# Differential Effects of the Muscarinic M$_1$ Receptor Agonist RS-86 and the Acetylcholine-Esterase Inhibitor Donepezil on REM Sleep Regulation in Healthy Volunteers

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Broad evidence from preclinical and clinical research indicates that cholinergic neurotransmission contributes significantly to the generation of rapid eye movement (REM) sleep. However, a potential role of different acetylcholine receptor (AChR) subtypes for the regulation of three main aspects of REM sleep, (1) REM onset, (2) REM maintenance, and (3) generation of REMs, are not clear. In the present double-blind, randomized and placebo-controlled study, we investigated the differential effects of the M$_1$ muscarinic AChR (mAChR) agonist RS-86 and the ACh-esterase inhibitor donepezil to further specify the AChR subtype function on REM sleep regulation in $n = 20$ healthy volunteers. We found that RS-86 selectively shortened REM latency (multivariate analysis of variance *post hoc* contrast $p = 0.024$ compared to placebo, not significant for donepezil) and that donepezil specifically enhanced the duration of REM sleep (% sleep period time, $p = 0.000$ compared to placebo; $p = 0.003$ compared to RS-86) and the number of REMs ($p = 0.000$ compared to placebo; $p = 0.000$ compared to RS-86). These results provide evidence that the onset of REM sleep is, in part, mediated by M$_1$ mAChR activity, whereas the maintenance of REM sleep and the number of REMs are mediated by non-M$_1$, but presumably M$_2$ mAChR activity. These findings are of interest for the understanding of sleep regulation and of neuropsychiatric disorders, such as Alzheimer's dementia and depressive disorders, whose etiopathology may involve alterations in cholinergic neurotransmission.
*Neuropsychopharmacology* (2006) **31**, 1294–1300. doi:10.1038/sj.npp.1300906; published online 28 September 2005

**Keywords:** RS-86; donepezil; muscarinic; acetylcholine; REM; sleep

## INTRODUCTION

Broad evidence from preclinical and clinical research indicates that cholinergic neurotransmission contributes significantly to the generation of rapid eye movement (REM) sleep. Preclinical studies show that the medial pontine reticular formation (mPRF) plays a key role in REM sleep generation. In this region, microinjection of cholinergic agonists causes a REM sleep-like state that is blocked by muscarinic acetylcholine receptor (mAChR) antagonists (reviewed in Baghdoyan and Lydic, 2002). In the same region, *in vivo* microdialysis data reveal that acetylcholine (ACh) release is enhanced during REM sleep (Leonard and Lydic, 1997). ACh release in the mPRF arises from the more rostral and dorsal cell groups comprising the laterodorsal (LDT) and the pedunculopontine tegmental (PPT) nuclei (Lydic and Baghdoyan, 1993). Consistently, LDT/PPT neurons demonstrate higher discharge rates during REM sleep (el Mansari *et al*, 1989). In humans, REM sleep is enhanced after administration of ACh-esterase inhibitors (Sitaram *et al*, 1976; Riemann *et al*, 1994) or cholinergic agonists (Spiegel, 1984; Riemann *et al*, 1988) and inhibited by anticholinergics (Sagales *et al*, 1969).

Recent studies show that different aspects of REM sleep, including (1) the number of rapid eye movements (number of REMs), (2) maintenance (REM sleep duration), and (3) onset (REM latency), can be individually evoked (eg Quattrochi and Hobson, 1999). In this process, differential activation of ACh receptor (AChR) subtypes may play a crucial role. In a widely accepted view, mAChRs are more relevant for REM sleep regulation than nicotinic AChRs (reviewed in Baghdoyan and Lydic, 2002). Actually, five mAChRs (M$_1$–M$_5$) have been molecularly and pharmacologically identified (Caulfield and Birdsall, 1998), with the M$_1$ and M$_2$ mAChRs being the most abundant subtypes in mammalian cortex (Levey *et al*, 1991). The lack of specific ligands for each subtype makes pharmacological studies

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difficult, but inferences can be made for a specific function by comparing the potencies of different compounds with known affinities for $M_1$–$M_5$ mAChRs.

