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Prefrontal Cortex Acetylcholine Release, EEG Slow Waves, and Spindles Are Modulated by M2 Autoreceptors in C57BL/6J Mouse

CHRISTOPHER L. DOUGLAS,[1,2] HELEN A. BAGHDOYAN,[1] AND RALPH LYDIC[1]
[1]*Department of Anesthesiology, University of Michigan, Ann Arbor, Michigan 48109; and* [2]*Department of Neuroscience and Anatomy, The Pennsylvania State University, College of Medicine, Hershey, Pennsylvania 17033*

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Douglas, Christopher L., Helen A. Baghoydan, and Ralph Lydic. Prefrontal cortex acetylcholine release, EEG slow waves, and spindles are modulated by M2 autoreceptors in C57BL/6J mouse. *J Neurophysiol* 87: 2817–2822, 2002; 10.1152/jn.01015.2001. Recent evidence suggests that muscarinic cholinergic receptors of the M2 subtype serve as autoreceptors modulating acetylcholine (ACh) release in prefrontal cortex. The potential contribution of M2 autoreceptors to excitability control of prefrontal cortex has not been investigated. The present study tested the hypothesis that M2 autoreceptors contribute to activation of the cortical electroencephalogram (EEG) in C57BL/6J (B6) mouse. This hypothesis was evaluated using microdialysis delivery of the muscarinic antagonist AF-DX116 (3 nM) while simultaneously quantifying ACh release in prefrontal cortex, number of 7- to 14-Hz EEG spindles, and EEG power spectral density. Mean ACh release in prefrontal cortex was significantly increased ($P < 0.0002$) by AF-DX116. The number of 7- to 14-Hz EEG spindles caused by halothane anesthesia was significantly decreased ($P < 0.0001$) by dialysis delivery of AF-DX116 to prefrontal cortex. The cholinergically induced cortical activation was characterized by a significant ($P < 0.05$) decrease in slow-wave EEG power. Together, these neurochemical and EEG data support the conclusion that M2 autoreceptor enhancement of ACh release in prefrontal cortex activates EEG in contralateral prefrontal cortex of B6 mouse. EEG slow-wave activity varies across mouse strains, and the results encourage comparative phenotyping of cortical ACh release and EEG in additional mouse models.

INTRODUCTION

The prefrontal cortex mediates higher-order cortical functions such as linking actions to consequences (Gaffan and Harrison 1988, 1989; Tanji and Hoshi 2001), relating arbitrary associations (Toni and Passingham 1999), and manipulating working memory (Gabrieli et al. 1998; Goldman-Rakic 1996). Deactivation of the prefrontal cortex is a defining characteristic of sleep (reviewed in Stickgold et al. 2001) and frontal lobe function is impaired by anesthetics (Andrade 1996). The prefrontal cortex also contributes to cardiopulmonary control (Groenewegen and Uylings 2000), and states of sleep and anesthesia are characterized by autonomic dysregulation. These findings encourage translational research aiming to specify prefrontal cortical function during sleep and anesthesia.

Cortical acetylcholine (ACh) release is low during non-rapid-eye-movement (NREM) sleep and highest during the electroencephalographic (EEG) activation of wakefulness and REM sleep (Jasper and Tessier 1971). Anesthetic agents long have been known to suppress EEG activation and cortical ACh release (Mitchell 1963). The volatile anesthetic halothane decreases cortical ACh (Damsma and Fibiger 1991; Ngai et al. 1978) and induces 7- to 14-Hz spindles in the cortical EEG that are similar to the EEG spindles of NREM sleep. Thus halothane anesthesia has been shown to be a useful tool for elucidating cholinergic mechanisms regulating EEG activity (Keifer et al. 1994, 1996).

Recent studies of halothane anesthetized mouse show that muscarinic autoreceptors of the M2 subtype modulate ACh release in prefrontal cortex (Douglas et al. 2001). The increase in ACh release mediated by M2 autoreceptors implies a functional role for autoreceptors in regulating cortical EEG. Mouse models have particular relevance for translational research on cortical excitability control. Examples include murine models of epilepsy caused by autosomal recessive inheritance of a single gene (Skradski et al. 2001), discovery of mouse genes modulating synaptic plasticity and memory (Thakker-Varia et al. 2001), and development of gene-based transgenic mouse models of Alzheimer's disease (Hock and Lamb 2001). The present study tested the hypothesis that muscarinic autoreceptor enhancement of ACh release in mouse prefrontal cortex causes EEG activation.

