Cholinergic basal forebrain structures are involved in the mediation of the arousal effect of noradrenaline

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SUMMARY
Cholinergic basal forebrain structures are implicated in cortical arousal and regulation of the sleep–wake cycle. Cholinergic neurones are innervated by noradrenergic terminals, noradrenaline excites them via alpha-1 receptors and microinjection of noradrenaline into the basal forebrain enhances wakefulness. However, it is not known to what extent the cholinergic versus non-cholinergic basal forebrain projection neurones contribute to the arousing effects of noradrenaline. To elucidate the roles of cholinergic basal forebrain structures we administered methoxamine, an alpha-1-adrenergic agonist into the basal forebrain, in intact animals and again after selective destruction of the basal forebrain cholinergic cells by 192 IgG-saporin. In eight male Han–Wistar rats implanted with electroencephalogram/electromyogram electrodes, a microdialysis probe targeted into the basal forebrain was perfused with artificial cerebrospinal fluid for 6 h on a baseline day, and with cerebrospinal fluid in the first and with methoxamine in the second 3-h period of the subsequent day. The sleep–wake activity was recorded for 24 h on both days. Saporin was then injected into the basal forebrain and 2 weeks later the same experimental schedule (with cerebrospinal fluid and methoxamine) was repeated. In the intact animals, methoxamine exhibited a robust arousing effect and non-rapid eye movement (NREM) and REM sleep was suppressed. Lesioning of the basal forebrain cholinergic neurones abolished almost completely the NREM sleep-suppressing effect of methoxamine, whereas the REM sleep-suppressing effect remained intact. Thus, the basal forebrain cholinergic neurones mediate, at least in part, cortical arousal and non-REM sleep-suppression, but they are not involved in the REM sleep-suppressing effects of noradrenaline.

INTRODUCTION

The basal forebrain (BF), including cholinergic, gamma-aminobutyric acid (GABA) ergic and glutamatergic cortically projecting neurones (Semba, 2000), is important in the regulation of the cortical activity and the sleep–wake cycle (Stenberg, 2007; Szymusiak, 1995). The neurones in this area display a broad spectrum of state-dependent activity patterns (Jones, 2005; Szymusiak, 1995; Szymusiak and McGinty, 2008). Some of these cells have the highest discharge rate during wakefulness (W) and rapid eye movement (REM) sleep which affords, at least in part, an explanation of the cortical arousal during these vigilance states. An interesting question concerns the nature of the regulatory mechanisms that activate the state-dependent changes in the vigilance state.

The BF has a particular role in induction of recovery sleep, which appears to be linked to the intact activity of the cortically projecting cholinergic neurones (Kalinchuk *et al.*, 2008; Porkka-Heiskanen and Kalinchuk, 2011; Porkka-Heiskanen *et al.*, 1997). Recovery sleep is the additional sleep experienced after a prolonged W period, and it is regarded as a sign of the homeostatic regulation of sleep (Borbély, 1982). In response to a lesion that specifically destroys the cholinergic cells in this area, a prolonged W period is no longer able to induce increases in either sleep duration or slow wave activity (Kalinchuk *et al.*, 2008; Kaur *et al.*, 2008).

The cholinergic BF cells participate in the induction of cortical arousal, and their activity is high during W and REM sleep, involving the release of acetylcholine to the cortex (Détári *et al.*, 1999; Jones, 2005; Lee *et al.*, 2005; Stenberg, 2007; Szerb, 1967; Szymusiak, 1995). However, cholinergic BF cells are not essential for arousal, as their destruction does not induce major changes in sleep–wake activity. Non-selective lesioning with ibotenic acid, administered into the cholinergic BF structures, results only in small and temporary declines in W and increases in electroencephalogram (EEG)-delta power, and selective destruction of cholinergic cells by local application of the immunotoxin 192 immunoglobulin (Ig)G-saporin (SAP) induces only small and temporary increases in non-REM (NREM) sleep and decreases in EEG gamma power (Berntson *et al.*, 2002; Kaur *et al.*, 2008; Lin, 2000).

The neurones in the BF receive rich innervations from the neuronal groups of the ascending activating system, including those from the noradrenergic locus coeruleus, serotonergic raphe nuclei, histaminergic tuberomamillary nuclei and orexin-containing neurones in the posterior hypothalamus (Jones, 2005; Szymusiak, 1995). These projections innervate most cell types in the BF (Berridge, 2008; Lin, 2000; Smiley *et al.*, 1999; Stenberg, 2007; Zaborszky *et al.*, 2004), and it is therefore difficult to evaluate whether the different neuronal subtypes have different profiles in producing cortical arousal.

