INTRODUCTION

Following discovery of the ascending reticular activating system (Moruzzi and Magoun 1949), ACh was postulated as a principal neurotransmitter regulating sleep–wake and anesthetically induced alterations in arousal (Domino et al. 1968; Krnjevic 1967). This postulate emerged from the findings that ACh excites cortical neurons (Krnjevic 1967) and that cortical levels of ACh are positively correlated with an activated EEG (Jasper and Tessier 1971; Marrosu et al. 1995). Volatile anesthetics that diminish behavioral arousal and deactivate the cortical EEG (Mitchell 1963) also decrease levels of ACh in the cortex (Griffiths et al. 1995).

Many effects of ACh in the neocortex are mediated by muscarinic cholinergic receptors (mAChRs). There are five subtypes of mAChRs identified pharmacologically and molecularly as M1–M5 (reviewed in Caulfield and Birdsall 1998). The M1 and M2 receptors are the most abundant subtypes in mammalian cortex (Levey et al. 1991), but the cellular distribution of these receptors has not been fully elucidated. Electron and light microscopic studies in nonhuman primates and in rat have localized some M2 receptors to the presynaptic terminals of cortical neurons (reviewed in Levey 1996). In mouse, in vivo (Douglas et al. 2001) and in vitro (Zhang et al. 2002) studies have provided evidence that the M2 subtype acts as a cholinergic autoreceptor in cortex. Further studies have demonstrated that one functional consequence of increasing ACh release by delivering an M2 antagonist into one prefrontal cortex is activation of the contralateral cortical EEG (Douglas et al. 2002). These data raise an important question. In prefrontal cortex ipsilateral to autoreceptor enhanced ACh release, what postsynaptic receptors mediate EEG activation in the contralateral prefrontal cortex? Therefore the present study tested the hypothesis that prefrontal cortical ACh activates the contralateral prefrontal EEG via postsynaptic M1 receptors.

METHODS

Animal preparation for simultaneous EEG recordings and microdialysis

All experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (National Academy of Sciences 1996). Fifteen adult male C57BL/6J (B6) mice (Jackson Labs, Bar Harbor, ME) were anesthetized with 2.5% halothane in 100% O2 and held in a David Kopf (Tujunga, CA) stereotaxic frame. Autonomic signs (core body temperature and respiratory rate) were monitored with an infrared thermometer and video camera throughout the experiments.

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monitored during anesthesia. Delivered halothane was maintained at 1.8% during all experiments as measured by a Raman spectrometer (Ohmeda Rascal II, Louisville, CO). A small craniotomy was created to provide access to the prefrontal cortex, identified as F2r in the stereotaxic atlas (Paxinos and Franklin 2001). A CMA/11 microdialysis probe (membrane: 1 mm long by 240-μm diam, 6 kDa cutoff; CMA Microdialysis, North Chelmsford, MA) was aimed for a site 3.0 mm anterior to bregma and 1.6 mm lateral to the midline. The vertical descent of the probe was guided visually such that the dialysis probe membrane rested fully within the cortex. Two additional craniotomies directly opposite the microdialysis probe (3.0 mm anterior to bregma and 0.5 and 1.5 mm lateral to the midline) allowed placement of two 0.13-mm-diam stainless steel wire (California Fine Wire Company, Grover City, CA) electrodes for recording prefrontal cortical EEG.

**Microdialysis and HPLC with electrochemical detection (EC)**

Ringer solution (147 mM NaCl, 2.4 mM CaCl₂, 4.0 mM KCl, and 10 μM neostigmine) constantly perfused the microdialysis probe at a rate of 2.0 μl/min. At intervals of 12.5 min, 25 μl dialysis samples from prefrontal cortex were injected into an HPLC/EC system [Bioanalytical Systems (BAS), West Lafayette, IN] for quantification of ACh. Following injection, a 50 mM Na₂HPO₄ mobile phase carried the microdialysis sample to a reversed-phase column that separated ACh and choline based on size and hydrophobicity. The separated ACh and choline then passed through an immobilized enzyme reaction column that converted them to equimolar amounts of hydrogen peroxide. An applied potential of 0.5 V (in reference to a Ag/AgCl electrode) ionized the hydrogen peroxide and the resulting signal created a chromatographic peak that was digitized using ChromGraph (BAS) software. Integration of the chromatogram yielded a measurement of the area under the chromatographic peaks and ACh was quantified as pmol/12.5 min using a standard curve produced before each experiment. The percentage of a known ACh solution recovered by each probe was measured before and after every experiment. If pre- and postexperimental probe recoveries were significantly different according to a t-test, the data were discarded. Cortical ACh release was quantified initially in each mouse during dialysis with Ringer solution alone (control). A CMA/110 liquid switch then was activated to deliver Ringer containing muscarinic antagonists. In this way, each mouse served as its own control for quantifying dependent measures.