METHODS

Experimental procedure

All experiments adhered to the *Guide for the Care and Use of Laboratory Animals* (National Academy of Sciences Press 1996). Adult male C57BL/6J mice (Jackson Labs, Bar Harbor, ME, $n = 11$) were anesthetized with 2–3% halothane in O_2 and placed in a David Kopf (Tujunga, CA) stereotaxic frame. Core body temperature and respiratory rate were monitored while anesthesia was maintained with halothane delivered at a mean concentration of 2.4% throughout the experiment. A small craniotomy allowed access to the cortex, and the microdialysis probe was aimed for prefrontal cortex (Uylings and van Eden 1990), designated as frontal association cortex (FrA) in the stereotaxic atlas (Paxinos and Franklin 2001). The aim site was 3.0 mm anterior to bregma and 1.6 mm lateral to the midline. The final dorsal-ventral position of each probe was determined visually to place the dialysis probe membrane fully within the cortex. EEG recordings

Address for reprint requests: R. Lydic, Dept. of Anesthesiology, University of Michigan, 7433 Med Sci I, 1150 W Medical Center Dr., Ann Arbor, MI 48109 (E-mail: rlydic@umich.edu).

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were obtained from two 0.13-mm-diam stainless steel wire (California Fine Wire, Grover City, CA) electrodes placed in the prefrontal cortex contralateral to the dialysis probe.

Microdialysis and high-performance liquid chromatography (HPLC)

Microdialysis probes (6-kDa cutoff; 1.0-mm length by 0.24-mm diam, CMA/Microdialysis, Acton, MA) were perfused continuously with Ringer solution (147 mM NaCl, 2.4 mM $CaCl_2$, 4.0 mM KCl, and 10 μM neostigmine) at 2.0 μl/min. Dialysis samples (25 μl) were collected every 12.5 min and injected into an HPLC/EC system [Bioanalytical Systems, (BAS), West Lafayette, IN] for quantification of ACh. Samples were carried by a 50 mM Na_2HPO_4 mobile phase to a column that separated ACh and choline. An immobilized enzyme reaction column produced H_2O_2 in amounts proportional to ACh and choline. The H_2O_2 was ionized with an applied potential of 0.5 V in reference to a Ag^+/AgCl electrode. The resulting signal created a chromatographic peak that was digitized using ChromGraph (BAS) software. The areas under the chromatographic peaks were integrated and ACh quantified as pmol/12.5 min using a standard curve produced on the day of each experiment. To verify that microdialysis probe membranes did not change during experiments, the percent recovery from a known ACh concentration was measured before and after each experiment. Experimental data were used only if pre- and postexperimental probe recoveries were not significantly different.

The muscarinic antagonist AF-DX116 was delivered via the same dialysis probe used to collect ACh. Muscarinic receptors exist as five subtypes (M1–M5), and the selectivity of muscarinic antagonists for these subtypes is concentration dependent (Caulfield and Birdsall 1998). By comparing relative potencies of different mAChR antagonists to block a response, it is possible to conclude which subtype mediates that response (Baghdoyan and Lydic 1999; Baghdoyan et al. 1998; Billard et al. 1995). For example, AF-DX116 has a relatively high and equal affinity for M2 and M4 receptors, whereas pirenzepine has a relatively high and equal affinity for M1 and M4 receptors (Caulfield and Birdsall 1998). Recent studies in B6 mouse found that dialysis delivery of 3 nM AF-DX116 or 300 nM pirenzepine increased prefrontal cortical ACh release (Douglas et al. 2001). Based on the known affinities of these antagonists for each of the five muscarinic receptor subtypes (Caulfield and Birdsall 1998), the finding that AF-DX116 was 100 times more potent than pirenzepine to increase ACh release indicates that the M2 subtype functions as an autoreceptor in mouse prefrontal cortex (Douglas et al. 2001). Thus the present study used AF-DX116 at a concentration of 3 nM to test the hypothesis that M2 autoreceptors modulate EEG activation.

