active waking and REM sleep, and their bursting discharge are synchronized with theta oscillations (Lee *et al.*, 2005). Selective

stimulation of the ChBF cells by neurotensin produced a dose-dependent decrease in delta and increase in both theta and gamma activity (Cape *et al.*, 2000). The lesion of ChBF cells using 192 IgG-saporin significantly reduces theta power (4–12Hz) during spontaneous REM sleep and wakefulness (Gerashchenko *et al.*, 2001) and increases delta power across all stages (Berntson *et al.*, 2002). This is in agreement with our results indicating saporin lesion of ChBF cells decreases activity in the entire theta range (5–9.2 Hz) during both spontaneous wakefulness and NREM sleep. Furthermore, we found that delta activity was increased at the selected frequency range (1.6–2.8Hz during wakefulness and 1.2–2.8Hz during NREM sleep), an observation not described in earlier reports (Kaur *et al.*, 2008; Fuller *et al.*, 2011).

The role of ChBF cells in linking biochemical and electrophysiological correlates of HSP during SD

Our data indicate that the SD-induced increases in the BF [NO]_{ex} and [AD]_{ex} correlate with the increase in cortical EEG low theta power, and that this correlation is impaired after the lesion of ChBF cells. This data indicate ChBF cells are important for triggering both the biochemical and EEG changes during SD. Also, our data indicate that ChBF cells are important for increasing the delta power/NREM sleep induced by the infusion of NO donor or AD (Kalinchuk *et al.*, 2008).

ChBF cells fire in rhythmic bursts in low theta range when depolarized from a hyperpolarized level via NMDA receptor stimulation (Khateb *et al.*, 1992; Khateb *et al.*, 1995; Cape & Jones, 2000). As the BF receives an important contingent of excitatory glutamatergic fibers from the wake-promoting brainstem, the cerebral cortex and the lateral hypothalamus (Carnes *et al.*, 1990; Jolkkonen *et al.*, 2001), it is possible that glutamate released from these fibers induces rhythmic bursting of ChBF neurons during wake (Khateb *et al.*, 1995). This bursting would provide a rhythmic modulation to target cortical neurons and potentially promote oscillations within a theta frequency range in cortical EEG.

We suggest that, during SD, ChBF neurons are stimulated, which is favorable for the sustained generation of theta activity. On the one hand, they experience excitatory influence from different wake-promoting mechanisms. Increase in the levels of NO and AD may lead to the hyperpolarization of ChBF neurons (Arrigoni *et al.*, 2006; Kang *et al.*, 2007). In SD, when the level of [AD]_{ex} and [NO]_{ex} increase, the level of hyperpolarization also increases and thereby the increase in low theta activity (5–7Hz).

In conclusion, we have presented data indicating an important causal role of ChBF cells in increasing biochemical levels of [AD]_{ex} and [NO]_{ex}, and EEG changes including an increase in low frequency theta associated with SD and the HSP. The activation of iNOS with SD, primarily in ChBF neurons, appears to be the key mechanism driving both the biochemical and EEG changes.