**Identifying functional roles of mAChR subtypes**

The goal of the present study was to use an in vivo pharmacological approach to identify the mAChR subtype or subtypes mediating the ACh-induced activation of prefrontal cortical EEG. One limitation for all studies aiming to identify functional roles of mAChRs is the lack of subtype-specific ligands. The selectivity of available muscarinic antagonists for the five mAChR subtypes is concentration dependent (Caulfield and Birdsell 1998). Thus, differences in mAChR subtypes require determining the relative potencies of a range of concentrations of different mAChR antagonists for eliciting the response of interest (Baghdoyan and Lydic 1999; Billard et al. 1995; Douglas et al. 2001).

A previous study used the approach described above to conclude that the M2 subtype functions as an autoreceptor in prefrontal cortex of B6 mouse (Douglas et al. 2001). In that study, the relative potencies of scopolamine, AF-DX 116, and pirenzepine for increasing ACh release were determined. Scopolamine is a muscarinic antagonist with equal and high affinity for the five mAChR subtypes (Billard et al. 1995; Jones et al. 1992) and therefore was expected to antagonize all five subtypes. Scopolamine (3 nM) increased ACh release in prefrontal cortex (Douglas et al. 2001). AF-DX 116 has relatively high affinity for the M2 or M4 subtype mAChRs and much lower affinity for M1, M3, and M5 receptors (Billard et al. 1995; Jones et al. 1992).

Pirenzepine has relatively high affinity for the M1 and M4 mAChRs and much lower affinity for the other mAChR subtypes (Billard et al. 1995; Jones et al. 1992). The lowest concentration of AF-DX 116 that increased ACh release was 3 nM, which is closest to its affinity for the M2 subtype (Billard et al. 1995). The lowest effective concentration of pirenzepine for increasing ACh release was 300 nM (Douglas et al. 2001), which also is closest to its affinity for the M2 subtype (Billard et al. 1995). Therefore increased ACh release was likely due to antagonism of the M2 receptor subtype.

In the present study, the muscarinic antagonists scopolamine methyl bromide (10 nM, Sigma-Aldrich Corp., St Louis, MO), pirenzepine dihydrochloride (3 nM, Sigma), and AF-DX 116 (3 nM, Boehringer-Ingelheim, Ridgefield, CT) were diluted in Ringer solution before beginning each experiment, as previously described (Douglas et al. 2001). Scopolamine was delivered at a concentration of 10 nM to ensure antagonism of all five mAChR subtypes. AF-DX 116 was used at a concentration of 3 nM to block M2 and M4 subtypes. Pirenzepine at 3 nM would be expected to block M1 and M4 subtypes. Muscarinic antagonists were delivered by reverse dialysis via the same probes used to collect ACh. This made it possible to determine the relative effects of each antagonist on ipsilateral ACh release and on activation of the contralateral cortical EEG.

**EEG spindle quantification and power spectral analysis**

The presence of 7- to 14-Hz spindles in the cortical EEG represents recurrent activity within a thalamocortical projection (Steriade et al. 1993a). Halothane-induced EEG spindles were detected in B6 mouse prefrontal cortex by the amplification and recording of signals from the two electrodes above the right prefrontal cortex using a Grass (West Warwick, RI) Model 15RXi digital polygraph and Polyview software. Spindle frequency was confirmed in the 7- to 14-Hz range via fast Fourier transformation (FFT) analysis. The number of EEG spindles/min and cortical ACh release were measured simultaneously. Signals from the same electrodes used to record EEG spindles were amplified and digitized with a sampling rate of 128 Hz. The EEG was filtered electronically with the Model 15RXi hardware at 0.3 and 30 Hz. The EEG was analyzed by FFT in 2-s bins. Analyses were conducted in 0.5-Hz increments for frequencies ranging from 0.5 to 25 Hz. The bins were averaged over 1-min intervals of EEG recordings. During microdialysis the total power (Vrms) for each frequency interval of prefrontal cortical EEG was quantified as power spectral density.