REMs represent higher level analogs of ponto-geniculo-occipital (PGO) waves (Pare et al, 1990). The number of REMs is predominantly determined by $M_2$ mAChRs. Microinjections of the unspecific cholinergic agonist carbachol in anterior parts of the caudal peribrachium (C-PB) of the rat enhance PGO wave activity (Datta et al, 1992; Calvo et al, 1992). Cholinergically evoked PGO waves were blocked by the $M_2$ mAChR antagonist methoctramine, suggesting mediation via this receptor subtype (Datta et al, 1993).

Also, REM sleep maintenance (REM duration) appears to be predominantly mediated by $M_2$ mAChRs. Microinjecting the ACh-esterase inhibitor neostigmine into medial regions of the mouse PRF causes a REM sleep-like state that is blocked by the $M_2/M_4$ antagonist methoctramine but not by the $M_1/M_4$ antagonist pirenzepine, indicating $M_2$ mAChR mediation (Coleman et al, 2004). Additionally, the $M_3$ mAChR subtype in the perilocus coeruleus alpha region of the cat pons contributed to REM sleep generation (Sakai and Onoe, 1997). Findings from studies in cats (Shuman et al, 1995; Baghdoyan and Lydic, 1999) and rats (Marks and Birabil, 2000) support that the cholinergically induced REM sleep-like state is mediated substantially by signal transduction pathways coupled to $M_2$ (and less $M_4$) but not $M_1$ mAChRs. Consistently, of the five mAChR subtypes ($M_1$–$M_5$), the $M_2$ subtype is predominant in the PRF of cats (Baghdoyan et al, 1994), rats (Baghdoyan, 1997), and humans (Cortes et al, 1986).

Owing to paucity of preclinical data, mAChR subtypes involved in the initiation of REM sleep (REM latency) are less well known. In humans, numerous ACh-esterase inhibitors (eg physostigmine: Sitaram et al, 1976; galanthamine: Riemann et al, 1994) and selective $M_1$ mAChR agonists, like pilocarpine (Berkowitz et al, 1990) or RS-86 (Riemann et al, 1988), shorten REM latency. Gillin et al (1991) demonstrated that low doses of the $M_1$ antagonist biperiden selectively enhanced REM latency without affecting REM duration. Only higher doses of biperiden associated with reduced $M_1$ selectivity and increasing $M_2$ antagonism decreased the duration of REM sleep. The number of REMs was consistently not altered by $M_1$-selective agents in healthy volunteers. These findings indicate that the initial occurrence of REM sleep during night sleep relates to $M_1$ mAChR activity. However, insufficient data are available to specify the impact of other mAChR subtypes.

Direct parallels between preclinical and human studies are not always possible. In preclinical studies, pharmacological agents are typically administered to discrete neuronal populations, such as the mPRF. In human studies, agents have to be administered systemically, making it more difficult to define the location of action. Given the decisive role of pontine $M_2$ mAChRs on REM sleep regulation in preclinical studies, we presume that orally administered cholinergic agents in humans exert parallel effects at the brainstem level. However, other effects may be mediated by AChRs located in other areas of the brain. Importantly, recent functional neuroimaging studies support a role for limbic and paralimbic structures in REM sleep function

(Nofzinger et al, 1997; Maquet et al, 1996; Braun et al, 1997), raising the possibility that mAChRs in these regions mediate, in part, the effects of systemically administered cholinergic agents. Notably, limbic and paralimbic areas are rich in $M_1$ mAChRs (Mash et al, 1988; Cortes et al, 1986), raising the possibility that $M_1$ mAChRs modulate REM sleep via an indirect pathway on limbic receptors.

The aim of the present study was to further specify the role of mAChR subtypes involved in REM sleep regulation in humans. For this purpose, RS-86 and donepezil were chosen as pharmacological agents due to their convenient pharmacokinetic and pharmacodynamic properties. RS-86 is a direct $M_1$ mAChR agonist with low affinities to $M_2$–$M_5$ mAChRs, nicotinic AchRs, or other receptors (in vitro and in vivo studies: Palacios et al, 1986; Wanibuchi et al, 1990; Rupniak et al, 1992). Donepezil enhances nonspecifically the neurotransmission on all $M_1$–$M_5$ mAChR and nicotinic AChR by inhibiting the Ach-esterase. Since specific $M_2$ mAChR agonists are lacking and given that nicotinic and $M_3$–$M_5$ AChRs are less involved in REM sleep regulation, the comparison of RS-86 and donepezil provides an indirect paradigm to investigate the effects of $M_1$ and $M_2$ mAChRs on REM sleep regulation in humans. Following the preclinical and clinical data presented above, we hypothesized that donepezil would shorten REM latency and enhance the duration of REM sleep and the number of REMs, and that RS-86 would selectively shorten REM latency but not affect REM sleep duration or the number of REMs.

