Behavioral/Systems/Cognitive

Histamine Release in the Basal Forebrain Mediates Cortical Activation through Cholinergic Neurons

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The basal forebrain (BF) is a key structure in regulating both cortical activity and sleep homeostasis. It receives input from all ascending arousal systems and is particularly highly innervated by histaminergic neurons. Previous studies clearly point to a role for histamine as a wake-promoting substance in the BF. We used *in vivo* microdialysis and pharmacological treatments in rats to study which electroencephalogram (EEG) spectral properties are associated with histamine-induced wakefulness and whether this wakefulness is followed by increased sleep and increased EEG delta power during sleep. We also investigated which BF neurons mediate histamine-induced cortical activation. Extracellular BF histamine levels rose immediately and remained constant throughout a 6 h period of sleep deprivation, returning to baseline levels immediately afterward. During the spontaneous sleep–wake cycle, we observed a strong correlation between wakefulness and extracellular histamine concentrations in the BF, which was unaffected by the time of day. The perfusion of histamine into the BF increased wakefulness and cortical activity without inducing recovery sleep. The perfusion of a histamine receptor 1 antagonist into the BF decreased both wakefulness and cortical activity. Lesioning the BF cholinergic neurons abolished these effects. Together, these results show that activation of the cholinergic BF by histamine is important in sustaining a high level of cortical activation, and that a lack of activation of the cholinergic BF by histamine may be important in initiating and maintaining nonrapid eye movement sleep. The level of histamine release is tightly connected to behavioral state, but conveys no information about sleep pressure.

Introduction

The basal forebrain (BF) plays a central role in regulating wakefulness and cortical arousal (Détári and Vanderwolf, 1987; Buzsaki et al., 1988). Through its direct and widespread cortical projections (Rye et al., 1984), the BF serves as an important ventral extrathalamic relay to the cortex, receiving input from all ascending arousal systems (Semba et al., 1989; Szymusiak, 1995; Jones, 2004), including the histaminergic system (Jones, 2003). Histaminergic neurons are located in the tuberomammillary nucleus and project to virtually all brain regions (Haas and Panula, 2003); the BF is particularly highly innervated (Panula et al., 1989).

Measurements and manipulations of histamine concentrations have consistently linked increased histaminergic transmission to increases in wakefulness, while decreased histamine concentrations increase non-rapid eye movement (NREM) sleep (Monti, 1993; Strecker et al., 2002; Lin et al., 2011; Thakkar, 2011), linking histamine levels to behavioral state. C-*fos* staining showed that histaminergic neurons are wake-active regardless of the time of day (Ko et al., 2003). Single-unit recordings in head-restrained mice showed that histamine neurons are active only during wakefulness, and that their activity is highest during attentive waking (Takahashi et al., 2006). When perfused into the BF, histamine dose dependently and site specifically increased wakefulness and decreased NREM sleep without affecting rapid eye movement (REM) sleep (Ramesh et al., 2004).

Work from our group emphasized the importance of the BF in regulating sleep homeostasis through cortically projecting cholinergic neurons (Porkka-Heiskanen et al., 1997, 2000; Porkka-Heiskanen and Kalinchuk, 2011). We proposed that the increase in extracellular adenosine in the BF during prolonged wakefulness promotes sleep through inhibitory A1 receptors (Gass et al., 2009). Studies on what mechanisms counteract the sleep-inducing effect of increasing BF adenosine concentrations during prolonged wakefulness revealed that BF neurons increase their firing rate, and that dopamine and serotonin metabolites increase during the first 3 h of prolonged wakefulness (Kostin et al., 2010; Zant et al., 2011), probably contributing to the ability of the animals to stay awake under increased sleep pressure. As a wake-promoting substance, we also expected histamine to contribute to this effect.

High-frequency theta (interpreted as high cortical activity) during wakefulness is followed by high delta activity (an electroencephalogram (EEG) correlate of sleep intensity) during subsequent recovery sleep (Wigren et al., 2009). We showed that in the BF, NMDA, but not AMPA glutamate receptor activation was required for high theta activation during wakefulness (Wigren et al., 2007). These experiments clarified that, although many acti-

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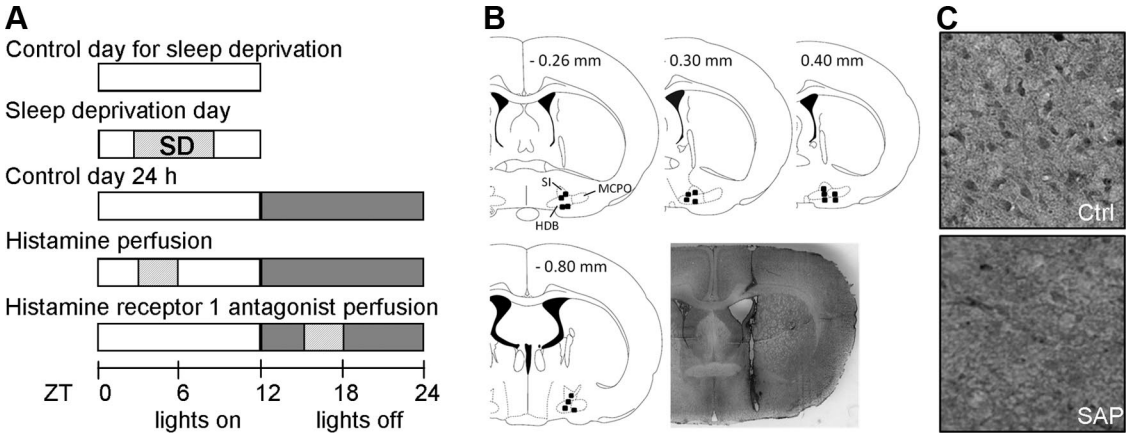

Figure 1. Experimental schedule, probe tip locations, and immunohistochemistry. ***A***, Experimental schedule. EEG and EMG were recorded continuously. On the control day before SD, aCSF perfusion began just after lights-on; perfusion stopped, and the animals were disconnected from the microdialysis tubing 30 min before lights-off. The SD experiment was similar to the control day, except for the 6 h SD by gentle handling (Franken et al., 1991), which began 2.5 h after lights-on. Microdialysis samples (flow 1 μl/min) were collected every 30 min. On the 24 h control day and during subsequent drug perfusion experiments, aCSF perfusion began 22–24 h before the control day began. Histamine perfusions were performed from ZT 3–6 (light period), and histamine receptor 1 antagonist perfusions took place between ZT 15 and 18 (dark period), after which aCSF perfusion remained continuous. ***B***, Probe tip locations were marked with ink injected through a modified microdialysis probe. All probe tips were located between −0.26 and −0.80 mm from bregma (Paxinos and Watson, 1998). Black squares indicate the location of the probe tip; if more than one tip was located in the same place, only one representative square is depicted. A representative coronal brain section showing the microdialysis probe track is shown in the lower right corner. ***C***, ChAT- positive neurons in the BF of a control animal (top) and after a local 192IgG-saporin lesion (bottom); 20× magnification. Ctrl, control; SAP, 192IgG-saporin lesion.

vating substances are able to increase cortical arousal through the BF, these activations have different EEG profiles and different effects on subsequent sleep.

In the present study, we characterized the type of wakefulness that histamine induces through BF activation. The specific questions, addressed using *in vivo* microdialysis combined with pharmacological treatments, were as follows: (1) Do histamine levels in the BF correlate with behavioral state? (2) Do elevated histamine levels in the BF induce wakefulness with high-frequency cortical theta activation followed by increased sleep intensity? (3) To what extent do BF cholinergic neurons mediate the effects of histamine on cortical activation?

Materials and Methods

Animals and surgery

Male Han-Wistar rats aged 3–4 months (300–400 g) were individually housed under constant temperature in a 12 h light/dark cycle. Food and water were provided *ad libitum*.