**Histological and statistical analyses**

Following each experiment, brains were removed and sectioned coronally at 40 μm on a Bright model OTF cryostat (Huntingdon, Cambridgeshire, England). Sections were mounted on gelatin-coated slides, fixed with paraformaldehyde vapors at 80°C, and stained with cresyl violet. Digitized images of the stained sections were used to localize microdialysis probe placements by comparison of probe center to a stereotaxic atlas (Paxinos and Franklin 2001). The alpha level for all analyses was P < 0.05. Descriptive statistics and repeated measures ANOVA with posthoc Tukey–Kramer statistic were used to analyze the dependent measures of ACh release and number of EEG spindles/min during the control and the three drug conditions. Drug effects on EEG frequency were analyzed by repeated-measures ANOVA and posthoc comparisons with Bonferroni correction using SAS (release 8.8, SAS Institute, Cary, NC). The numerator degrees of freedom (df) equals the number of conditions minus one and the denominator df equals the product of the numerator df and the number of frequency bins minus one.

**RESULTS**

Microdialysis and EEG data were included in the present study only if dialysis sites were histologically localized to be
fully within prefrontal cortex. Figure 1A schematizes the relative locations of cortical EEG electrodes and a microdialysis probe in the prefrontal cortex of B6 mouse. Figure 1B illustrates how histological localization of microdialysis probe-induced lesions confirmed that ACh measures were obtained from prefrontal cortex. The representative polygraphic record in Fig. 1C was taken during microdialysis with Ringer solution and illustrates halothane-induced EEG spindles. A reduced number of EEG spindles caused by dialysis delivery of the mAChR antagonist AF-DX 116 during the same experiment is illustrated in Fig. 1D.

Mean prefrontal cortical ACh release was increased by dialysis delivery of mAChR antagonists. Percentage change in ACh release after antagonist delivery is shown graphically in Fig. 2A. Repeated-measures ANOVA comparing the effects of Ringer (control) and Ringer containing muscarinic antagonists showed a significant drug main-effect on ACh release ($P = 100.1; \text{df} = 3, 132; P < 0.0001$). Posthoc Tukey–Kramer statistic revealed that AF-DX 116, scopolamine, and AF-DX 116 plus pirenzepine significantly increased ACh release over Ringer levels ($P < 0.01$). Increases in ACh release caused by the three antagonist treatment conditions were not significantly different from each other.

Figure 2B summarizes the number of halothane-induced EEG spindles/min during control and drug delivery conditions.
for the entire recording period (75 min). A significant drug main-effect was revealed by repeated-measures ANOVA ($F = 282; \text{df} = 3, 321; P < 0.0001$). Posthoc Tukey–Kramer statistic revealed that only AF-DX 116 caused a significant ($P < 0.01$) decrease in halothane-induced EEG spindles/min compared with control dialysis. The number of EEG spindles/min during dialysis delivery of AF-DX 116 also was significantly lower than during dialysis delivery of either scopolamine ($P < 0.01$) or AF-DX 116 plus pirenzepine ($P < 0.01$).

The plots of power spectral densities in Fig. 3 represent the averaged results of EEG recordings during 15 experiments. Scopolamine did not cause a decrease in EEG power at any frequency (Fig. 3A). Analysis of variance revealed that there was a significant drug main-effect on EEG power caused by AF-DX 116 ($F = 9.78; \text{df} = 49, 245; P = 0.004$, Fig. 3B, gray line). Post hoc multiple comparisons tests with Bonferroni correction showed that AF-DX 116 caused a significant ($P < 0.05$) decrease in slow-wave EEG power at the 0.5, 1.0, 1.5, 2.0, 2.5, 3.5, and 4.5 Hz frequencies. Interestingly, this decrease in EEG power included the frequencies described as slow oscillations in feline cortex (Steriade et al. 1993b). The decrease in slow-wave EEG power caused by AF-DX 116 was blocked by the addition of pirenzepine to the microdialysis solution (Fig. 3B, dashed line).