## SUBJECTS AND METHODS

### Subjects

Twenty healthy volunteers, 10 men and 10 women, aged 21–37 years (mean $\pm$ standard deviation (SD), $27.1 \pm 4.6$ years), were included in the present study. All participants underwent an extensive physical examination, including an electrocardiogram (ECG), electroencephalogram (EEG), and routine laboratory screening to rule out any somatic disorder. In addition, a urine drug screening demonstrated that all participants were free of any benzodiazepines, barbiturates, amphetamines, or opiates. Only women with a negative pregnancy test and sufficient contraception were included. No participant presented any contraindication for cholinergic drugs. All subjects underwent an extensive psychiatric evaluation, including a Structured Clinical Interview for Diagnosis (SCID) and a thorough clinical evaluation of the family history by an experienced psychiatrist. Subjects with a personal or family history of psychiatric disorders or primary sleep disorders according to DSM-IV were excluded. All subjects were completely free of any medication, drank no alcohol during the period of the study, and were nonsmokers. Caffeine intake was limited to one cup of coffee in the morning. A sleep diary ensured that subjects' typical sleep schedules approximated the imposed sleep schedule in the laboratory. All participants were informed in detail and provided their written and valid informed consent prior to the onset of the study. The study had been approved by the local ethic committee and has been carried out in accordance with the Declaration of Helsinki.

## Experimental Design

All participants spent two blocks of three nights in the sleep laboratory (blocks I and II). Between the two blocks in the sleep laboratory, participants slept at home for 2 weeks. The first night in each block served as adaptation to the sleep laboratory conditions. Furthermore, the first night in the sleep laboratory was used to rule out any sleep abnormalities. In the second and third night of each block, participants received 1.5 mg RS-86 or placebo (block I) and 10 mg donepezil or placebo (block II) at 2200 hours, 1 h prior to 'light-off' time at 2300 hours. The administration of RS-86/placebo and donepezil/placebo followed a randomized double-blind protocol within each block. For statistical analyses of EEG sleep data, the placebo night of block I, the RS-86 night of block I, and the donepezil night of block II were used. Owing to the long half-life of donepezil (approximately 70 h), the placebo condition of block II was excluded from analyses. Since there was no intervention between blocks I and II in the sleep laboratory that might affect baseline EEG sleep measures, we used the placebo night of block I as a control night of sleep for both the RS-86 and donepezil intervention nights.

## Substance Description and Properties

*RS-86.* The spiro-piperidyl derivate RS-86 is a specific and direct $M_1$ mAChR agonist with low or no affinities to other receptors (*in vitro* and *in vivo* studies: Palacios *et al*, 1986; Wanibuchi *et al*, 1990; Rupniak *et al*, 1992). It is efficiently absorbed with oral administration, reaches peak plasma concentrations in 2–3 h, and has an elimination half-life of 6–8 h (Spiegel, 1984). Owing to its pharmacodynamic profile, RS-86 causes no or only minor peripheral side effects in doses up to 2 mg. Side effects in higher doses include cholinomimetic symptoms, such as increased salivation, sweating, nausea, or diarrhea. No serious side effects were observed in studies using up to 20 mg RS-86 (Spiegel, 1984). Based on pharmacological data and following previous studies (eg Riemann *et al*, 1988), 1.5 mg RS-86 was administered orally at 2200 hours, 1 h prior to 'light-off' time at 2300 hours.

*Donepezil.* Donepezil is widely used in the treatment of Alzheimer's dementia. It is a piperidine derivate that selectively and reversibly inhibits the enzyme ACh-esterase enhancing neurotransmission on all AChR subtypes. Donepezil is well absorbed with oral administration, reaches peak plasma concentrations in 3–4 h, and has an elimination half-life of around 70 h. Potential side effects include cholinomimetic symptoms, like nausea, vomiting, and diarrhea (in up to 5% of subjects). Following pharmacological properties and in parallel to RS-86 (double-blind design), 10 mg donepezil were administered orally at 2200 hours, 1 h prior to 'light-off' time at 2300 hours.