The animals were habituated to handling beginning at least 4 d before surgery. Under general anesthesia (2.5 mg/kg, i.p. diazepam; 0.4 mg/kg, i.p. medetomidine + 60 mg/kg, i.p. ketamine), the rats were placed in a stereotaxic device (Kopf Instruments). After exposing, cleaning and disinfecting the skull bone, two gold-coated screws were fitted into the skull for frontoparietal epidural bipolar recording of the EEG. To record the electromyogram (EMG), two silver wire electrodes were inserted into the neck musculature. A unilateral guide cannula for the microdialysis probe (CMA 11 Guide; CMA/Microdialysis) was placed 3 mm above the BF area, including the horizontal diagonal band of Broca (HDB), substantia innominata (SI), and magnocellular preoptic area (MCPO). The coordinates respective to bregma were as follows: anterior = −0.3 mm; lateral = 2.0 mm; vertical = −5.5 mm (Paxinos and Watson, 1998). Finally, the guide cannula, screw electrodes and supporting screws were secured in place with acrylic dental cement. After recovery and adaptation to the recording conditions (one week), a 3 d recording was taken from each rat to ensure that the rats had fully adapted to the conditions.

The Ethics Committee for Animal Experiments at the University of Helsinki and the provincial government of Southern Finland approved all experiments, which were performed in accordance with the laws of Finland and the European Union. We made every effort to minimize the number of animals used and their suffering.

In vivo microdialysis

Microdialysis probes (CMA 11, CMA/Microdialysis; membrane length 2 mm, diameter 0.24 mm, ~15% mean recovery rate for histamine) were inserted through the guide cannula 22–24 h before the first experiment.

Sleep deprivation experiment

Control day for SD. The control day served as a reference to which we compared subsequent microdialysis samples and EEG recordings obtained during the sleep deprivation day. During the control day, the animals were allowed to sleep and wake undisturbed. For a schematic representation of the experimental schedule, see Figure 1*A*. The rats were connected to microdialysis tubing and EEG/EMG recording leads just after lights-on, after which we began continuous EEG and EMG recording, and perfusion (1 μl/min) of artificial CSF (aCSF) (147 mM NaCl, 3 mM KCl, 1.2 mM $CaCl_2$, 1 mM $MgCl_2$). Samples were collected at 30 min intervals. The samples were stored at −80°C until assayed. The perfusion was stopped, and the animals were disconnected from the microdialysis tubing 30 min before lights-off.

Sleep deprivation day. Sleep deprivation (SD) was performed on the day after the control day. The SD experiment was similar to the control day, except for 6 h of SD by gentle handling (Franken et al., 1991) beginning 2.5 h after lights-on to allow time to connect the animals and obtain a pre-SD baseline. The animals were kept awake by introducing novel objects into the home cage.

Drug perfusion experiments

Control day 24 h. During the control day, the animals were allowed to sleep and wake undisturbed. Microdialysis samples were collected at 30 min intervals by a fraction collector to assess histamine release in the BF over a 24 h period. In addition, the control day served as a reference to which we compared subsequent EEG recordings obtained during the drug perfusion days. The aCSF perfusion was continuous and began immediately after probe insertion (22–24 h before the experiment began). EEG and EMG were recorded continuously.

Drug perfusions. Perfusions of either histamine (histamine base; Sigma-Aldrich) at three different concentrations (100 μM, 500 μM, or 1000 μM) or pyrilamine (10 mM), a histamine receptor 1 antagonist (pyrilamine maleate salt; Sigma-Aldrich), were performed following the control day up to 5 d after the control day. All drug solutions were prepared in aCSF. The concentrations of histamine were based on the concentrations used in a study by Ramesh et al. (2004) in which they

studied the wakefulness-inducing effects of histamine. To further investigate these effects, we used the same concentrations in our study, as in their experiment. The final concentration of 10 mM pyrilamine was based on preliminary testing using concentrations of 15 μM, 100 μM, and 10 mM, of which only the 10 mM concentration clearly reduced wakefulness.

On each day, a 3 h baseline period of aCSF perfusion (same-day baseline) preceded the 3 h of drug perfusion. Histamine was perfused between 3 and 6 h after lights-on (zeitgeber time (ZT) 3–6, light period). The histamine receptor 1 antagonist was perfused from 3–6 h after lights-off (ZT 15–18, dark period). After drug perfusion, the perfusion medium was reverted back to aCSF and the perfusion continued nonstop. Drug perfusions were performed at least 24 h apart in a counterbalanced design.

To verify the stability of the recovery of the microdialysis probes over several days, we performed an *in vivo* recovery experiment. We measured the concentration of histamine in 30 min samples on the control day and again after 5 d of continuous perfusion and then compared the maximum concentrations; we found no significant difference (data not shown).

Unilateral local cholinergic lesion experiments

Unilateral local cholinergic lesions, modified from Kalinchuk et al. (2008), were performed using the immunotoxin 192IgG-saporin (Millipore) in a separate group of animals ($n = 5$); 192IgG is a monoclonal antibody that binds to the p75 nerve growth factor receptor located on cholinergic BF neurons. When linked to saporin, the newly formed 192IgG-saporin complex acts as a selective immunotoxin, destroying cholinergic BF neurons (Book et al., 1992; Heckers et al., 1994).

During the surgery for EEG/EMG placement and microdialysis guide cannula implantation, 192IgG-saporin (0.23 μg/μl) was injected locally through a modified microdialysis probe, with the membrane tip cut off, connected to a 10 μl Hamilton syringe. It was injected at a flow rate of 0.1 μl/min into the HDB/SI/MCPO for 10 min to reach a total injection volume of 1 μl. After the injection, the modified probe remained in place for 5 min and was then carefully removed.

Two weeks after the lesion, the EEG and EMG were recorded during a control day and during the drug perfusion experiments as described in the previous section. For histamine perfusions, we used only the highest dose (i.e.,1000 μM).

EEG recording and analysis

The EEG and EMG signals were amplified (gain 5000), filtered (high pass: 0.3 Hz; low pass 100 Hz), and sampled at 271 Hz. EEG recordings were manually scored in 4 s epochs for wakefulness, NREM sleep, and REM sleep in the Spike2 program (version 6, Cambridge Electronic Devices) using the script Sleepscore v1.01.

The vigilance states were scored according to standard criteria. Wakefulness was scored for low-amplitude desynchronized EEG activity in combination with activity in the EMG. To score low and high EMG-activity wakefulness, the maximum EMG amplitude was determined per 24 h file. Epochs were scored as low EMG-activity wakefulness when the amplitude of the EMG in the entire epoch did not rise >25% of the maximum amplitude. High EMG-activity wakefulness was scored when the EMG amplitude rose >25% of the maximum amplitude. NREM sleep was determined by high-amplitude delta (0.5–4 Hz) waves in the EEG and low-amplitude or absent EMG. REM sleep was identified by regular theta (5–9 Hz) activity in the EEG and low or absent EMG activity.

Brief awakenings were scored for brief periods of wakefulness lasting no more than 16 s. Epochs containing artifacts or more than one vigilance state were excluded from further analysis.

EEG power spectra were calculated within the 0.5–50 Hz frequency range by fast Fourier transform (FFT = 256; Hanning window, 0.5 Hz resolution).

High-performance liquid chromatography

The concentration of histamine was determined using high-performance liquid chromatography (HPLC) combined with fluorescence detection. The HPLC system consisted of four Shimadzu LC20AD pumps, an autosampler SIL-20AC, a fluorescence detector RF-10Axl, and a controller CBM-20A. LCSolution 1.21 software was used for system control and data collection/processing. The dialysis samples were analyzed without prior purification.