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References

- Arrigoni E, Chamberlin NL, Saper CB, McCarley RW. Adenosine inhibits basal forebrain cholinergic and noncholinergic neurons in vitro. *Neuroscience*. 2006; 140:403–413. [PubMed: 16542780]
- Basheer R, Porkka-Heiskanen T, Stenberg D, McCarley RW. Adenosine and behavioral state control: adenosine increases c-Fos protein and AP1 binding in basal forebrain of rats. *Brain Res Mol Brain Res*. 1999; 73:1–10. [PubMed: 10581392]
- Basheer R, Strecker RE, Thakkar MM, McCarley RW. Adenosine and sleep-wake regulation. *Prog Neurobiol*. 2004; 73:379–396. [PubMed: 15313333]
- Berntson GG, Shafi R, Sarter M. Specific contributions of the basal forebrain corticopetal cholinergic system to electroencephalographic activity and sleep/waking behaviour. *Eur J Neurosci*. 2002; 16:2453–2461. [PubMed: 12492440]
- Borbely AA. A two process model of sleep regulation. *Hum Neurobiol*. 1982; 1:195–204. [PubMed: 7185792]
- Brown RE, Basheer R, McKenna JT, Strecker RE, McCarley RW. Control of sleep and wakefulness. *Physiol Rev*. 2012; 92:1087–1187. [PubMed: 22811426]
- Caesar K, Hashemi P, Douhou A, Bonvento G, Boutelle MG, Walls AB, Lauritzen M. Glutamate receptor-dependent increments in lactate, glucose and oxygen metabolism evoked in rat cerebellum in vivo. *J Physiol*. 2008; 586:1337–1349. [PubMed: 18187464]
- Cape EG, Jones BE. Effects of glutamate agonist versus procaine microinjections into the basal forebrain cholinergic cell area upon gamma and theta EEG activity and sleep-wake state. *Eur J Neurosci*. 2000; 12:2166–2184. [PubMed: 10886356]
- Cape EG, Manns ID, Alonso A, Beaudet A, Jones BE. Neurotensin-induced bursting of cholinergic basal forebrain neurons promotes gamma and theta cortical activity together with waking and paradoxical sleep. *J Neurosci*. 2000; 20:8452–8461. [PubMed: 11069953]
- Cardenas A, Moro MA, Hurtado O, Leza JC, Lorenzo P, Castrillo A, Bodelon OG, Bosca L, Lizasoain I. Implication of glutamate in the expression of inducible nitric oxide synthase after oxygen and glucose deprivation in rat forebrain slices. *J Neurochem*. 2000; 74:2041–2048. [PubMed: 10800947]
- Carnes KM, Fuller TA, Price JL. Sources of presumptive glutamatergic/aspartatergic afferents to the magnocellular basal forebrain in the rat. *J Comp Neurol*. 1990; 302:824–852. [PubMed: 1982006]
- Celesia GG, Jasper HH. Acetylcholine released from cerebral cortex in relation to state of activation. *Neurology*. 1966; 16:1053–1063. [PubMed: 5950916]
- Daan S, Beersma DG, Borbely AA. Timing of human sleep: recovery process gated by a circadian pacemaker. *Am J Physiol*. 1984; 246:R161–183. [PubMed: 6696142]
- Dworak M, McCarley RW, Kim T, Kalinchuk AV, Basheer R. Sleep and brain energy levels: ATP changes during sleep. *J Neurosci*. 2010; 30:9007–9016. [PubMed: 20592221]
- Franken P, Dijk DJ, Tobler I, Borbely AA. Sleep deprivation in rats: effects on EEG power spectra, vigilance states, and cortical temperature. *Am J Physiol*. 1991; 261:R198–208. [PubMed: 1858947]
- Fuller PM, Sherman D, Pedersen NP, Saper CB, Lu J. Reassessment of the structural basis of the ascending arousal system. *J Comp Neurol*. 2011; 519:933–956. [PubMed: 21280045]
- Gerashchenko D, Salin-Pascual R, Shiromani PJ. Effects of hypocretin-saporin injections into the medial septum on sleep and hippocampal theta. *Brain Res*. 2001; 913:106–115. [PubMed: 11532254]
- Hawryluk JM, Ferrari LL, Keating SA, Arrigoni E. Adenosine inhibits glutamatergic input to basal forebrain cholinergic neurons. *J Neurophysiol*. 2012; 107:2769–2781. [PubMed: 22357797]