## Sleep Recordings

Sleep recordings were performed from 2300 to 0700 hours and scored according to standard criteria (Rechtschaffen and Kales, 1968) by experienced and blinded raters. All raters participate in weekly meetings discussing and solving scoring problems. Inter-rater reliability for all technicians involved in scoring polysomnograms is checked bimonthly. Coefficients of agreement between two raters are required to be higher than 85%. The following variables of *sleep continuity and architecture* were assessed: sleep onset latency was defined as the period between when the lights were turned out and the first 30 s epoch of sleep stage 2 (sleep latency); sleep period time was defined as the period between sleep onset and the final awakening (SPT); sleep efficiency was defined as the ratio of time in bed to total sleep time × 100% (SE); time spent in waking referred to SPT in percent, waking (%SPT); and time spent in sleep stages 1, 2, and slow wave sleep (SWS) (combined stages 3 and 4) referred to SPT in percent, 1 (%SPT), 2 (%SPT), and SWS (%SPT). *REM sleep variables*: REM sleep duration in percent referred to sleep period time REM (%SPT); averaged duration of single REM sleep periods (DREMPs); number of REMs across the whole night (number of REMs); REM density was calculated as the ratio of 3 s miniepochs per REM period including REMs to the total amount of miniepochs per REM period × 100% (REMD); REM sleep latency was defined as the period between sleep onset and the occurrence of the first 30 s epoch of REM sleep, including intermittent waking times (REM latency); and number of REM/NREM cycles (REM/NREM). Note that the number of REM/NREM cycles requires the occurrence of NREM sleep after REM sleep and may, thus, be lower (−1) than the number of REM periods when REM sleep was the last sleep stage of a night.

## Data Analysis

Descriptive presentation of the data includes the mean values and SDs. To evaluate the effects of RS-86 and donepezil on sleep variables in comparison with the placebo, a multivariate analysis of variance (MANOVA) was conducted, treating the three conditions (placebo, RS-86, and donepezil) as a repeated measure. To determine differences between the conditions, *post hoc* contrasts were calculated for the three conditions. For the exploratory analysis of the latencies of subsequent REM periods (Figure 4), *t*-tests for dependent samples were used. All statistical analyses were performed with the SPSS 12 program package. The level of significance was set at $p < 0.05$ (two-tailed).

## RESULTS

### REM Sleep Parameters

A repeated measures ANOVA revealed a significant intervention effect (placebo, RS-86, and donepezil) for multivariate testing (Wilk's Lambda, F(12,66) = 5.7; $p = 0.000$). As can be seen in Table 1, Donepezil enhanced the *duration of REM sleep* referred to sleep period time (SPT REM%) across the whole night and the *number of REMs* (REMs) compared to both placebo and RS-86. Furthermore, donepezil increased the averaged *duration of single REM sleep periods* (DREM period) in comparison to RS-86, but not to placebo. In contrast, RS-86 did not alter the parameters of REM sleep duration or the number of REMs. As the increase in the number of REMs after administration of donepezil exceeded

**Table 1** REM Sleep Parameters[a], Mean ± SD, Multivariate Repeated Measures ANOVA with Factor Substance[b], Univariate Tests, and *Post Hoc* Contrasts

| | Placebo | RS-86 | Donepezil | Substance | | RS-86 Placebo | Donepezil Placebo | RS-86 Donepezil |
|---|---|---|---|---|---|---|---|---|
| | | | | F(2,38) | *p* | *p* | *p* | *p* |
| SPT REM% | 21.7 ± 4.5 | 22.8 ± 4.8 | 26.7 ± 3.9 | 13.59 | **0.000** | 0.213 | **0.000** | **0.003** |
| DREM period | 29.7 ± 11.1 | 24.6 ± 7.0 | 32.9 ± 6.9 | 7.2 | **0.003** | 0.058 | 0.145 | **0.000** |
| REMs | 494.4 ± 139.7 | 513.2 ± 176.4 | 680.7 ± 210.6 | 26.0 | **0.000** | 0.480 | **0.000** | **0.000** |
| REM density | 25.2 ± 5.7 | 24.3 ± 6.2 | 28.3 ± 8.5 | 4.30 | **0.023** | 0.476 | **0.041** | **0.020** |
| REM latency | 85.3 ± 49.5 | 62.4 ± 42.0 | 67.3 ± 27.1 | 3.87 | **0.032** | **0.024** | 0.063 | 0.513 |
| REM/NREM | 3.2 ± 0.9 | 3.8 ± 0.8 | 3.4 ± 0.8 | 4.05 | **0.026** | **0.014** | 0.262 | 0.090 |