The histamine analysis method was based on the online postcolumn derivatization with *o*-phthalaldehyde, as described by Yamatodani et al. (1985). Briefly, samples were separated on a 4.6*50 mm, 5 μm SCX Phenomenex Luna column equipped with a SecurityGuard SCX 4*3 mm precolumn cartridge (Phenomenex); the mobile phase consisted of 0.25 M KH_2PO_4/0.75 mM NaN_3 (flow rate 0.6 ml/min), samples were then automatically derivatized by online mixing with 0.1% OPA/2 M NaOH/0.2M H_3BO_3 reagent in a reaction coil incubated at 45°C and, finally, stabilized with 3M H_3PO_4. Fluorescence was measured at Ex360, Em 450 nm.

Histological verification of probe locations

After the experiments, animals received a lethal dose of pentobarbital (100 mg/kg). To verify the probe locations, ink was injected through a modified microdialysis probe inserted into the guide cannula. The brains were then removed, frozen on dry ice, and stored at −80°C. Sections (20 μm) were cut on a freezing microtome, stained with toluidine blue, and visually inspected under a light microscope. Only data from animals with probe tips located within the limits of the BF region (HDB, MCPO, and SI) were included in the analysis.

Choline acetyltransferase immunohistochemistry to verify local cholinergic lesions

Animals used in the unilateral local cholinergic lesion experiments (five lesioned, four control) were transcardially perfused under deep anesthesia (pentobarbital 100 mg/kg, buprenorphine 0.02 mg/kg) with 20 ml 0.9% saline followed by 180 ml 4% paraformaldehyde (PFA) in 0.1 M PBS, pH 7.4. The brains were then removed and postfixed overnight in the same PFA solution. The brains were then submerged in a 30% sucrose solution at 4°C for 4 d for cryoprotection. After the brains sank, they were frozen and stored at −80°C. Coronal sections (40 μm) were cut all the way through the BF area on a freezing microtome and collected into PBS for immunohistochemical staining for choline acetyltransferase (ChAT). The sections were washed in PBS, after which endogenous peroxidase activity was blocked by incubation with 0.3% hydrogen peroxide (H_2O_2) in PBS for 30 min at room temperature. The sections were then treated with blocking solutions (0.5% Triton X-100 in PBS for 2 h; 3% donkey serum in PBS for 1 h) and incubated overnight at 4°C with a rabbit anti-ChAT primary antibody (1:1000; Millipore). The following day, the sections were incubated with the secondary antibody (biotinylated donkey anti-rabbit IgG 1:300; Millipore) for 2 h at room temperature and treated with avidin–biotin complex (ABC; Vector Elite Kit, Vector Laboratories). Diaminobenzidine tetrahydrochloride (DAB; peroxidase substrate kit DAB, Vector Laboratories) was used for visualization. After staining, sections were mounted on microscope slides (Menzel-Gläser) and coverslipped with Eukitt quick-hardening mounting medium (Fluka; Sigma-Aldrich).

The ChAT-positive cells were counted unilaterally in three sections (interval 200 μm) per rat using a light microscope (Nikon) at a 10× magnification (Fig. 1*C*); a 4× magnification was used to determine the outline of the BF. The cell counts of these three sections were averaged and compared between the lesion and control groups.

Data analysis and statistics

All values are shown as a mean ± SEM. Statistical analyses were performed using SigmaStat 3.1 (SPSS Science Software). For analysis of the histamine concentration during SD, histamine levels were averaged in 1 h bins. We used a one-way repeated-measures ANOVA with multiple comparisons versus the control group (same day baseline sample BL1) and the Holm–Sidak method as a *post hoc* test, to test for significant differences during the SD day. For the SD control day, we used a one-way repeated-measures ANOVA on ranks with multiple comparisons versus the control group and Dunn's *post hoc* method. To compare histamine levels from the 24 h control day with the percentage of time spent in wakefulness, we matched histamine levels in the BF from a 30 min mi-

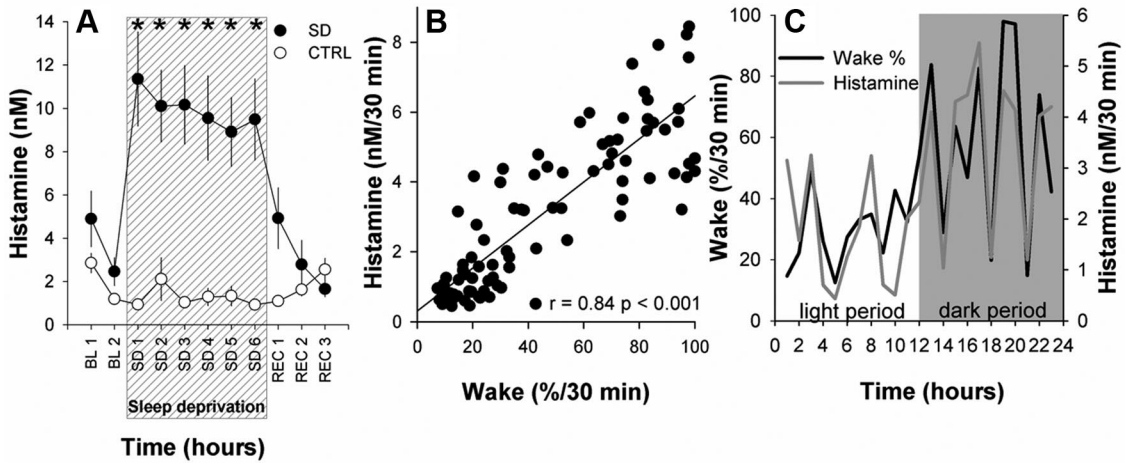

Figure 2. Effect of wakefulness on histamine levels in the BF. ***A***, Effect of 6 h SD on extracellular histamine levels in the BF. Time course of histamine levels on the SD (closed circles) and control (open circles) days (nM). Circles indicate mean levels ± SEM in 1 h time bins. The hatched area indicates the SD period. BL, same day baseline; CRTL, control day; SD, sleep deprivation day/period; REC, recovery sleep. *Significant difference from BL (SD day only; $n = 8$; one-way repeated-measures ANOVA $p < 0.001$). ***B***, We compared histamine levels from a 24 h period with the percentage of time spent in wakefulness. We matched histamine levels in the BF from a 30 min microdialysis sample with the percentage of wakefulness in their corresponding 30 min time bin. We compared samples from four rats, analyzed one 30 min sample per hour over a 24 h period (six samples were lost). A simple regression line is drawn across the plot ($n = 90$ $r = 0.84$ Pearson product moment correlation $p \leq 0.001$). ***C***, Histamine levels in the BF (dark gray line, right *y*-axis) and percentage of wakefulness (black line, left *y*-axis) from a representative animal over a 24 h period. The shaded area indicates the dark period.

crodialysis sample with the percentage of wakefulness in their corresponding 30 min time bin and analyzed this using the Pearson Product Moment Correlation.

To test the effect of the different concentrations of histamine and histamine receptor 1 antagonist on vigilance state, we used a two-way repeated-measures ANOVA per vigilance state with factors "concentration" and "time." To test the effect of drug perfusion after the cholinergic lesion, we used factors "vigilance state" and "time."

Power spectra were calculated in 3 h bins (same-day baseline, perfusion, recovery 1, recovery 2, and recovery 3). For statistical analysis, we normalized power spectra during perfusion, recovery 1, recovery 2, and recovery 3 against same-day baseline on the drug perfusion day as well as on the control day. To test the effect of the different concentrations of histamine on the EEG power spectra, we used a two-way repeated-measures ANOVA per vigilance state per time point with factors "concentration" and "frequency."

To assess the effect of histamine perfusion on the amounts of NREM, brief awakenings, and NREM bouts, we used a two-way repeated-measures ANOVA with factors "concentration" and "time."

We also used a two-way repeated-measures ANOVA with factors "concentration" and "type of wakefulness" to investigate relative changes in the amount of low- and high-EMG-activity wakefulness.