- Heckers S, Ohtake T, Wiley RG, Lappi DA, Geula C, Mesulam MM. Complete and selective cholinergic denervation of rat neocortex and hippocampus but not amygdala by an immunotoxin against the p75 NGF receptor. *J Neurosci.* 1994; 14:1271–1289. [PubMed: 8120624]
- Himmelheber AM, Sarter M, Bruno JP. Increases in cortical acetylcholine release during sustained attention performance in rats. *Brain Res Cogn Brain Res.* 2000; 9:313–325. [PubMed: 10808142]
- Holmes CJ, Jones BE. Importance of cholinergic, GABAergic, serotonergic and other neurons in the medial medullary reticular formation for sleep-wake states studied by cytotoxic lesions in the cat. *Neuroscience.* 1994; 62:1179–1200. [PubMed: 7845593]
- Jolkkonen E, Pikkarainen M, Kemppainen S, Pitkanen A. Interconnectivity between the amygdaloid complex and the amygdalostriatal transition area: a PHA-L study in rat. *J Comp Neurol.* 2001; 431:39–58. [PubMed: 11169989]
- Jones BE. Activity, modulation and role of basal forebrain cholinergic neurons innervating the cerebral cortex. *Prog Brain Res.* 2004; 145:157–169. [PubMed: 14650914]
- Kahana MJ, Seelig D, Madsen JR. Theta returns. *Curr Opin Neurobiol.* 2001; 11:739–744. [PubMed: 11741027]
- Kalinchuk AV, Lu Y, Stenberg D, Rosenberg PA, Porkka-Heiskanen T. Nitric oxide production in the basal forebrain is required for recovery sleep. *J Neurochem.* 2006a; 99:483–498. [PubMed: 17029601]
- Kalinchuk AV, McCarley RW, Porkka-Heiskanen T, Basheer R. Sleep deprivation triggers inducible nitric oxide-dependent nitric oxide production in wake-active basal forebrain neurons. *J Neurosci.* 2010; 30:13254–13264. [PubMed: 20926651]
- Kalinchuk AV, McCarley RW, Porkka-Heiskanen T, Basheer R. The time course of adenosine, nitric oxide (NO) and inducible NO synthase changes in the brain with sleep loss and their role in the non-rapid eye movement sleep homeostatic cascade. *J Neurochem.* 2011; 116:260–272. [PubMed: 21062286]
- Kalinchuk AV, McCarley RW, Stenberg D, Porkka-Heiskanen T, Basheer R. The role of cholinergic basal forebrain neurons in adenosine-mediated homeostatic control of sleep: lessons from 192 IgG-saporin lesions. *Neuroscience.* 2008; 157:238–253. [PubMed: 18805464]
- Kalinchuk AV, Stenberg D, Rosenberg PA, Porkka-Heiskanen T. Inducible and neuronal nitric oxide synthases (NOS) have complementary roles in recovery sleep induction. *Eur J Neurosci.* 2006b; 24:1443–1456. [PubMed: 16987226]
- Kalinchuk AV, Urrila AS, Alanko L, Heiskanen S, Wigren HK, Suomela M, Stenberg D, Porkka-Heiskanen T. Local energy depletion in the basal forebrain increases sleep. *Eur J Neurosci.* 2003; 17:863–869. [PubMed: 12603276]
- Kang Y, Dempo Y, Ohashi A, Saito M, Toyoda H, Sato H, Koshino H, Maeda Y, Hirai T. Nitric oxide activates leak K⁺ currents in the presumed cholinergic neuron of basal forebrain. *J Neurophysiol.* 2007; 98:3397–3410. [PubMed: 17928563]
- Kaur S, Junek A, Black MA, Semba K. Effects of ibotenate and 192IgG-saporin lesions of the nucleus basalis magnocellularis/substantia innominata on spontaneous sleep and wake states and on recovery sleep after sleep deprivation in rats. *J Neurosci.* 2008; 28:491–504. [PubMed: 18184792]
- Khateb A, Fort P, Serafin M, Jones BE, Muhlethaler M. Rhythmical bursts induced by NMDA in guinea-pig cholinergic nucleus basalis neurones in vitro. *J Physiol.* 1995; 487(Pt 3):623–638. [PubMed: 8544126]
- Khateb A, Muhlethaler M, Alonso A, Serafin M, Mainville L, Jones BE. Cholinergic nucleus basalis neurons display the capacity for rhythmic bursting activity mediated by low-threshold calcium spikes. *Neuroscience.* 1992; 51:489–494. [PubMed: 1488109]
- Kuhr WG, Korf J. N-methyl-D-aspartate receptor involvement in lactate production following ischemia or convulsion in rats. *Eur J Pharmacol.* 1988; 155:145–149. [PubMed: 2854069]
- Lee MG, Hassani OK, Alonso A, Jones BE. Cholinergic basal forebrain neurons burst with theta during waking and paradoxical sleep. *J Neurosci.* 2005; 25:4365–4369. [PubMed: 15858062]
- Mackenzie IM, Ekangaki A, Young JD, Garrard CS. Effect of renal function on serum nitrogen oxide concentrations. *Clin Chem.* 1996; 42:440–444. [PubMed: 8598110]
- Magistretti PJ, Pellerin L, Rothman DL, Shulman RG. Energy on demand. *Science.* 1999; 283:496–497. [PubMed: 9988650]