[a]SPT REM% = REM sleep (% sleep period time); DREM period = averaged duration of REM sleep periods in minutes; REMs = number of rapid eye movements; REM/NREM = number of REM/NREM cycles.
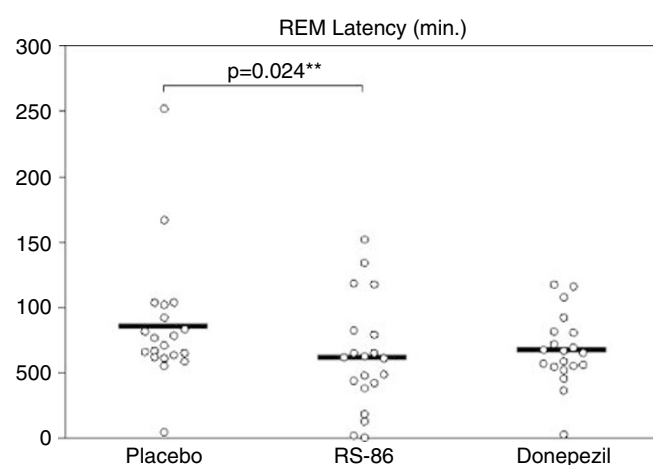[b]Placebo, RS-86. and donepezil.
Significance of $p < 0.05$ is given in bold.
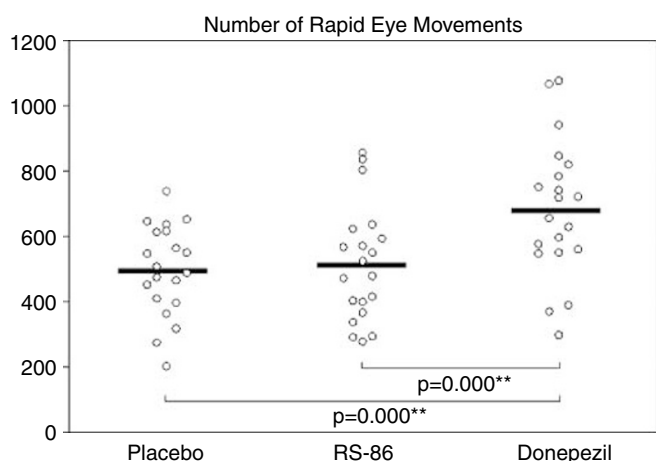


**Figure 1** Effects of RS-86 and donepezil on the number of rapid eye movements (REMs), single values and means.
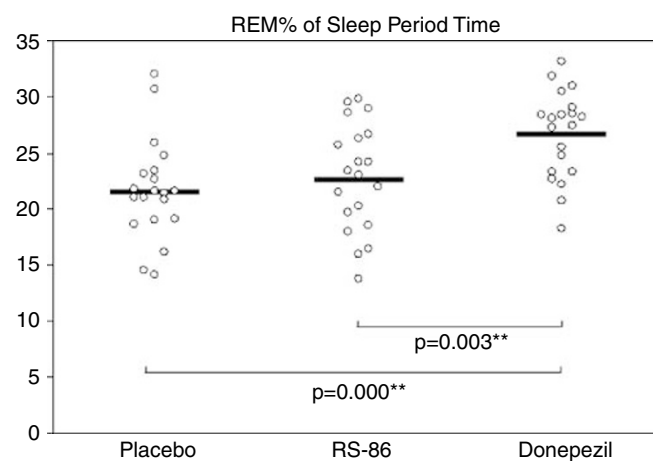


**Figure 2** Effects of RS-86 and donepezil on the percentage of REM sleep referred to sleep period time, single values and means.

the increase in REM sleep duration, *REM density* was significantly increased in the donepezil condition in relation to placebo and RS-86. RS-86, but not donepezil, significantly reduced *REM latency* relative to the placebo night (averaged reduction 22.9 min), but not to the donepezil night. This effect persisted after the exclusion of a REM latency in one subject (250 min) that exceeded a normal range (*post hoc* contrasts placebo-RS-86: $p = 0.044$; placebo–donepezil: $p = 0.113$). Subjects in the RS-86 condition had an increased *number of REM/NREM cycles* compared to the placebo, but not to the donepezil condition.