EEG spindle quantification

Signals from the prefrontal cortical electrodes were amplified and recorded using a Grass (West Warwick, RI) Model 7 polygraph. The EEG signal was visualized on a Tektronix (Beaverton, OR) dual beam storage oscilloscope to confirm spindle frequency in the 7- to 14-Hz range. Initial EEG spindle quantification and cortical ACh measurement was made during dialysis delivery of Ringer alone (control). A CMA/110 liquid switch then was activated to deliver Ringer containing AF-DX116. During dialysis delivery of both Ringer alone and Ringer plus AF-DX116, simultaneous measures were taken of ACh release and number of EEG spindles/min.

EEG power spectral analysis

Signals from the recording electrodes were amplified, filtered at 0.3 and 30 Hz, and captured digitally with a sampling rate of 128 Hz using a Grass model 15RXi digital polygraph and Polyview software. Fast Fourier transform analyses were conducted on two-second bins between 0.5 and 25 Hz in 0.5-Hz increments. The bins were averaged over 1-min intervals of EEG recordings. The total power for each frequency interval of prefrontal cortical EEG was determined for the duration of microdialysis with Ringer and for the duration of microdialysis delivery of AF-DX116.

Histology

Following each experiment, brains were removed for histological verification of microdialysis and recording sites. Brains were rapidly frozen, then sectioned coronally at 40 μm, 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. Microdialysis and EEG data were analyzed only if dialysis sites were localized fully within FrA by comparison to a stereotaxic atlas (Paxinos and Franklin 2001).

Statistical analyses

The dependent measures of ACh release and number of EEG spindles were analyzed using descriptive statistics and *t*-test. Repeated-measures ANOVAs and post hoc comparisons with Bonferroni correction evaluated drug main-effect on EEG frequency. The alpha level was $P < 0.05$.

RESULTS

Histological analyses revealed that all microdialysis probes were placed fully within prefrontal cortex. Figure 1*A* schematizes placement of a microdialysis probe in the prefrontal cortex and EEG electrodes in the contralateral prefrontal cortex. Figure 1*B* shows a representative polygraphic record of EEG spindling in a mouse. Spindles were induced by delivery of 2.1% halothane. Figure 1*C* shows an EEG tracing from the same mouse during the same experiment after changing the dialysate to Ringer containing the mAChR antagonist AF-DX116.

Figure 2 plots mean prefrontal cortical ACh release from 11 mice and EEG spindle counts averaged from seven mice. Microdialysis delivery of AF-DX116 significantly increased ACh release ($t = 4.2$; df = 32; $P < 0.0002$; Fig. 2*A*) and significantly decreased the number of halothane-induced EEG spindles ($t = 15.2$; df = 111; $P < 0.0001$; Fig. 2*B*). Oscilloscope images confirmed EEG spindle frequency in the 7- to 14-Hz range. The decrease in EEG spindles occurred within the first 12.5 min after switching to AF-DX116 and endured for as long as drug was administered (37.5 min). Autonomic signs were monitored constantly during the recording period, and no change was observed in core body temperature, respiratory rate, or response to hindlimb pinch.

Figure 3 summarizes the averaged EEG power spectra for four mice. The power spectral density is presented as mean ± SE from 0.5 to 25 Hz in 0.5-Hz intervals. ANOVA revealed a significant main-effect on EEG power of drug ($F = 54.22$; df = 1,3; $P = 0.0052$), of EEG frequency ($F = 33.18$; df = 49,147; $P < 0.0001$), and a drug by frequency interaction ($F = 15.51$; df = 49,147; $P < 0.0001$). Post hoc multiple comparison statistics with Bonferroni correction showed that dialysis delivery of AF-DX116 to the prefrontal cortex significantly ($P < 0.05$) reduced EEG power in the contralateral prefrontal cortex for the 0.5-, 1.0-, 1.5-, 2.0-, 2.5-, and 3.5-Hz bins.