Noradrenaline (NA) plays an important role in cortical activation (Berridge, 2008; Jones, 2005; Stenberg, 2007) and the microinjection of NA into the BF enhances W (Berridge and O'Neill, 2001; Cape and Jones, 1998). Cholinergic cells in the BF are innervated by noradrenergic fibres (Smiley *et al.*, 1999) and can be excited by NA via alpha-1 receptors (Fort *et al.*, 1995). Because non-cholinergic BF cells also project to the cortex (Gritti *et al.*, 1997; Lin, 2000; Manns *et al.*, 2001; Semba, 2000) and possess noradrenergic receptors (Berridge, 2008; Zaborszky *et al.*, 2004), it is not clear to what extent the arousing effect of NA is mediated through the BF cholinergic cells.

The aim of the present study was to clarify whether BF cholinergic cells mediate the arousing effects of NA on cortical activity. We administered methoxamine (MTX), an alpha-1-adrenergic agonist, locally into the BF in the vicinity of the horizontal diagonal band (HDB), substantia innominata (SI) and magnocellular preoptic nucleus (MCPO) of intact animals and measured their sleep–wake cycle before and after the administration of MTX. Analogous experiments in the same animals were carried out after selective destruction of the BF cholinergic cells by means of a microinjection of the immunotoxin SAP.

MATERIALS AND METHODS

Animals and surgery

The experiments were carried out on male Han–Wistar rats (370–400 g, $n = 8$; Harlan Laboratories, Venray, the Netherlands). All procedures were approved by the Ethics Committee for Animal Experiments at the University of Helsinki and the provincial Government of Uusimaa, Finland (permission number: STH207A), and were in accordance with Finnish and the European Union laws. All efforts were made to minimize the number of animals used and their suffering. Under general anaesthesia [3 mg kg^{-1} intraperitoneal (i.p.) diazepam; Actavis Oy, Espoo, Finland; 50 mg kg^{-1} i.p. ketamine; Pfizer Oy, Helsinki, Finland; 0.5 mg kg^{-1} subcutaneous (s.c.) medetomidine, Orion Pharma Oy, Espoo, Finland], rats were implanted with EEG and electromyogram (EMG) electrodes. The EEG electrodes (gold-plated screws) were implanted epidurally over the frontal (2 mm rostral and 2 mm lateral to the bregma) and parietal (4 mm rostral and 1 mm lateral to the lambda) cortices. The EMG recording electrodes (silver wires covered by Teflon®, A-M Systems, Inc., Carlsborg, WA, USA) were implanted into the neck muscles. For the local administration of MTX and SAP, a unilateral microdialysis guide cannula (CMA/11 Guide; CMA/Microdialysis, Stockholm, Sweden) was implanted in such a way that the tip was located 2 mm above the target area in the BF (HDB/SI/MCPO; AP = −0.3; ML = 2.0; V = 6.5; Paxinos and Watson, 1998). After surgery, the rats were housed individually. They were kept under conditions of controlled temperature (22 ± 1 °C) and lighting (12-h light–dark cycle, lights-on in the period 8:30–20:30 hours), with access to food and water *ad libitum*.

Recording

The rats were connected to the recording leads and habituated to the experimental conditions before the recording sessions. The EEG/EMG signals were amplified and sampled at 104 Hz. EEG recordings were scored manually with the aid of the Spike 2 program (version 5.11; Cambridge Electronic Devices, Cambridge, UK) over 30-s epochs. The percentage of time spent in each state of vigilance during a 3-h period was determined.

In-vivo microdialysis, MTX administration

The protocols for microdialysis were similar to those described by Kalinchuk *et al.* (2008). In brief, microdialysis probes (CMA 11, CMA/microdialysis, 6000 Daltons, membrane length 2 mm, diameter 0.24 mm, ~10% mean recovery rate) were inserted through the guide cannula into the HDB/SI/MCPO (AP = −0.3; ML = 2.0; V = 8.5; Paxinos and Watson, 1998) at least 20 h before the start of the first experiment (Porkka-Heiskanen *et al.*, 1997). The rats were connected to the microdialysis tubing between 09:30 and 10:00 hours, and continuous perfusion was then started. Artificial cerebrospinal fluid (CSF; NaCl 147 mM; KCl 3 mM; $CaCl_2$ 1.2 mM; $MgCl_2$ 1.0 mM) or MTX (20 mg mL^{-1}; Sigma-Aldrich Co., St Louis, MO, USA) was pumped through the microdialysis probe (1 μL min^{-1}) according to the experimental schedule (see below).