We tested for changes in the EEG power spectra of low- and high-EMG-activity wakefulness during histamine perfusion using a two-way repeated-measures ANOVA with factors "concentration" and "frequency."

All two-way repeated-measures ANOVAs were followed by Holm–Sidak *post hoc* tests.

Results

Histology and immunohistochemistry

Histological analyses confirmed the location of the microdialysis probe tip within the limits of the BF region in all animals included in the analyses. The probe tip location data are summarized in the schematic drawing of coronal brain sections (Fig. 1*B*). To assess the effect of the unilateral local cholinergic lesions, ChAT-positive cells were counted unilaterally and compared with the control group. Compared with the control group (*t* test, $t_{(8)} = 31.92$, $p < 0.001$), 192IgG-saporin lesions (Fig. 1*C*) caused a significant loss of cholinergic neurons of 83.3 ± 1.69%. The effect of this lesion was in accordance with previous results (Kalinchuk et al., 2008; Kaur et al., 2008).

Histamine levels in the BF correlate with wakefulness

To assess whether histamine transfers information about sleep pressure to the BF, we measured extracellular histamine levels in the BF during SD and on a control day.

SD by "gentle handling " increased the extracellular levels of histamine during the 6 h SD period, as compared with baseline. The histamine release increased immediately from the first hour of SD, remaining elevated until the last hour of SD. We found no progressive increase in extracellular histamine levels. Recovery sleep was associated with an immediate decrease in the histamine concentration to the baseline level (Fig. 2*A*; $n = 8$; one-way repeated-measures ANOVA multiple comparisons versus control *post hoc* Holm–Sidak, $F_{(10,60)} = 16.034$; $p < 0.001$). The levels of extracellular histamine during SD were somewhat higher than those observed during natural waking periods probably due to higher levels of attentive wakefulness that our SD method elicited, since histaminergic activity is highest during attentive wakefulness (Takahashi et al., 2006).

To investigate whether histamine release in the BF correlates with the percentage of wakefulness, as was shown for histamine release in the rat frontal cortex (Chu et al., 2004), histamine levels from a 24 h period were compared with the percentage of time spent in wakefulness. Histamine levels from a 30 min microdialysis sample were matched with the percentage of wakefulness in their corresponding 30 min time bin. We compared samples from four rats and analyzed one 30 min sample per hour over a 24 h period (six samples were lost). Figure 2*B* shows the strong positive correlation ($r = 0.84$) between extracellular histamine levels in the BF and the percentage of wakefulness in the corresponding 30 min bin. A simple regression line is drawn across the plot ($n = 90$; Pearson product moment correlation $p < 0.001$). When analyzed separately for the light and dark periods, the *r* values were 0.703 and 0.737, respectively ($n = 45$; Pearson product moment correlation $p < 0.001$).

The histamine levels and wakefulness percentages of a representative animal during a 24 h period are shown in Figure 2*C*, demonstrating the strikingly similar pattern.

Histamine perfusion in the BF increases the percentage of wakefulness

To confirm the wakefulness-promoting effects of histamine in the BF observed by Ramesh et al. (2004), three different concentrations (100, 500, and 1000 μM) were perfused into the BF during the second 3 h of the light period. All concentrations of histamine perfused into the BF significantly increased wakefulness (Fig. 3; $n = 6$, two-way repeated-measures ANOVA *post hoc* Holm–Sidak, $F_{(5,70)} = 25.897$, $p < 0.001$) and decreased NREM sleep (Fig. 3; $n = 6$, two-way repeated-measures ANOVA *post hoc* Holm–Sidak, $F_{(5,70)} = 30.170$, $p < 0.001$), as compared with the control day. REM sleep remained unaffected by all three concentrations. In contrast with the results from Ramesh et al. (2004), we found no significant dose–response effect on the vigilance state. Since the measured extracellular histamine concentrations in the BF range from 6 to 100 nM (corrected for 15% probe recovery), the effect of our lowest dose (15 μM when corrected for probe recovery) could already be inducing the maximal possible increase in wakefulness. A strain difference between Han–Wistar (used in this study) and Sprague Dawley (used in Ramesh et al., 2004) may account for this dissimilarity.

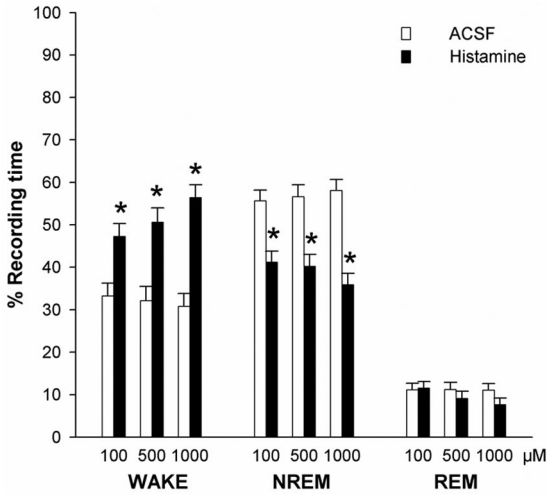

Figure 3. Effect of BF histamine perfusion during the light period on vigilance state. Effect of 3 h BF perfusion during the light period at three different concentrations (100, 500, 1000 μM) of histamine on vigilance state. Bars represent the mean ± SEM on the control day (open bars) or drug perfusion day (black bars) during the 3 h perfusion period. *Significant difference from control day ($n = 6$; two-way repeated-measures ANOVA $p < 0.001$).

Histamine perfusion into the BF increases EEG arousal

To examine the effect of extracellular histamine in the BF on cortical activation, we computed EEG power spectra using FFT.

The 3 h perfusion of histamine into the BF during the light period caused significant changes in the EEG power spectra across vigilance states.

Wakefulness-specific EEG power spectra effects of histamine perfusion

During the perfusion of 1000 μM histamine, we found a significant decrease in the delta range (1.5–2 Hz) and low theta range (5–6.5 Hz) of the EEG power spectra (Fig. 4*A*,*D*). In addition, we observed a significant increase in the high theta range (7.5–8 Hz for the 100 μM concentration; 8–8.5 Hz for the 1000 μM concentration; $n = 6$, two-way repeated-measures ANOVA *post hoc* Holm–Sidak, $F_{(100,3000)} = 3.836$, $p < 0.001$). During the recovery period, the wakefulness-specific EEG power spectra returned to control day levels for all concentrations.

NREM sleep-specific EEG power spectra effects of histamine perfusion

NREM sleep-specific power spectra showed decreased power in the delta and low theta range for all three concentrations (Fig. 4*B*,*E*) (0.5–4.5 Hz for 100 μM; 0.5–5 Hz for 500 μM; 0.5–5.5 Hz for 1000 μM) as well as a decrease in the high theta, sigma, and higher frequency ranges (8.5–15 Hz for 100 μM; 7.5–20.5 Hz for 500 μM; 8–19.5 Hz for 1000 μM; $n = 6$, two-way repeated-measures ANOVA *post hoc* Holm–Sidak, $F_{(100,3000)} = 66{,}802$, $p < 0.001$).