**DISCUSSION**

The diverse regulatory roles subserved by the prefrontal cortex (Groenewegen and Uylings 2000) justify efforts to understand control of prefrontal cortical excitability. The prefrontal cortex is involved in memory formation (Gabrieli et al. 1998), forward planning (Gaffan and Harrison 1989; Tanji and Hoshi 2001), and relation of arbitrary associations (Toni and Passingham 1999). Cardiopulmonary control and the emotional content of perception are modulated by prefrontal cortical projections (reviewed in Groenewegen and Uylings 2000). The prefrontal cortex is particularly vulnerable to the effects of anesthesia, and memory-blocking drugs commonly used during surgery disrupt prefrontal cortical function (Andrade 1996; Casele-Rondi 1996). Dysfunctions of the prefrontal cortex also are implicated in neurodegenerative diseases such as Alzheimer’s and Parkinson’s, psychiatric disorders such as schizophrenia, and postoperative delirium in the elderly population (reviewed in Groenewegen and Uylings 2000).

The homology of the rodent prefrontal cortex to human prefrontal cortex has been questioned (Preuss 1995). Alternatively, brain regions in rodents that have not evolutionarily differentiated into highly complex structures may indeed be homologous to brain regions in primates (Uylings and van Eden 1990). When the prefrontal cortex is defined by the major projection field of the mediodorsal thalamus and by interconnectivity with other brain regions (Groenewegen and Uylings 2000; Öngür and Price 2000), these anatomical criteria suggest that the mouse prefrontal cortex is homologous to primate prefrontal cortex. All of the present results were from the prefrontal cortex of B6 mouse (Fig. 1, A and B) as verified by histological examination. The results provide the first evidence that endogenous ACh in one prefrontal cortex activates the EEG in the contralateral prefrontal cortex via postsynaptic M1 receptors.

**Pre- and postsynaptic muscarinic mechanisms modulate ACh release and excitability in prefrontal cortex**

Numerous studies substantiate the present in vivo pharmacological approach for identifying mAChR subtypes (Baghdoyan et al. 1998; Baghdoyan and Lydic 1999; Billard et al. 1995; Douglas et al. 2001, 2002). Recent studies of B6 mouse...
found that scopolamine and AF-DX 116 are equipotent for increasing ACh release and that pirenzepine is 100-fold less potent than scopolamine or AF-DX 116 (Douglas et al. 2001). Those data supported the interpretation that M2 mAChRs function as presynaptic autoreceptors in prefrontal cortex of B6 mouse (Douglas et al. 2001). A subsequent study (Douglas et al. 2002) found that one functional consequence of enhancing ACh release in ipsilateral prefrontal cortex is activation of the contralateral prefrontal cortical EEG. No data were available to identify the postsynaptic receptors through which endogenous ACh could activate EEG in contralateral prefrontal cortex. Therefore the present study evaluated the hypothesis that the postsynaptic M1 receptor is one site at which ACh activates prefrontal cortical EEG.

Characterizing brain neurochemistry relative to various mouse strains and other species is an important goal for mouse phenotyping. Previous work on feline EEG analysis has shown a fast component in the gamma (30–40 Hz) range (Steriade and Amzica 1996). Therefore additional FFT analyses were performed in the present study across the EEG frequency spectrum \( \leq 60 \) Hz. These analyses did not reveal any prominent power in the 30- to 40-Hz range. This lack of gamma activity may be one reason most studies of mouse EEG have focused on frequencies ranging from 0 to 25 Hz (Franken et al. 1998; Huber et al. 2000; Tobler et al. 1997; Veasey et al. 2000; Zhang et al. 1996).

When delivered to prefrontal cortex by microdialysis, 3 nM AF-DX 116 increased ipsilateral cortical ACh release (Fig. 2A) and activated the EEG in the contralateral prefrontal cortex (Figs. 2B and 3B). Cortical activation was quantified by a reduction in the number of EEG spindles (Fig. 2B) and by a decrease in EEG slow waves as determined by FFT analysis (Fig. 3B, solid gray line). AF-DX 116 has higher affinity for M2 and M4 subtypes than for M1, M3, or M5 subtypes (Billard et al. 1995; Caulfield and Birdsal 1998; Jones et al. 1992). Taken together, these findings indicate that contralateral EEG activation (Figs. 2B and 3B) was mediated by a non-M2/M4 mAChR subtype.