DISCUSSION

The results demonstrate for the first time that halothane-induced EEG spindles and EEG slow waves in mouse prefron-

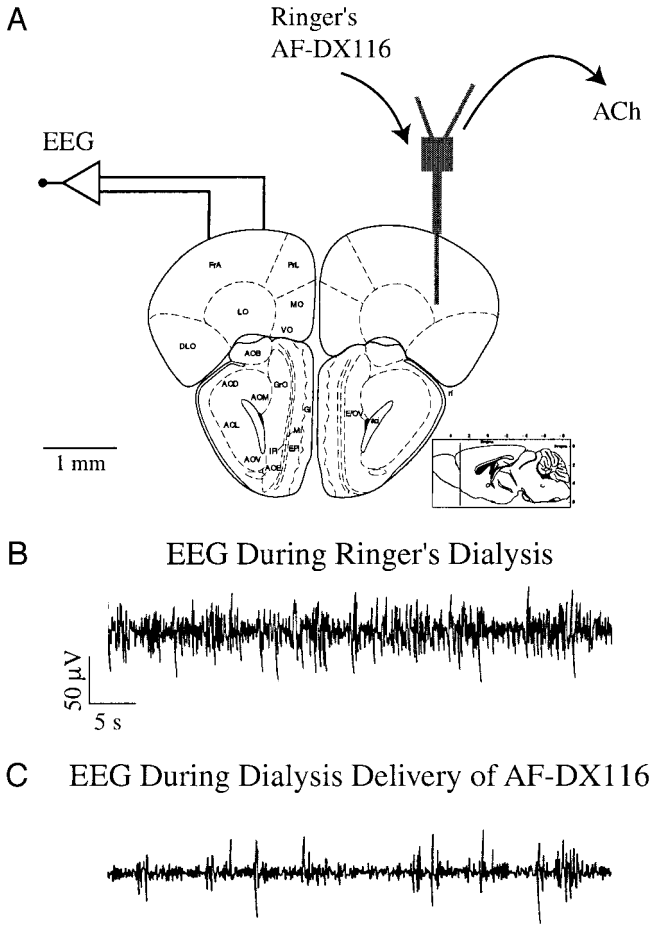

FIG. 1. Microdialysis and electroencephalographic (EEG) measures from prefrontal cortex of B6 mouse. *A*: schematic of coronal section from mouse brain atlas (Paxinos and Franklin 2001) showing technique for simultaneous measures of EEG and acetylcholine (ACh) release. *Inset*: midsagittal perspective with vertical line denoting level of coronal section along the rostral (left) to caudal (right) axis. Mouse brain schematic modified with permission from (Paxinos and Franklin 2001). *B*: EEG spindles during dialysis delivery of Ringer. *C*: decreased EEG spindles caused by dialysis delivery of AF-DX116. *Bottomn left*: calibration scales of *B* also apply to *C*.

tal cortex are significantly decreased by microdialysis delivery of the muscarinic receptor antagonist AF-DX116. The findings are discussed in relation to ongoing efforts to understand mechanisms by which cortical cholinergic transmission contributes to EEG activation.

EEG spindle suppression by blocking muscarinic autoreceptors in prefrontal cortex

Figures 1 and 2 provide novel evidence that ACh release evoked by blocking M2 autoreceptors modulates EEG spindle generation. Recent pharmacological data (Douglas et al. 2001) support the conclusion that AF-DX116 causes increased ACh release (Fig. 2*A*) by blocking M2 autoreceptors. The finding of a decreased number of EEG spindles (Figs. 1, *B* and *C*, and 2*B*) in association with increased ACh release is consistent with evidence showing cholinergic activation of EEG. For example, during wakefulness and REM sleep, there is a significant increase in cortical ACh above NREM levels (Celesia and Jasper 1966; Jasper and Tessier 1971) and a high-frequency (16–25 Hz) low-amplitude (10–30 μV) activated EEG (Steriade 1999). In contrast, during NREM sleep the cortical EEG is characterized by periodic oscillations of medium-frequency (7–14 Hz) high-amplitude (80–120 μV) spindles (Steriade et al. 1993).