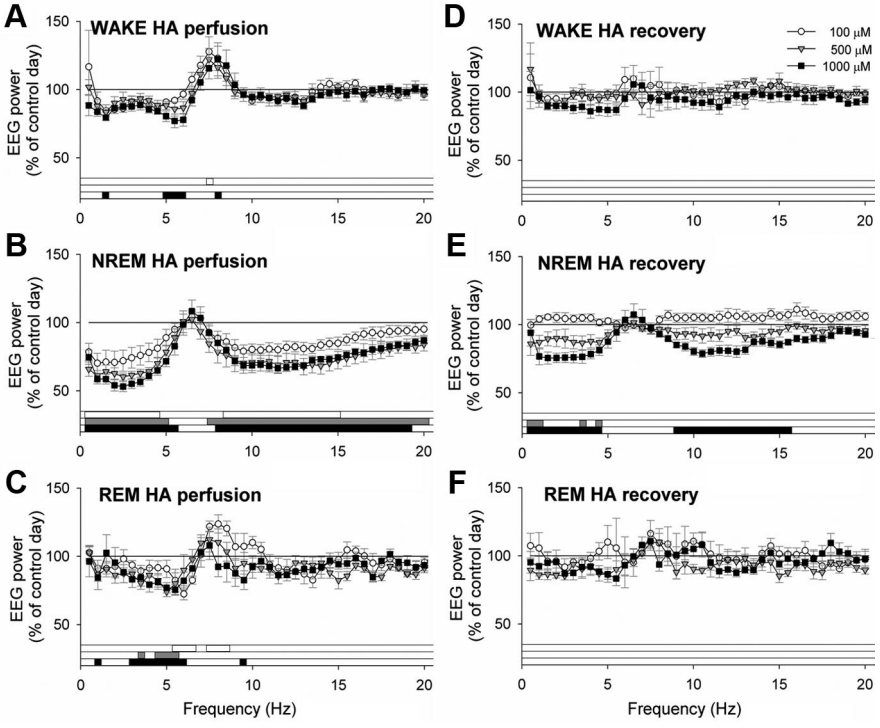

Figure 4. Effect of BF histamine perfusion during the light period on EEG power spectra. Effect of 3 h BF histamine perfusion during the light period at three different concentrations (100, 500, 1000 μM) on wakefulness, NREM, and REM sleep EEG power spectra during the 3 h perfusion period (***A–C***) or during the 3 h after perfusion (recovery, ***D–F***). Circles (100 μM), triangles (500 μM), and squares (1000 μM) represent the group mean ± SEM of EEG power expressed as a percentage of the EEG power at the same time on the control day, and the control day level is indicated by the horizontal 100% reference line. Bars above the *x*-axis indicate the frequency bins per concentration that significantly differ from those at the same time on the control day ($n = 6$, two-way repeated-measures ANOVA $p < 0.001$).

NREM sleep-specific EEG power spectra from the rats that received the 500 and 1000 μM concentrations showed a delayed return to control day levels. During the first 3 h recovery period, the delta, low theta, sigma, and higher frequency ranges of these power spectra remained decreased (0.5–1.5, 3.5–4, and 4.5–5 Hz for 500 μM; 0.5–5 and 9–16 Hz for 1000 μM; $n = 6$, two-way repeated-measures ANOVA *post hoc* Holm–Sidak, $F_{(100,3000)} = 48{,}404$, $p < 0.001$). The power spectra from these concentrations

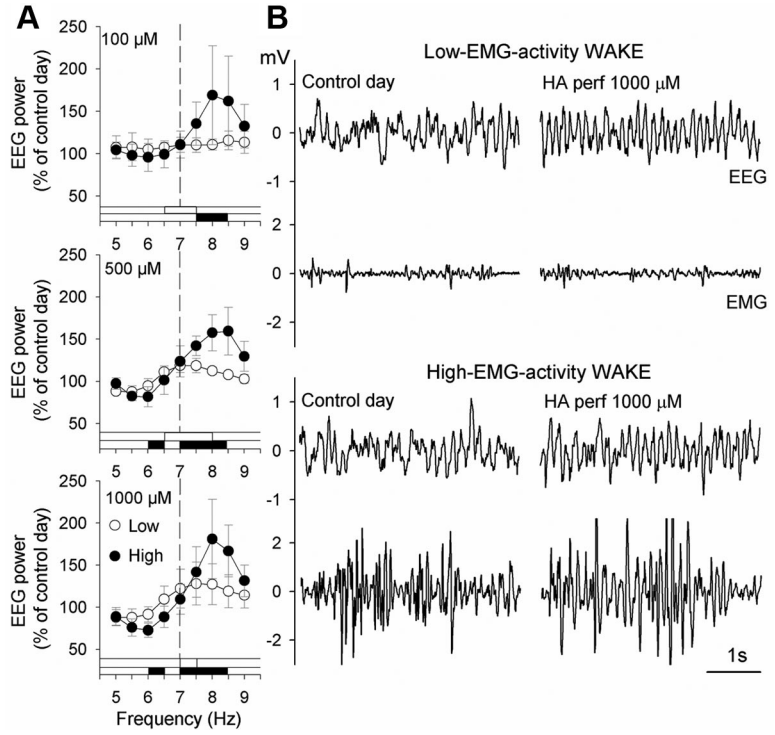

Figure 5. Effect of BF histamine perfusion during the light period on low and high EMG-activity wakefulness EEG power. ***A***, Effect of 3 h BF perfusion during the light period at three different concentrations (100, 500, and 1000 μM) of histamine on low (open circles) and high (closed circles) EMG-activity wakefulness EEG power spectra during the 3 h perfusion period. Circles represent the group mean ± SEM of EEG power expressed as a percentage of the EEG power at the same time on the control day. Bars above the *x*-axis indicate the frequency bins that differ significantly from those at the same time on the control day ($n = 6$, for 500 μM $n = 5$, two-way repeated-measures ANOVA $p < 0.001$). ***B***, Traces of a 4 s EEG/EMG recording of both the control day and histamine perfusion day (1000 μM). Top: Low EMG-activity wakefulness. Bottom: High EMG-activity wakefulness.

returned to control day levels in the second 3 h recovery period (data not shown).

REM sleep-specific EEG power spectra effects of histamine perfusion

The REM sleep-specific EEG power spectra revealed a decrease in the high delta and low theta range, in addition to an increase in the high theta range for the 100 μM concentration (5.5–7 and 7.5–9 Hz for 100 μM; 3.5–4 and 4.5–6 Hz for 500 μM; 1–1.5, 3.5–6.5, and 9.5–10 Hz for 1000 μM; $n = 6$, two-way repeated-measures ANOVA *post hoc* Holm–Sidak, $F_{(100,3000)} = 3.787$, $p < 0.001$) (Fig. 4*C*,*F*). No significant differences were found during the recovery period.

Histamine perfusion in the BF promotes attentive wakefulness

Histamine perfusion in the BF led to an increase in wakefulness (Fig. 3) and in high theta power during wakefulness, which was clearly evident in the EEG recording (Fig. 5*B*). To assess whether the increase in wakefulness and high theta elicited by histamine perfusion resulted purely from an increase in motor activity, we examined the distribution of low and high EMG-activity wakefulness. Although histamine perfusion increased wakefulness at all three concentrations, we found no significant differences in the distribution of low and high EMG-activity wakefulness. A trend emerged toward a significant increase of high EMG-activity wakefulness at 100 μM ($p = 0.099$; $n = 6$, two-way repeated-measures ANOVA). In addition, we compared the wakefulness-specific EEG power spectra in the theta range separately for low and high EMG-activity wakefulness for all three histamine perfusion concentrations. At all three concentrations, we found a significant increase in the higher theta range for both low and high EMG-activity wakefulness. This increase was of a greater magnitude and occurred in higher frequencies during high EMG-activity wakefulness, than during low EMG-activity wakefulness (Fig. 5*A*). For low EMG-activity wakefulness: 6.5–7.5 Hz for 100 μM ($n = 6$, $F_{(12,60)} = 35.062$), 6.5–8 Hz for 500 μM ($n = 5$, $F_{(12,48)} = 19.059$), and 7–7.5 Hz for 1000 μM ($n = 6$, $F_{(12,60)} = 26.039$); for high EMG-activity wakefulness: 7.5–8.5 Hz for 100 μM ($n = 6$, $F_{(12,60)} = 21.580$), 7–8.5 Hz for both 500 ($n = 5$, $F_{(12,48)} = 12.48$), and 1000 μM ($n = 6$, $F_{(12.60)} = 18.698$). Both the 500 and 1000 μM concentrations also elicited a decrease in the lower theta range (6–6.5 Hz), but only for high EMG-activity wakefulness (two-way repeated-measures ANOVA *post hoc* Holm–Sidak $p < 0.001$).