The present results suggest that nicotinic cholinergic receptors did not cause EEG activation. Scopolamine is a cholinergic antagonist that blocks all five mAChR subtypes (Jones et al. 1992). Microdialysis delivery of scopolamine increased ipsilateral ACh release (Fig. 2A) but did not activate the contralateral EEG (Figs. 2B and 3A). The increased ACh release caused by scopolamine would be expected to activate nicotinic cholinergic receptors. There was no contralateral prefrontal cortical activation associated with scopolamine-evoked ACh release. The present data therefore are consistent with the interpretation that the contralateral cortical activation associated with increased ACh release was mediated by muscarinic, and not nicotinic, ACh receptors.

Microdialysis delivery of pirenzepine was used to test the hypothesis that M1 mAChRs mediate ACh-induced activation of contralateral cortex. Previous studies have shown that 3 nM pirenzepine does not alter ACh release when delivered by microdialysis to prefrontal cortex (Douglas et al. 2001). At a concentration of 3 nM, pirenzepine should block M1 and M4 receptors (Jones et al. 1992). In the current study, 3 nM pirenzepine was added to Ringer containing 3 nM AF-DX 116 for dialysis delivery to prefrontal cortex. The combination of antagonists increased ACh release at the dialysis site (Fig. 2A) but no more effectively than did 3 nM AF-DX 116 alone (Fig. 2A). A key finding was that pirenzepine blocked the AF-DX 116-induced activation of the contralateral prefrontal cortical EEG (Figs. 2B and 3B). Microdialysis delivery of AF-DX 116 plus pirenzepine failed to decrease the number of EEG spindles/min (Fig. 2B) and likewise failed to decrease EEG slow waves (Fig. 3B, dashed line). The only change resulting from the addition of pirenzepine to the AF-DX 116 was antagonism of M1 receptors. Therefore the AF-DX 116 plus pirenzepine data are consistent with the interpretation that postsynaptic M1 mAChRs contribute to cholinergically enhanced EEG activation in contralateral prefrontal cortex.

**Limitations and conclusions**

The mouse is an important model for mechanistic studies relevant to human disease (Behringer 2001; Hock and Lamb...
2001; Skradski et al. 2001), and it has been noted that “every human gene has a mouse homologue” (O’Brien et al. 1999). The present results are limited to B6 mouse and additional data will be needed to determine the extent to which these results are generalizable to other mammalian brains. The present studies do not address the issue of connectivity between the two hemispheres of prefrontal cortex. Although most likely, it is not known for certain that the putative M1 receptor mediating cortical EEG activation resides on processes innervating the contralateral cortex through the corpus callosum. The present data also do not address the question of which transmitter or receptor in the contralateral cortex mediated cortical activation. Close inspection of Fig. 3 reveals that EEG spindles do not appear as a prominent feature of the EEG power spectra. Available data suggest that the lack of a 7- to 14-Hz peak reflects limitations of FFT analysis (Douglas et al. 2002). FFT analysis assumes that the signal being analyzed does not change frequency content during the epoch being analyzed. This assumption is violated by biological signals such as EEG. Furthermore, EEG spindles comprised <8.5% of the total EEG, therefore contributing relatively little to total EEG power. This interpretation is supported by FFT analyses that were focused on EEG frequencies in the 7- to 14-Hz range (data not shown). FFT analyses of the 7- to 14-Hz frequency range did not unmask peaks in EEG amplitude. Therefore number of EEG spindles/min (Fig. 2B) provided an additional quantitative index of EEG activation.

In conclusion, the present study is the first to provide evidence that one functional consequence of increased cortical ACh release, activation of contralateral cortical EEG, is mediated partly by postsynaptic M1 receptors (Fig. 4). Thus enhancing the release of ACh by blocking M2 autoreceptors and/or by activating postsynaptic M1 receptors may help to alleviate deficits in cholinergic neurotransmission characteristic of dementing diseases (Carey et al. 2001; Kitai et al. 1999). The present results also are relevant for efforts to understand the similarities and differences in the neuronal mechanisms regulating sleep and anesthesia. During sleep, EEG spindles block sensory input to cortex (Steriade 2000). During halothane anesthesia, EEG spindle generation is enhanced in B6 mouse (Figs. 1C and 2B), cat (Keifer et al. 1996), and human (Yli-Hankala et al. 1989). During sleep and halothane anesthesia, EEG spindles are suppressed by increasing cholinergic neurotransmission at the level of the brain stem (Keifer et al. 1996; Steriade 2000) and, as shown here, by activating M1 mAChRs in the cortex.

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