Figures 1, 2 and 3 depict single values of the main findings of the present study: the number of REMs, REM duration (%SPT), and REM latency.

Figure 4 demonstrates the cyclic pattern of REM/NREM sleep regulation. All time spans from sleep onset to the occurrence of the first (see above) and subsequent REM sleep periods (REM latency 2, 3, and 4) were significantly shortened after the administration of RS-86 compared to baseline, but not compared to donepezil (Table 2). However, the calculated multiple *t*-tests for dependent samples have just exploratory but not confirmative value. Given that RS-86 did not alter the averaged duration of single REM



**Figure 3** Effects of RS-86 and donepezil on REM latency, single values and means.

sleep periods, RS-86 appeared to advance the onset of the REM/NREM cycle. Donepezil did not significantly influence the temporal distribution of single REM sleep periods.

**Table 2** Time Spans from Sleep Onset to the Occurrence of the Second and Subsequent REM Sleep Periods[a], Mean $\pm$ SD, and Multiple *t*-Tests for Dependent Samples

| | Placebo | RS-86 | Donepezil | RS-86 Placebo *p* | Donepezil Placebo *p* | RS-86 Donepezil *p* |
|---|---|---|---|---|---|---|
| REM latency 2 | $187.5 \pm 61.4$ | $154.4 \pm 44.5$ | $165.6 \pm 39.2$ | **0.016** | 0.082 | 0.307 |
| REM latency 3 | $298.0 \pm 42.6$ | $246.3 \pm 55.1$ | $269.7 \pm 46.0$ | **0.003** | 0.080 | 0.106 |
| REM latency 4 | $399.2 \pm 39.8$ | $341.7 \pm 59.7$ | $374.8 \pm 42.2$ | **0.033** | 0.277 | 0.074 |

[a]REM latencies 2, 3, and 4.
Significance of $p < 0.05$ is given in bold.
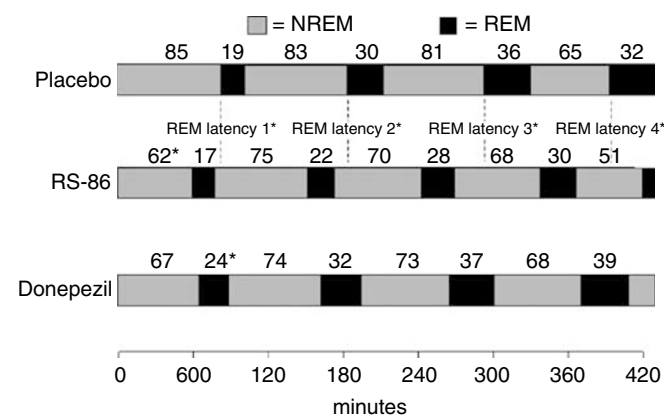


**Figure 4** Cyclic pattern of REM/NREM sleep regulation, time in minutes.

**Table 3** Sleep Continuity and Architecture[a], Mean $\pm$ SD, Multivariate Repeated Measures ANOVA with Factor Substance[b], and Univariate Tests

| | | | | Substance | |
|---|---|---|---|---|---|
| | Placebo | RS-86 | Donepezil | F(2,38) | *p* |
| Sleep latency | $21.3 \pm 21.1$ | $15.0 \pm 8.1$ | $16.5 \pm 8.3$ | 1.13 | 0.317 |
| SPT | $453.6 \pm 27.0$ | $462.7 \pm 12.7$ | $455.2 \pm 30.7$ | 1.21 | 0.309 |
| SE | $89.4 \pm 6.4$ | $91.2 \pm 3.9$ | $89.2 \pm 8.1$ | 0.85 | 0.421 |
| SPT W% | $5.6 \pm 3.3$ | $5.5 \pm 3.2$ | $6.1 \pm 4.5$ | 0.24 | 0.736 |
| SPT 1% | $8.6 \pm 3.9$ | $8.3 \pm 3.9$ | $9.6 \pm 4.6$ | 1.61 | 0.217 |
| SPT 2% | $54.0 \pm 6.2$ | $55.7 \pm 7.2$ | $49.4 \pm 5.0$ | 12.39 | **0.000** |
| SPT SWS% | $10.1 \pm 7.4$ | $7.7 \pm 7.8$ | $8.1 \pm 6.9$ | 2.68 | 0.101 |

[a]SPT = sleep period time; SE = sleep efficiency; SPT W% = waking (%SPT); SPT 1% = stadium 1 (%SPT); SPT 2% = stadium 2 (%SPT); SPT SWS% = slow wave sleep (%SPT).
[b]Placebo, RS-86, and donepezil.
Significance of $p < 0.05$ is given in bold.