Histamine perfusion in the BF elicits no rebound sleep

The 3 h perfusion of histamine in the BF reduced NREM sleep (Fig. 3) and NREM delta power (Fig. 4*B*), but increased high theta power during wakefulness (Figs. 4*A*, 5). However, we observed no increase in subsequent NREM delta power during the recovery period (Fig. 4*D*). To investigate this further, we compared the amount of NREM sleep and two measures for sleep pressure; namely, brief awakenings (≤16 s) and the number of NREM bouts during the recovery period from the 1000 μM histamine perfusion to control day amounts. During the recovery period, one time point showed an increase in NREM sleep after histamine perfusion (ZT 16–18) as compared with the same time on the control day (Fig. 6*A*; $n = 6$, two-way repeated-measures ANOVA *post hoc* Holm–Sidak, $F_{(7,35)} = 22.987$, $p < 0.001$). We found no differences in the number of brief awakenings between the perfusion and control day (Fig. 6*B*). The number of NREM bouts increased at two time points (ZT 16–18 and 19–21; Fig. 6*C*; $n = 6$, two-way repeated-measures ANOVA *post hoc* Holm– Sidak, $F_{(7.35)} = 17.699$, $p < 0.001$).

Histamine receptor 1 antagonist perfusion in the BF decreases the amount of wakefulness

The histamine receptor 1 antagonist pyrilamine (10 mM) was perfused into the BF during the second 3 h of the dark period to assess its somnogenic effects. The perfusion reduced wakefulness and increased NREM sleep significantly compared with the same time on the control day (Fig. 7*A*; $n = 6$, two-way repeated-measures ANOVA *post hoc* Holm–Sidak, $F_{(10,50)} = 6.824$, $p < 0.001$).

Histamine receptor 1 antagonist perfusion in the BF decreases cortical arousal

The 3 h perfusion of a histamine receptor 1 antagonist (pyrilamine; 10 mM) into the BF during the dark period resulted in significant changes in the EEG power spectra across vigilance

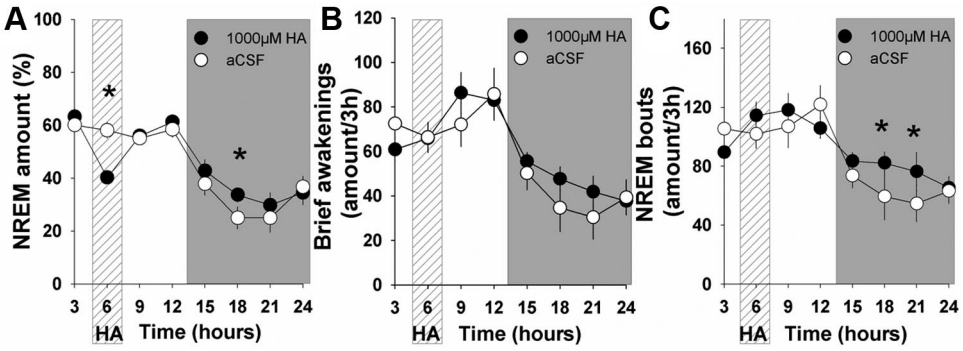

Figure 6. Effect of BF histamine perfusion during the light period on subsequent NREM and sleep pressure. Effect of 3 h BF perfusion of histamine (HA) during the light period (1000 μM) on the amount of (***A***) NREM, (***B***) brief awakenings, and (***C***) NREM bouts. Circles indicate mean levels ± SEM in 3 h time bins on the control day (open circles) and on the histamine perfusion day (closed circles). The hatched area indicates the histamine (HA) perfusion, and the shaded area indicates the dark period. *Significant difference from control day ($n = 6$, two-way repeated-measures ANOVA $p < 0.001$).

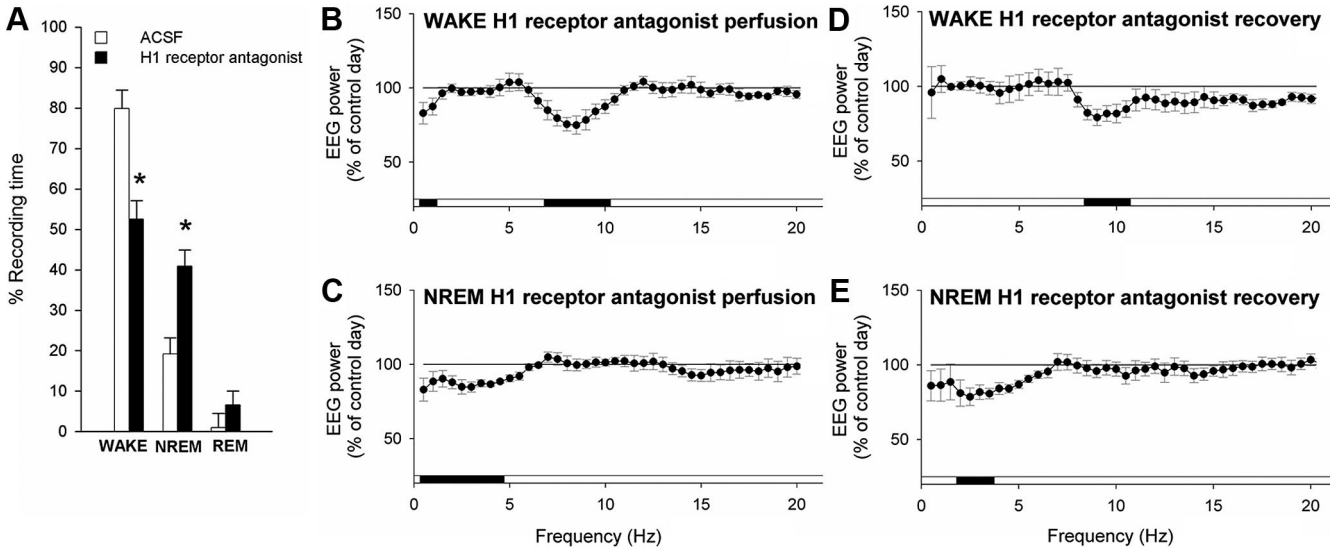

Figure 7. Effect of BF histamine receptor 1 antagonist perfusion during the dark period on vigilance state and EEG power spectra. ***A***, Effect of 3 h BF perfusion of histamine receptor 1 antagonist during the dark period (pyrilamine, 10 mM) on vigilance state. Bars represent the mean ± SEM on the control day (open bars) or drug perfusion day (black bars) during the 3 h perfusion period. *Significant difference from control day ($n = 6$, two-way repeated-measures ANOVA $p < 0.001$). ***B***, ***C***, Effect of 3 h BF perfusion of histamine receptor 1 antagonist during the dark period (10 mM pyrilamine) on wakefulness and NREM sleep EEG power spectra during the 3 h perfusion period or (***D***, ***E***) during the 3 h after perfusion (recovery). Circles represent the group mean ± SEM of EEG power expressed as a percentage of the EEG power at the same time on the control day, and the horizontal 100% reference line indicates the control day level. Bars above the *x*-axis indicate the frequency bins that differ significantly from those at the same time on the control day ($n = 6$, two-way repeated-measures ANOVA $p < 0.001$).

states. We could not assess REM sleep spectra, however, because the amount of REM sleep at this time is typically very low (1% or less), and the number of rats who spent time in REM sleep during the perfusion period on the control day and also on the perfusion day was too small to compare EEG power spectra.