- Maloney KJ, Cape EG, Gotman J, Jones BE. High-frequency gamma electroencephalogram activity in association with sleep-wake states and spontaneous behaviors in the rat. *Neuroscience*. 1997; 76:541–555. [PubMed: 9015337]
- Marrosu F, Portas C, Mascia MS, Casu MA, Fa M, Giagheddu M, Imperato A, Gessa GL. Microdialysis measurement of cortical and hippocampal acetylcholine release during sleep-wake cycle in freely moving cats. *Brain Res*. 1995; 671:329–332. [PubMed: 7743225]
- McCarley RW. Neurobiology of REM and NREM sleep. *Sleep Med*. 2007; 8:302–330. [PubMed: 17468046]
- Metherate R, Cox CL, Ashe JH. Cellular bases of neocortical activation: modulation of neural oscillations by the nucleus basalis and endogenous acetylcholine. *J Neurosci*. 1992; 12:4701–4711. [PubMed: 1361197]
- Methippara MM, Kumar S, Alam MN, Szymusiak R, McGinty D. Effects on sleep of microdialysis of adenosine A1 and A2a receptor analogs into the lateral preoptic area of rats. *Am J Physiol Regul Integr Comp Physiol*. 2005; 289:R1715–1723. [PubMed: 16109808]
- Murillo-Rodriguez E, Blanco-Centurion C, Gerashchenko D, Salin-Pascual RJ, Shiromani PJ. The diurnal rhythm of adenosine levels in the basal forebrain of young and old rats. *Neuroscience*. 2004; 123:361–370. [PubMed: 14698744]
- Paxinos, G.; Watson, C. The rat brain in stereotaxic coordinates. Academic Press; San Diego: 1998.
- Pellerin L, Magistretti PJ. Glutamate uptake into astrocytes stimulates aerobic glycolysis: a mechanism coupling neuronal activity to glucose utilization. *Proc Natl Acad Sci U S A*. 1994; 91:10625–10629. [PubMed: 7938003]
- Pizzo DP, Waite JJ, Thal LJ, Winkler J. Intraparenchymal infusions of 192 IgG-saporin: development of a method for selective and discrete lesioning of cholinergic basal forebrain nuclei. *J Neurosci Methods*. 1999; 91:9–19. [PubMed: 10522820]
- Porkka-Heiskanen T, Kalinchuk AV. Adenosine, energy metabolism and sleep homeostasis. *Sleep Med Rev*. 2011; 15:123–135. [PubMed: 20970361]
- Porkka-Heiskanen T, Strecker RE, Thakkar M, Bjorkum AA, Greene RW, McCarley RW. Adenosine: a mediator of the sleep-inducing effects of prolonged wakefulness. *Science*. 1997; 276:1265–1268. [PubMed: 9157887]
- Prichard J, Rothman D, Novotny E, Petroff O, Kuwabara T, Avison M, Howseman A, Hanstock C, Shulman R. Lactate rise detected by ¹H NMR in human visual cortex during physiologic stimulation. *Proc Natl Acad Sci U S A*. 1991; 88:5829–5831. [PubMed: 2062861]
- Schurr A, Payne RS. Lactate, not pyruvate, is neuronal aerobic glycolysis end product: an in vitro electrophysiological study. *Neuroscience*. 2007; 147:613–619. [PubMed: 17560727]
- Shram N, Netchiporouk L, Cespuglio R. Lactate in the brain of the freely moving rat: voltammetric monitoring of the changes related to the sleep-wake states. *Eur J Neurosci*. 2002; 16:461–466. [PubMed: 12193189]
- Thakkar MM, Delgiacco RA, Strecker RE, McCarley RW. Adenosinergic inhibition of basal forebrain wakefulness-active neurons: a simultaneous unit recording and microdialysis study in freely behaving cats. *Neuroscience*. 2003; 122:1107–1113. [PubMed: 14643776]
- Urrila AS, Hakkarainen A, Heikkinen S, Vuori K, Stenberg D, Hakkinen AM, Lundbom N, Porkka-Heiskanen T. Metabolic imaging of human cognition: an fMRI/¹H-MRS study of brain lactate response to silent word generation. *J Cereb Blood Flow Metab*. 2003; 23:942–948. [PubMed: 12902838]
- Vinogradova OS. Expression, control, and probable functional significance of the neuronal theta-rhythm. *Prog Neurobiol*. 1995; 45:523–583. [PubMed: 7624485]
- Vyazovskiy VV, Tobler I. Theta activity in the waking EEG is a marker of sleep propensity in the rat. *Brain Res*. 2005; 1050:64–71. [PubMed: 15975563]
- Wigren HK, Rytönen KM, Porkka-Heiskanen T. Basal forebrain lactate release and promotion of cortical arousal during prolonged waking is attenuated in aging. *J Neurosci*. 2009; 29:11698–11707. [PubMed: 19759316]
- Wigren HK, Schepens M, Matto V, Stenberg D, Porkka-Heiskanen T. Glutamatergic stimulation of the basal forebrain elevates extracellular adenosine and increases the subsequent sleep. *Neuroscience*. 2007; 147:811–823. [PubMed: 17574765]