Lesioning of BF cholinergic structures by administration of the immunotoxin SAP

SAP (1 μL, at a concentration of 0.23 μg μL^{-1}; Chemicon International, Inc., Temecula, CA, USA; batch no. 0703054253) was injected into the HDB/SI/MCPO (AP = −0.3; ML = 2.0; V = 8.5 (Paxinos and Watson, 1998) at a flow rate of 0.1 μL min^{-1} through microdialysis probes, which were modified by removing the microdialysis membrane from the tip. This has been reported to provide selective lesioning of the BF cholinergic cells (Kalinchuk *et al.*, 2008; Kaur *et al.*, 2008; Pizzo *et al.*, 1999).

Experimental schedule

After surgery, the rats were allowed 10 days of recovery. The microdialysis probe targeted into the BF was perfused with CSF for 6 h, starting at 10:00 hours on the baseline day, and with CSF in the first and MTX in the second 3-h periods (i.e. 10:00–13:00 and 13:00–1600 hours, respectively) on the subsequent MTX day. The sleep–wake activity was recorded for 24 h, starting at 10:00 hours on both days. SAP was next injected into the BF, and the experimental schedule with CSF and MTX was repeated in the same animals 2 weeks later (Fig. 1).

Histological verification of probe locations

For confirmation of the locations of the probe tips, the animals received a lethal dose of pentobarbital (100 mg kg^{-1} Mebunat®, i.p.; Orion Pharma Oy, Espoo, Finland) after the final experiment. Coloured 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. Twenty-micrometre sections were cut on a freezing microtome, stained with toluidine blue and inspected visually under a light microscope to verify the location of the probe tip. Only animals with probe tips located in the vicinity of the HDB, MCPO or SI in the BF were included in the subsequent analysis.

Statistics

All statistical analyses were performed with Statistica 9 (StatSoft, Inc., Tulsa, OK, USA). The 3-h values of the states of vigilance were compared by means of two-way analysis of variance (ANOVA) for repeated measures between the baseline and MTX days before and after lesioning with SAP. The treatment effect (MTX versus baseline) and the time effect (variations in 3-h values across the day) were the two factors included in the ANOVA. In general, *F*-statistics are reported only for the treatment effect and for the interactions between the treatment and the time factors when the differences proved to be statistically significant. The Tukey test was used for *post-hoc* comparisons to test the effects of MTX in the various 3-h time bins. Because MTX suppressed NREM sleep in the second and third, and REM sleep in the second, third and fourth 3-h periods, we also compared these average 6- and 9-h values, respectively, by means of two-way ANOVA for repeated measures between the baseline and MTX days in the intact and the SAP lesioned animals (factors: treatment and lesion effects). To test the effects of SAP lesioning on baseline sleep–wake activity, vigilance state values were also compared by means of two-way ANOVA for repeated measures between the baseline days before and after the SAP lesion (factors: lesion effect and time effect). A value of $P < 0.05$ was considered significant.

RESULTS

In the intact animals, MTX exhibited a robust arousal effect (Fig. 2). W was enhanced (ANOVA, $F_{(1, 7)} = 5.7853$, $P < 0.05$), the increase proving significant in the second and third 3-h periods (Tukey: $P < 0.05$), i.e. during the perfusion and the following 3 h. The daily distribution of NREM sleep was changed significantly (ANOVA, time × treatment interaction: $F_{(8, 49)} = 8.2865$, $P < 0.05$). NREM sleep was suppressed for 6 h by MTX in the second and third 3-h periods (Tukey: $P < 0.05$), i.e. during the MTX perfusion and the following 3-h period. REM sleep was also suppressed by MTX (ANOVA: $F_{(1, 7)} = 34.116$, $P < 0.05$); the decrease in REM sleep was

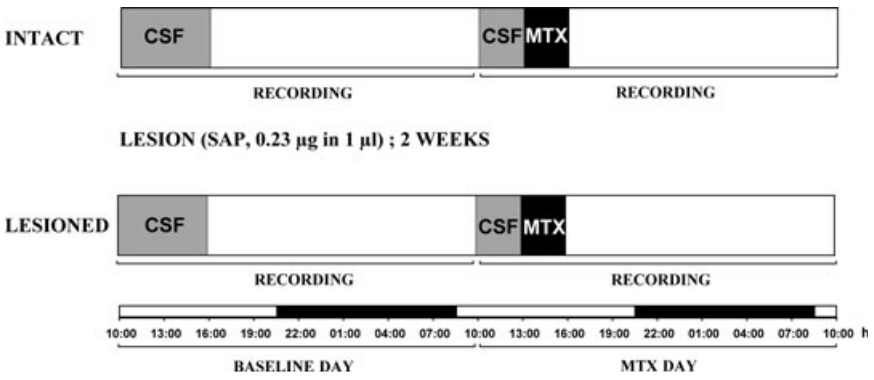

Figure 1. Experimental schedule.

significant in the second, third and fourth 3-h periods (Tukey: $P < 0.05$), i.e. during perfusion and the following 6 h. This arousal effect was followed by an opposite reaction, resulting in tendencies to decrease in W and increase significantly in REM sleep (Tukey: $P < 0.05$).