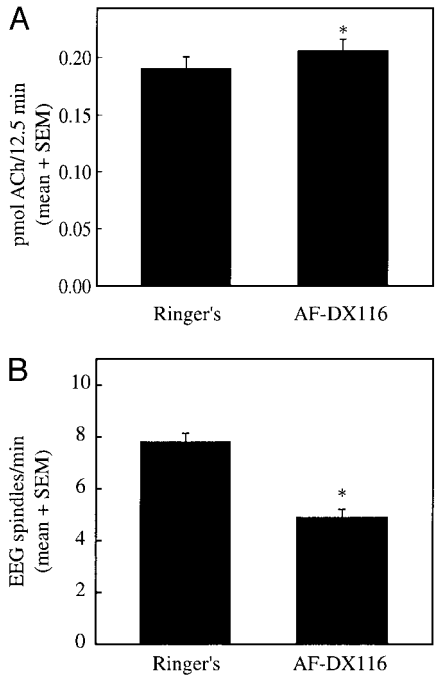

FIG. 2. Dialysis delivery of AF-DX116 to prefrontal cortex significantly (*) increased ACh release by 9.5% (*A*) and significantly decreased the number of EEG spindles/min by 37% (*B*).

There is good agreement across species that halothane anesthesia enhances EEG spindle activity. Halothane anesthesia causes spindle activity in human EEG (reviewed in Clark and

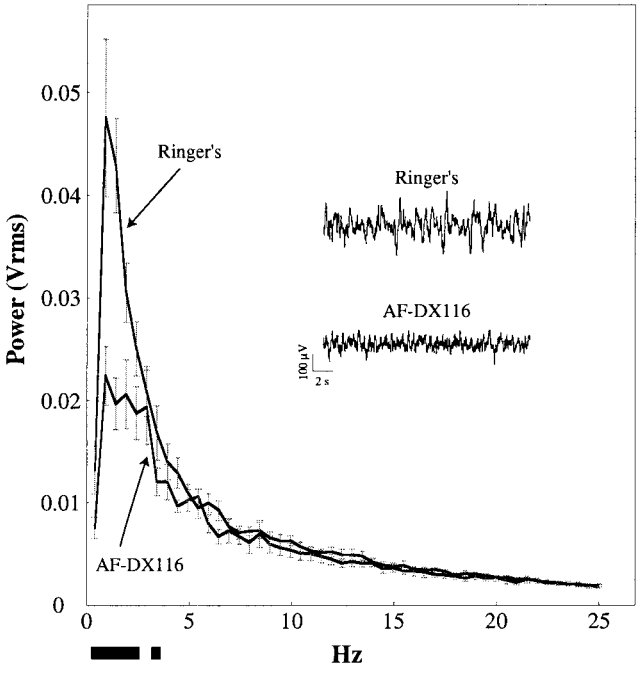

FIG. 3. Average prefrontal cortical EEG power during microdialysis with Ringer and Ringer containing AF-DX116. ■, EEG frequency bins at which AF-DX116 caused a significant decrease in EEG power. *Inset*: digital EEG recordings from mouse prefrontal cortex.

Rosner 1973). Previous studies of feline EEG have shown that the 7- to 14-Hz cortical EEG spindles caused by halothane anesthesia are not significantly different in waveform, amplitude, or number from the EEG spindles of NREM sleep (Keifer et al. 1996). Microinjection of the cholinergic agonist carbachol into the feline medial pontine reticular formation (mPRF) decreases halothane-induced EEG spindles (Keifer et al. 1996). In the present study, cortical delivery of AF-DX116 significantly increased ACh release (Fig. 2*A*) and significantly decreased EEG spindles (Fig. 2*B*), showing that EEG activation can be elicited by antagonizing M2 autoreceptors.

Cholinergic suppression of EEG slow waves

Fourier analysis of the digitized EEG showed that dialysis delivery of AF-DX116 to the prefrontal cortex caused a significant decrease in the EEG power of slow-wave (delta) activity (Fig. 3). This finding provides further support for the interpretation that autoreceptor modulation of cortical ACh release alters cortical excitability. The present EEG results obtained during halothane anesthesia fit well with EEG data of NREM sleep. The prefrontal cortex is particularly vulnerable to the effects of both sleep deprivation (Harrison and Horn 1997; Horne 1993) and anesthesia (Andrade 1996; Casele-Rondi 1996). Halothane anesthesia deactivates the EEG and suppresses sensory responsiveness. During NREM sleep, as EEG delta power (0.5–2 Hz) is enhanced, responsiveness to sensory stimulation decreases (Borbely et al. 1981). Studies of brain energy metabolism show that regions of human prefrontal cortex are deactivated in NREM sleep (Andersson et al. 1998; Braun et al. 1997). During NREM sleep, decreased cholinergic input is a requirement for the generation of slow-wave EEG activity (reviewed in Steriade 1999). Thus the AF-DX116-induced increase in ACh release and decrease in EEG slow wave activity (Fig. 3) are consistent with cholinergic activation of cortical EEG.