- Wisor JP, Rempe MJ, Schmidt MA, Moore ME, Clegern WC. Sleep slow-wave activity regulates cerebral glycolytic metabolism. *Cereb Cortex*. 2013; 23:1978–1987. [PubMed: 22767634]
- Young CK, McNaughton N. Coupling of theta oscillations between anterior and posterior midline cortex and with the hippocampus in freely behaving rats. *Cereb Cortex*. 2009; 19:24–40. [PubMed: 18453538]
- Zant JC, Rozov S, Wigren HK, Panula P, Porkka-Heiskanen T. Histamine release in the basal forebrain mediates cortical activation through cholinergic neurons. *J Neurosci*. 2012; 32:13244–13254. [PubMed: 22993440]

Figure 1.

Comparison of SD-induced biochemical changes in BF before (control) and after saporin lesions (saporin). A, B) The levels of [NO_x]_{ex} and [AD]_{ex} were significantly increased during 3h SD before saporin lesions; however, these increases were blocked after lesions. C, D) Saporin lesions did not affect SD-induced increases in [Lac]_{ex} and [Pyr]_{ex} levels. The levels of [NO_x]_{ex}, [AD]_{ex}, [Lac]_{ex} and [Pyr]_{ex} were calculated by normalizing the average of 3h SD values (N=6) to the average of 2h pre-SD BL values (N=4). *, value significantly differs from pre-SD baseline, $p < 0.05$; #, value significantly differs from control condition, $p < 0.05$.

Figure 2.

Comparison of EEG power spectra in spontaneous wakefulness and NREM sleep before and after saporin lesions. Power spectra were calculated from recordings of EEG BL days corresponding to 3h SD (9AM–12PM) or 3h NREM RS (12PM–3PM) on SD days. Left panels illustrate the EEG power spectra during spontaneous wakefulness (A) or NREM sleep (B) in pre-injection control condition (control baseline, black dots) and in post-injection saporin condition (saporin baseline, gray dots). Right panels illustrate the percent change of EEG power during wakefulness (A) or NREM sleep (B) after saporin injection versus pre-injection condition. White dots indicate significantly different values. A) During

wakefulness, there was a significant increase in EEG delta power (1.6–2.8Hz) and significant decrease in theta power, both low and high (6–9.2Hz). B) During NREM sleep, after saporin lesions, we also observed a significant increase in delta power (1.2–2.8Hz) and significant decrease in theta power (6–8Hz).

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Figure 3.