Wakefulness-specific EEG power spectra effects of histamine receptor 1 antagonist perfusion

During the histamine receptor 1 antagonist perfusion, we observed a significant decrease in the delta range (0.5–1.5 Hz) and the high theta range (7–10.5 Hz) of the EEG power spectra during wakefulness (Fig. 7*B*; $n = 6$, two-way repeated-measures ANOVA *post hoc* Holm–Sidak, $F_{(100,500)} = 7.399$, $p < 0.001$). During the first 3 h recovery period, a significant decrease in the high theta range remained in the wakefulness-specific EEG power spectrum (8.5–10.5 Hz; Fig. 7*D*; $n = 6$, two-way repeated-measures ANOVA *post hoc* Holm–Sidak, $F_{(100,500)} = 3.237$, $p < 0.001$).

We found no significant differences from the second 3 h recovery period onwards.

NREM sleep-specific EEG power spectra effects of histamine receptor 1 antagonist perfusion

The histamine receptor 1 antagonist affected NREM sleep-specific EEG power spectra in the delta and low theta range (0.5–4.5 Hz; Fig. 7*C*; $n = 6$, two-way repeated-measures ANOVA *post hoc* Holm–Sidak, $F_{(100,500)} = 6.631$, $p < 0.001$). We observed a sustained decrease in the delta range (2–3.5 Hz; Fig. 7*E*; $n = 6$, two-way repeated-measures ANOVA *post hoc* Holm–Sidak, $F_{(100,500)} = 3.109$, $p < 0.05$) during the first 3 h recovery period, after which the power spectra values returned to control day levels.

The observed decrease in the delta and low theta ranges during histamine receptor 1 antagonist perfusion and recovery could be due to a reduction of high theta frequencies during wakefulness that would otherwise increase delta power in subsequent NREM sleep. The additional NREM sleep during the perfusion and the recovery period most likely consisted of light NREM sleep in which the delta range is less pronounced than in deep NREM sleep, which could also explain the ob-

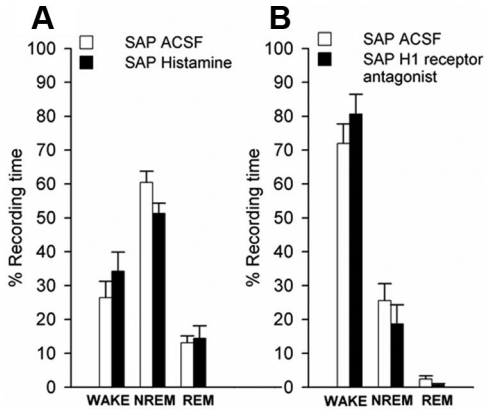

Figure 8. No effect of BF histamine or histamine receptor 1 perfusion on vigilance state remained after local cholinergic lesions. No significant effect of 3 h BF perfusion of (***A***) histamine during the light period (1000 μM) and (***B***) histamine receptor 1 antagonist during the dark period (pyrilamine, 10 mM) on vigilance state remained after local cholinergic lesions. Bars represent the mean ± SEM on the control day (open bars) or drug perfusion day (black bars) during the 3 h perfusion period as a percentage of total wakefulness ($n = 5$; two-way repeated-measures ANOVA). SAP, 192IgG-saporin.

served decrease in delta power compared with the baseline day power spectra.

Cholinergic BF neurons mediate the arousal effect of histamine

We used unilateral local BF cholinergic lesions (Kalinchuk et al., 2008) to examine the role of these neurons in mediating histamine-induced cortical arousal. Two weeks after the cholinergic lesions, we repeated the histamine (1000 μM) and histamine receptor 1 antagonist (10 mM) perfusions; now, although both treatments resulted in a modest increase in wakefulness, we observed no significant effect on vigilance states (Fig. 8; $n = 5$; two-way repeated-measures ANOVA). The remaining cholinergic neurons may have mediated this small residual activating effect of histamine perfusion, because the lesion reduced the number of neurons by ~83%. However, we observed the small increase in wakefulness after both histamine and histamine receptor 1 antagonist perfusion, and it is not necessarily a result of these treatments. The cholinergic lesion per se results in a small transient decrease in wakefulness, which recovers between days 11 and 17 (Kaur et al., 2008). Since our recordings took place between days 14 and 17, it is possible that the observed effect may be due to the recovery to normal wakefulness levels after our baseline recording took place.

Because intraparenchymal injections of immunotoxin 192IgG-saporin selectively lesion BF cholinergic neurons without affecting the number of parvalbumin- and GAD-immunopositive neurons (Pizzo et al., 1999; Kalinchuk et al., 2008; Kaur et al., 2008), this method demonstrates that cholinergic BF neurons mediate histamine-induced cortical arousal. The lack of significant effect of histamine or histamine receptor 1 antagonist perfusion on the vigilance state after the cholinergic lesion also excludes the possibility that the observed effect of perfusion may result from diffusion away from the BF, because the extent of this lesion is localized to the BF, thereby sparing, for instance, the septo-hippocampal cholinergic system (Berntson et al., 2002).

Discussion

The main findings of the present study were that histamine induces theta-enriched cortical activation through the cholinergic neurons of the BF, and that this activation does not lead to increased sleep pressure.

We also observed a strong correlation between behavioral state and BF histamine concentrations, a correlation unaffected by the time of day, thus suggesting that histamine release is regulated primarily by vigilance state rather than by circadian timing.

Perfusing a histamine receptor 1 antagonist into the BF had a strong negative effect on cortical activation and increased the amount of NREM sleep. As this effect disappeared after a cholinergic lesion, we conclude that histamine-mediated cortical activation is profoundly relayed through the cholinergic neurons in the BF. A summary of our current findings and the existing literature is shown in Figure 9.

Diurnal, behavioral-state-associated release of histamine in the BF

We found a remarkably high correlation between histamine levels in the BF and wakefulness when the animals were left undisturbed during both the light and dark periods. Previous work showed a similar relationship with cortical histamine levels (Chu et al., 2004). The overall lower BF histamine levels during the light period (sleep period for rodents) were interspersed with increases during periods of waking, suggesting that the vigilance state overrules the potential circadian component in the regulation of brain histamine levels; in other words, during waking, histamine levels increase regardless of the time of day.

Histamine perfusion into the BF induces cortical activity through cholinergic neurons

Histamine perfusion increased wakefulness and reduced NREM sleep at all three concentrations. Histamine perfusion increased the high theta and decreased the delta and low theta ranges in the wakefulness-specific EEG power spectra. During NREM sleep, the delta, low theta, and sigma ranges were decreased. In rodents, the theta range varies between 4 and 12 Hz depending on the animal, species, and recording method (Winson, 1972; van Lier et al., 2003). The high theta range (>7 Hz) is associated with active exploratory behavior and attentive wakefulness (Kramis et al., 1975; Lancel, 1993). The delta and low theta ranges are associated with increased sleep pressure and drowsiness during wakefulness. During NREM sleep, the delta range is a measure of sleep intensity, whereas the sigma range corresponds to sleep spindles found during the lighter stages of NREM sleep (Dijk, 2009). The BF modulates EEG activity through cholinergic, GABAergic, and glutamatergic cortical projections (Henny and Jones, 2008). The experiments following the 192IgG-saporin lesion demonstrated that cholinergic BF neurons mediate histamine-induced cortical activation. In guinea pig basal forebrain slices, Khateb et al. (1995) showed that cholinergic neurons are activated mainly through histamine receptor 1, and histamine perfusion in the BF of freely moving rats increased acetylcholine release in the cortex (Khateb et al., 1995; Cecchi et al., 2001). While the actions of histamine on GABAergic and glutamatergic neurons in the cortical projecting part of the BF are poorly characterized, histamine can excite GABAergic neurons in the adjacent medial septum/diagonal band of Broca that projects to the hippocampus via both histamine receptors 1 and 2 (Xu et al., 2004).