## Sleep Continuity and Sleep Architecture of NREM Sleep

A repeated measures ANOVA revealed a significant substance effect (placebo, RS-86, and donepezil) for multivariate testing (Wilk's Lambda, F(12,64) = 5.6; $p = 0.000$). As can be seen in Table 3, parameters of sleep continuity (sleep latency, sleep period time, sleep efficiency, waking time %) were not relevantly altered after the administration of RS-86 or donepezil. The only significant effect on NREM sleep architecture was observed for donepezil, leading to a significant reduction of stage 2 (% SPT) referred to placebo and RS-86 (*post hoc* contrasts $p = 0.003$; $p = 0.000$, respectively).

## DISCUSSION

The present study was designed to further specify the role of AChR subtypes on different components of human REM sleep by using the ACh-esterase inhibitor donepezil and the $M_1$ mAChR agonist RS-86 as pharmacological challenges in comparison to placebo. The results support the initial hypotheses that the *number of* REMs and the *duration of REM sleep* are predominantly mediated by non-$M_1$, but presumably $M_2$ mAChR activation and that the *initiation of REM sleep* (REM latency) is, to a significant extent, mediated by activation of $M_1$ mAChRs.

Prior to discussing these effects on REM sleep regulation in more detail, it is important to note that no changes in sleep continuity, such as sleep onset latency, sleep period time, or awakenings, were observed. The following alterations in REM sleep parameters, therefore, are due to REM sleep-generating mechanisms and not to alterations in sleep continuity.

Evidence that the number of REMs and REM sleep maintenance (REM sleep duration of the whole night and averaged duration of single REM sleep periods) are mediated by AChRs others than $M_1$ mAChRs is that in the present study, the nonspecific activation of AChRs by donepezil largely enhanced the number of REMs and REM sleep maintenance, whereas the selective $M_1$ mAChR agonist RS-86 did not alter these parameters (Figures 1 and 2). These findings are consistent with those from previous clinical (Sitaram *et al*, 1976; Riemann *et al*, 1994; Schredl *et al*, 2001; Kanbayashi *et al*, 2002) and preclinical studies (eg Calvo *et al*, 1992). They showed an increase of the number of REMs and REM sleep maintenance after the administration of numerous ACh-esterase inhibitors. Also, preclinical (Velazquez-Moctezuma *et al*, 1991; Coleman *et al*, 2004) and clinical (Riemann *et al*, 1988; Gillin *et al*, 1991) studies have shown that compounds with a selective affinity for $M_1$ mAChRs did not affect these parameters. In contrast, the ACh-esterase inhibitor tacrin did not significantly affect REM density or duration (Riemann *et al*, 1996). This raises the possibility that differences between

compounds with the same pharmacodynamic profile might be related to differences in pharmacokinetic properties between agents, such as the short half-life of tacrin (2 h). Furthermore, the frequently used parameter REM density is less adequate to reflect alterations in the generation of REMs itself since it depends on the REM sleep duration. Given that the $M_1$ and $M_2$ mAChR subtype are the most abundant AChR subtypes in the mammalian cortex (Levey et al, 1991), and given that there is broad evidence from preclinical studies indicating that the number of REMs and REM maintenance are predominantly mediated by $M_2$ mAChR activity in the brain stem (Velazquez-Moctezuma et al, 1991; Coleman et al, 2004), it is likely to assume that the increases in the number of REMs and REM sleep duration observed after the administration of donepezil in the present study are mediated, to a large extent, by $M_2$ mAChR activation at the brain stem level.