Comparison of EEG power spectra during forced wakefulness (SD) and NREM RS vs. BL before and after saporin lesions. This comparison was performed between corresponding time periods of SD days and EEG BL days. Left panels show EEG power spectra during spontaneous wakefulness (A,B) or NREM sleep (C,D) (baseline, black dots) and 3h SD (A,B) or NREM RS (C,D) (gray dots) before (A,C) or after saporin lesions (B,D). Right panels show the percent changes in EEG power during SD (A,B) or NREM RS (C,D), compared to their respective baselines. A) During SD, in control pre-injection conditions before saporin lesions, there was a significant increase in EEG theta power (4.8–9.2Hz), decrease in delta power (0.4–4.4Hz), and increase in sigma (12–14Hz) power. B) During SD after saporin lesions, there was a significant decrease in the delta range (0.4–4.4Hz) and increase in high theta range (6.8–9.2Hz). C) During recovery NREM sleep in the pre-injection control condition, there was a significant increase in delta and theta (0.4–9.2Hz) power. D) After saporin lesions, the increase in delta power seen during recovery NREM sleep was eliminated.

Figure 4.

The correlation between changes in EEG theta power during SD and delta power during NREM RS, before and after saporin lesions. A) In the control pre-injection condition, SD induced increases in total theta power (5–9Hz), low theta (5–7Hz) and high theta (7–9Hz). After saporin lesions, increases in total and high theta power were still significant but smaller as compared to control condition. The increase in low theta power was completely eliminated. *, value significantly differs from baseline, $p < 0.05$; #, value significantly differs from control conditions, $p < 0.05$. B) In the control pre-injection condition, SD led to an increase in subsequent 3h of NREM delta power. This increase was completely blocked after saporin lesions. C) Correlation analysis between theta power during SD and delta power during NREM RS revealed a strong positive correlation between low theta and delta

(middle panel), but not between total theta and delta (left panel) and high theta and delta (right panel). D) After saporin lesions, the correlation between low theta and delta power is eliminated.

Figure 5.

Correlation between biochemical and electrophysiological markers of HSP. A) There was a positive correlation between the increase in total theta and increase in [Lac]_{ex}, but not between total theta and [NO]_x_{ex}, [AD]_{ex}, or [Pyr]_{ex}. B) There was a strong correlation between low theta and [NO]_x_{ex} or [AD]_{ex}. However, there was no correlation between low theta and [Lac]_{ex} or [Pyr]_{ex}. C) There was no positive correlation between high theta and [NO]_x_{ex} or [AD]_{ex}. However, there was a positive correlation between high theta and [Lac]_{ex} or [Pyr]_{ex}.

Figure 6.

Comparison of the effects of DETA NONOate infusion into BF before (control) and after saporin lesions (saporin). In the control condition, DETA NONOate infusion induced a significant increase in both NREM sleep (A) and NREM EEG delta power (B) within 18h after the infusion. These effects were eliminated after saporin lesions of cholinergic neurons. BL=pre-infusion baseline (2h), D=DETA NONOate infusion (3h). *, value significantly differs from baseline day, $p < 0.05$; #, value significantly differs from control conditions, $p < 0.05$.

Figure 7.

Comparison of iNOS expression in ChAT+ and non-ChAT+ cells after SD and in sleep-wake controls. For each animal, ChAT+, iNOS+, ChAT+/iNOS+ and ChAT-/iNOS+ cells were counted in 5 sections collected throughout the BF. Counts from 5 sections were summarized to yield a total number of cells for each rat. A) Number of iNOS+ cells was significantly higher in SD group compared to WC and SC groups; however, the number of ChAT+ cells did not differ. B) In the SD group, the majority of iNOS+ cells were ChAT+ (74%), and only 26% of cells were ChAT-/iNOS+. The number of ChAT+/iNOS+ cells significantly increased after SD, while the number of non-ChAT+/iNOS+ did not differ significantly between SD, WC and SC groups. C) Double-labeling with markers for ChAT+ and Parv+ wake-active BF neurons showed that some iNOS+ cells were Parv+, while the majority were ChAT+/iNOS+. White arrows indicate cells which were double-labeled for ChAT+/iNOS+ or Parv+/iNOS+. *, value significantly differs from control, $p < 0.05$.

Figure 8.

The correlation between iNOS induction in ChBF cells during SD and EEG low theta power. A) EEG theta power was increased during SD, but not during SC or WC (N=6). B) There was a strong positive correlation between iNOS intensity and EEG low theta power in the SD group. *Value significantly differs from baseline, $P=0.001$