To clarify the connection between motor activity and histamine-induced high theta activity, we calculated the distribution of low and high EMG-activity wakefulness during the histamine perfusion and found that histamine perfusion did not increase the relative amount of high EMG-activity wakefulness. In addition, the EEG power spectra for both low and high EMG-

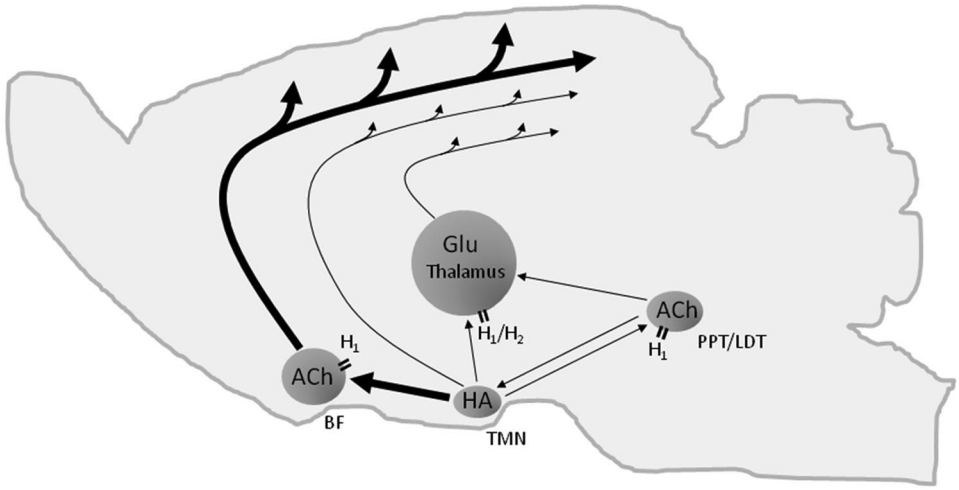

Figure 9. Schematic representation of histaminergic projections and mechanisms involved in wakefulness and cortical activation. Represented are the four main ascending and descending excitatory histaminergic pathways involved in wakefulness and cortical activation. The putative neurotransmitters and receptors involved are shown in each structure. The results from previous studies and the current study show that the perfusion of histamine into the BF results in wakefulness and cortical activation, whereas antagonizing BF histamine receptor 1 decreases cortical arousal. A specific lesion of the BF cholinergic cells abolishes these effects, suggesting that the corticopetal pathway through the BF is the main route for histamine to activate the cortex. ACh, acetylcholine; BF, basal forebrain; Glu, glutamate; H_1, histamine receptor 1; H_2, histamine receptor 2; HA, histamine; PPT/LDT, pedunculopontine tegmental nucleus and laterodorsal tegmental nucleus of the mesopontine tegmentum.

activity wakefulness showed increases in the mid to high theta range during histamine perfusion. Therefore, the increase in wakefulness and in the high theta range did not result from an increase in motor activity. This finding agrees with those of Anaclet et al. (2009), who showed that histamine promotes wakefulness without increasing motor activity in a wheel-running task. Thus, the perfusion of histamine into the BF not only increases wakefulness, but also seems to specifically promote attentive wakefulness.

Interestingly, the histamine perfusions did not affect REM sleep, as Ramesh et al. (2004) previously observed. This result is in contrast with the effect of other activating transmitters: the administration of glutamatergic agonists or noradrenalin completely abolished REM sleep (Cape and Jones, 1998; Wigren et al., 2007). These results further emphasize the different actions that the transmitters of the ascending arousal systems adopt in regulating sleep and sleep homeostasis.

Sleep homeostasis and histamine

SD

The BF is a key site for the regulation of cortical activation and sleep homeostasis, and is one of the main targets of histaminergic neurons (Panula et al., 1989). Contrary to our expectations, extracellular histamine concentrations did not progressively increase during the 6 h SD period. Instead, we observed an almost immediate increase in histamine concentration at the initiation of the SD, which remained stable throughout the entire SD period, indicating that sleep pressure does not affect histamine release in the BF. We previously suggested that an increase in sleep pressure could lead to an increase in the activity of ascending arousal systems, which could contribute to counteracting the effect of sleep pressure in the BF. In contrast to the profile of histamine release, we found progressive increases in the extracellular levels of dopamine and serotonin metabolites in the BF during the 6 h SD period (Zant et al., 2011). Strecker et al. (2002) showed that sleep pressure does not influence histamine levels in the preoptic/anterior area of the hypothalamus of the cat. Together with our finding that sleep pressure has no effect on histamine release in the BF of the rat, the evidence suggests that histamine release is not regulated by sleep pressure, and does not transfer information about sleep pressure.

Homeostatic response after histamine perfusions

Despite the considerable increase in wakefulness induced by all concentrations of histamine, we observed only a small increase in the amount of NREM sleep 10–12 h after the end of perfusion of the highest concentration of histamine. This increase did not correspond with increases in sleep intensity or other measures of sleep pressure. NREM sleep delta power showed no increase, nor did the number of brief awakenings and NREM sleep fragmentation decrease, thus indicating that histamine-induced prolonged wakefulness is not followed by the classic homeostatic response. This conclusion is in agreement with previous work showing no increase in sleep after BF histamine perfusion (Ramesh et al., 2004) or after intraperitoneal administration of histamine receptor 3 antagonists (Parmentier et al., 2007).

Considering the huge and long-lasting decrease in NREM delta power during the perfusion and its persistence during the first 3 h recovery period for the higher concentrations, the lack of NREM delta rebound is somewhat surprising. Wigren et al. (2007) used several glutamate agonists to stimulate the BF and to induce prolonged wakefulness. All agonists prolonged wakefulness equally, although only NMDA increased high theta activity during wakefulness (Wigren et al., 2007). This was also observed after microinjections of NMDA in the BF (Cape and Jones, 2000). A homeostatic response, measured by NREM delta power, was observed only after NMDA administration, suggesting that the homeostatic response was associated with the high level of cortical activation. Even though histamine perfusion led to increased high theta activity during wakefulness, no homeostatic response was induced.

One explanation for the lack of NREM delta rebound after histamine perfusion could be the different way in which histamine and NMDA activate the BF. Histamine depolarizes BF cholinergic neurons and increases their tonic firing (Khateb et al.,

1995), whereas NMDA promotes rhythmic bursting (Khateb et al., 1997). This difference in BF firing mode could result in a different build-up of extracellular adenosine and sleep pressure. Another explanation could be that during the histamine perfusion, the increase in theta power (20–30%) was not high enough, or that the increase in the amount of wakefulness was not sufficient to elicit a homeostatic response.

Effect of antagonizing BF histamine on cortical activity

Antagonizing the histamine 1 receptors by pyrilamine perfusion into the BF decreased wakefulness dramatically, even though the perfusion was performed unilaterally and reached only one of the major brain regions that can activate the cortex. Since histamine can activate the cortex both directly and via the BF, these data suggest that the BF is a key site for histamine to promote cortical activation and wakefulness. Localized commissural neurons connect the two sides of the BF (Semba et al., 1988). Through these connections, the BF may be able to send information about activation to the contralateral side, possibly recruiting the other side to work in parallel, which could explain why unilateral BF manipulations can affect cortical activity so substantially.

The high theta range, associated with attentive wakefulness, decreased in the wakefulness-specific EEG during histamine receptor 1 antagonist perfusion, further highlighting the importance of histaminergic transmission in the BF in maintaining a high vigilance state.

Together, these results show that activation of the cholinergic BF by histamine is important in sustaining a high level of cortical activation, and that a lack of activation of the cholinergic BF by histamine may be important in initiating and maintaining NREM sleep. The level of histamine release is tightly connected to behavioral state, but conveys no information about sleep pressure.

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