Evidence that the onset of REM sleep is mediated, at least in part, by $M_1$ mAChR activity is that the selective $M_1$ mAChR agonist RS-86 significantly shortened REM latency compared to placebo (mean reduction 22.9 min). This finding is consistent with those of previous studies investigating the effects of RS-86 on REM latency in healthy subjects (Riemann et al, 1988; Lauer et al, 2004). The finding that numerous ACh-esterase inhibitors shorten REM latency (eg physostigmine; Sitaram et al, 1976) is consistent but does not further contribute to the discussion of the involvement of AChR subtypes. As in previous studies in younger healthy subjects (Kanbayashi et al, 2002; Perlis et al, 2002), donepezil did not significantly alter REM sleep latency in comparison to placebo. The tendency towards a reduction in REM latency by donepezil in our sample ($p = 0.063$) may reflect a weak activation of $M_1$ mAChRs that may reach significance if a larger sample size were studied. The absence of a statistically significant reduction in REM latency by donepezil, therefore, may represent a false-negative result, or a type II error. The finding that donepezil clearly shortened REM latency in depressed subjects (Perlis et al, 2002) points to a differential responsiveness to cholinergic agents based on history of depressive disorder. Interestingly, RS-86, after advancing the onset of the first REM period, also appeared to advance the occurrence of succeeding REM periods relative to sleep onset even when the duration of each REM/NREM cycle was unaffected. This constellation resulted in a significant increase of the number of REM/NREM cycles per night in the RS-86 compared to placebo condition (Table 1). This advance in the REM/NREM cycle without affecting the phase duration was also observed in a previous RS-86 study (Riemann et al, 1988). In this study, the observed increase in averaged REM sleep duration was clearly related to the advancement of the REM sleep cycle and the resulting increase of the number of REM/NREM cycles, rather than to an increase in the duration of single REM sleep periods. However, the observation that the $M_1$ mAChR agonism by RS-86 advances the onset of succeeding REM periods was in our study tested only on an exploratory level (repeated t-tests for nonprimary hypotheses). This finding requires replication.

The present study has several limitations: First, as specific ligands for each nicotinic and $M_1$–$M_5$ mAChR subtype are lacking, the present study provides direct evidence only for the $M_1$ mAChR function. Owing to this, it seemed plausible to link the donepezil data of the present study to preclinical findings and postulate indirectly a mediation of REMs and REM sleep maintenance by $M_2$ mAChRs. However, other nicotinic or mAChRs may be involved. Secondly, the finding that donepezil did not significantly shorten REM latency may be related to its pharmacokinetic properties since it reaches plasma peak concentrations 3–4 h after administration (RS-86: 2–3 h). This pharmacokinetic profile makes it even more plausible that the absence of a statistically significant reduction in REM latency by donepezil represents a false-negative result. However, it is important to note that the pharmacokinetic properties of RS-86 and donepezil do not impede the main conclusions of the present study ($M_1$ mAChR involvement in REM sleep onset; non-$M_1$ but presumably $M_2$ mAChR involvement in REM sleep maintenance and generation of REMs). The third limitation is that it is not possible to determine the location of action of the systematically administered compounds. $M_2$ mAChRs are predominantly located in the brainstem leading to the hypothesis that the effects observed in the present study are, in part, evoked in this area. However, $M_2$ mAChRs are located in other areas of the brain and have been localized not only postsynaptically but also to the presynaptic terminals of cortical neurons (reviewed in Levey, 1996). Interestingly, recent functional neuroimaging studies have demonstrated that limbic and paralimbic structures are activated during REM sleep (Nofzinger et al, 1997; Maquet et al, 1996; Braun et al, 1997). Studies using receptor autoradiography show that these areas are rich in $M_1$ mAChR sites (Mash et al, 1988; Cortes et al, 1986). Given the consistently demonstrated reduction of REM latency after administration of $M_1$ AChR agonists, it may be that these limbic and paralimbic areas play an important role in the onset of REM sleep. This hypothesis seems worth further testing in preclinical studies. As a minor limitation, it is to note that RS-86 and donepezil were administered in a fixed order (block I: RS-86; block II: donepezil). However, it is plausible to assume that the resulting uncontrolled exposure to the laboratory setting did not specifically influence the main outcome parameters of REM sleep regulation. With regard to the clinic, the specification of AChR subtype function is of interest to further elucidate the presumed cholinergic alterations in various disorders that may have distinct patterns of alterations in different aspects of REM sleep, or that may be differentially affected by cholinergic agents with varying levels of specificity for AChR subtypes, such as Alzheimer's dementia and depressive disorders.

In conclusion, the present study suggests that $M_1$ mAChRs are mediating, in part, the onset of REM sleep in healthy human subjects and that the activation of non-$M_1$, but presumably $M_2$ mAChRs, maintains REM sleep and evokes the occurrence of REMs.

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