The lesioning of the BF cholinergic structures by SAP did not change the baseline sleep–wake activity significantly. However, the arousing effect of MTX was diminished significantly by the lesioning. MTX did not suppress NREM sleep significantly after the SAP treatment. REM sleep was also decreased by MTX (ANOVA: $F_{(1, 7)} = 9.2837$, $P < 0.05$) in the lesioned animals; the suppression was significant in the second, third and fourth 3-h periods (Tukey: $P < 0.05$), i.e. during perfusion and the following 6 h. Some late changes, i.e. tendencies to decrease in W and increase in REM sleep, were still present after the lesion. Unlike in intact animals, there was also a late, significant increase in NREM sleep at the last recording period.

As MTX suppressed NREM sleep in the intact rats in the second and third 3-h periods, these 6-h average NREM values (in the intact rats baseline day: 59 $\pm$ 1%, MTX day: 34 $\pm$ 3%; in the lesioned animals baseline day: 56.5 $\pm$ 2%, MTX day: 50.3 $\pm$ 3.9%) were compared by ANOVA in the intact and lesioned animals on the baseline (CFS) and MTX days. MTX suppressed NREM sleep significantly ($F_{(1, 7)} = 44.956$, $P < 0.05$) and this suppression was influenced significantly by the SAP lesion, as indicated by the interaction between the factors treatment and lesion ($F_{(1, 7)} = 6.614$, $P < 0.05$). As MTX suppressed REM sleep in the second, third and fourth 3-h periods in the intact rats, these 9-h average REM sleep values (in the intact rats baseline day: 13 $\pm$ 0.7%, MTX day: 1.1 $\pm$ 0.3%; in the lesioned animals baseline day: 11.9 $\pm$ 0.8%, MTX day: 1.5 $\pm$ 0.5%) were compared by ANOVA in the intact and lesioned animals on the baseline (CFS) and MTX days. MTX suppressed REM sleep significantly ($F_{(1, 7)} = 223.2736$, $P < 0.05$), but the lesioning with SAP did not influence this suppression significantly.

DISCUSSION

The main finding of the present study was that selective lesioning of the BF cholinergic cells abolishes almost completely the W-promoting and NREM sleep-suppressing effects of MTX, an alpha-1-adrenergic agonist, but has no effect on its REM sleep-suppressing effect.

Our findings are consistent with earlier observations that the local administration of NA or adrenergic agonists into the BF enhanced the cortical activity (Berridge and O'Neill, 2001; Cape and Jones, 1998). The present results indicate that these effects might be mediated to a considerable extent through alpha-1 receptors on BF cholinergic cells.

The immunotoxin SAP is a monoclonal antibody which binds specifically to the p75 nerve growth factor receptors on the cholinergic neurones and destroys them (Book *et al.*, 1992; Pizzo *et al.*, 1999). We have shown previously that both the systemic and the local administration of SAP to the

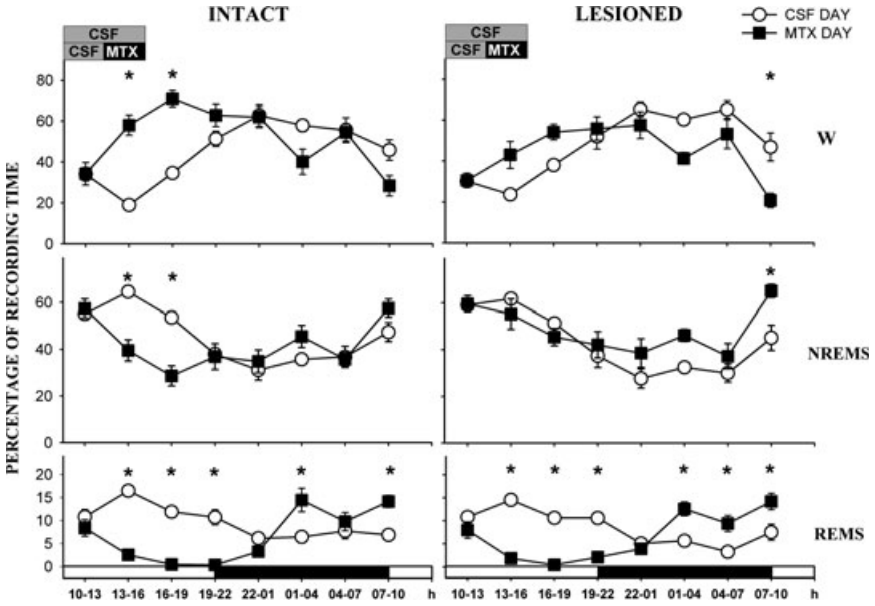

Figure 2. Effects of methoxamine (MTX) perfusion on sleep–wake activity in intact rats and after the selective lesioning of the cholinergic cells in the basal forebrain (BF) by the microinjection of saporin (SAP). The BF was perfused with cerebrospinal fluid (CSF) in the first 6-h period of the baseline day, and with CSF in the first and with MTX (20 mg mL^{-1}) in the second 3-h period of the subsequent MTX day. The horizontal grey and black bars at the top indicate CSF and MTX perfusions, respectively (upper bars: baseline day; lower bars MTX day). The curves display the daily patterns of wakefulness (W), non-rapid eye movement sleep (NREMS) and rapid eye movement sleep (REMS). The data are computed for consecutive 3-h bins and expressed as percentages of the recording time (mean values $\pm$ standard error). Open symbols: baseline (CSF) day; closed symbols: MTX day. The light and dark horizontal bars on the *x*-axis indicate light and dark phases, respectively. Asterisks denote significant differences from the baseline (Tukey, $P < 0.05$).

BF destroy effectively and selectively the cholinergic cells in the BF (Kalinchuk *et al.*, 2008). In the present study, the same dose of SAP was administered locally to the same site and by exactly the same procedure. We developed a procedure, which was also used in the present study, where *in-vivo* microdialysis studies can be combined with simultaneous EEG recordings before and after lesioning in the same animals. By reducing the individual variations, the method greatly improves the reliability of the measurements and also reduces the number of animals necessary for the studies.

Somewhat surprisingly, the REM sleep-suppressing effect of MTX was not influenced by SAP treatment, indicating that while NA in the BF has a suppressive effect on REM sleep, this effect is not mediated by cholinergic neurones in the BF but by some other type(s) of cell(s). Two other types of cortically projecting cells have been described in the BF: GABAergic and non-cholinergic, non-GABAergic, presumably glutamatergic neurones (Jones, 2005; Szymusiak, 1995; Szymusiak and McGinty, 2008). The activity of the pontine cholinergic neurones has been regarded traditionally as central in the regulation of REM sleep (McCarley, 2007), whereas the importance of the cholinergic neurones in the BF in this process has been disregarded. However, the cholinergic cells in the BF fire maximally during W and REM sleep (Lee *et al.*, 2005) and their acetylcholine release is higher during REM sleep than during W (Vazquez and Baghdoyan, 2001), which also indicates a role in this state. The present results indicate that this function might not involve regulation of the REM sleep state itself. This would be in line with evidence indicative of the importance of non-cholinergic neurones in the regulation of REM sleep, which has been described in detail in pontine areas and also in the hypothalamus (Luppi *et al.*, 2011), but not within the BF–cortex connection.

The local administration of MTX, an alpha-1-adrenergic agonist into the BF, evoked a marked increase in W at the expense of NREM sleep and REM sleep, which were suppressed significantly in the intact rats. NA can enhance cortical activity via several routes: it can enhance the activities of both cholinergic and non-cholinergic W-active cells in the BF, it can inhibit sleep-active neurones, it can excite thalamocortical neurones, etc. (Berridge, 2008; Jones, 2005, 2008; Szymusiak and McGinty, 2008). Because lesioning of the BF cholinergic structures by SAP abolished the W-inducing and NREM sleep-suppressing effects of MTX almost completely, it can be concluded that cholinergic neurones in the BF are involved heavily in the mediation of the cortical activation induced by NA.

Basal forebrain cholinergic cells were found to be excited by the stimulation of alpha- but not beta-adrenergic receptors (Fort *et al.*, 1995). However, both alpha- and beta-adrenergic receptors in the BF are involved in the mediation of the wake-promoting action of noradrenaline (Berridge, 2008). This raises the possibility that non-cholinergic structures are involved in the mediation of the wake-promoting action of BF beta-adrenergic receptor excitation. Also, the very late rebound-like effects observed in the lesioned animals, a decrease in W compared to the baseline at the end of the recording period and an increase in NREM sleep at the same time-point might evidence such a non-cholinergic effect.

CONFLICT OF INTEREST

No conflicts of interest declared.

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