Limitations and conclusions

One limitation of the present study stems from the fact that these experiments were conducted using halothane anesthesia. While halothane is useful in reproducing one trait of NREM sleep (7- to 14-Hz EEG spindles), general anesthesia is not equivalent to the state of NREM sleep. Dialysis delivery of muscarinic antagonists to prefrontal cortex of intact, unanesthetized mice may alter EEG and cortical ACh release in a manner that is quantitatively or qualitatively different from reported here for anesthetized mice. Characterizing such possible differences is open to future investigation.

A second limitation concerns the inability of the power spectral analysis (Fig. 3) to convey the significant decrease in EEG spindles/min caused by AF-DX116 (Fig. 2*B*). One assumption of FFT is that the signal being analyzed is stationary and does not change frequency content during the epoch of analysis. Failure of most biological signals to meet this assumption is a classic limitation for FFT. Additionally, the EEG spindles comprised only a small portion of the EEG signal being analyzed. For example, even when the number of spindles per minute was maximal (Fig. 2*B*, Ringer) these 7- to 14-Hz waves comprised only 8.3% of a 1-min EEG segment. Because power is energy per unit time, the lack of a prominent peak in the 7- to 14-Hz frequency band (Fig. 3) may reflect the relatively small contribution made by EEG spindles to total EEG power. Power analyses focusing on a limited portion of a frequency range can sometimes unmask peaks not seen on a broad frequency spectrum (see Fig. 2 of Dworkin et al. 2000). At all integers of EEG frequency from 7 to 14 Hz, the EEG amplitude (square root of power) during Ringer dialysis was not significantly different from EEG amplitude during dialysis delivery of AF-DX116. The foregoing points reinforce the appropriateness of using EEG spindles/min to quantify EEG activation caused by AF-DX116 (Fig. 2).

By what mechanisms might antagonism of muscarinic receptors in mouse prefrontal cortex activate the EEG? Answers to this question will require additional studies to overcome gaps in existing knowledge concerning: the origin of cholinergic input to mouse prefrontal cortex, contributions of cortical efferents to EEG activation, and pre- versus postsynaptic actions of ACh.

Data on the origin of cholinergic input to prefrontal cortex in different mouse strains are not available. Cholinergic neurons in the basal forebrain and brain stem receive projections from prefrontal cortex (Groenewegen and Uylings 2000). Choline acetyltransferase (ChAT) mapping of mouse forebrain (Kitt et al. 1994) shows that cortical association layers II and III receive the majority of ACh inputs from basal forebrain. These studies of ChAT-positive neurons in mouse forebrain further note that "there do not appear to be many, if any, cholinergic neurons in mouse cortex" (Kitt et al. 1994). Immunocytochemical (Kitt et al. 1994) and pathway mapping (Groenewegen and Uylings 2000) data suggest that ACh in B6 mouse prefrontal cortex arises mostly from basal forebrain cholinergic neurons. Therefore the majority of muscarinic autoreceptors in prefrontal cortex through which AF-DX116 enhances ACh release (Fig. 2) (Douglas et al. 2001) are likely to reside on terminals projecting to prefrontal cortex from cholinergic basal forebrain neurons.

There is limited information on how corticofugal projections contribute to generation of EEG spindles. Removal of corticothalamic projections by transection disrupts EEG spindle generation in feline brain (Contreras et al. 1996). Intracellular recordings from cortical neurons indicate long-lasting hyperpolarization during NREM sleep, suggesting disfacilitation of thalamocortical synapses by decreased cholinergic activity (Timofeev et al. 2001). The present finding that M2 autoreceptors in one prefrontal cortex can significantly alter EEG in the contralateral prefrontal cortex may indicate functionally significant cortical efferent pathways in B6 mouse.

The effects of AF-DX116 on mouse EEG spindles and slow-wave activity may include mechanisms in addition to presynaptic autoreceptors. The increased ACh release caused by blocking muscarinic autoreceptors would be anticipated to activate postsynaptic cholinergic receptors. In mature rat cortex, non-M2 muscarinic (Vidal and Changeux 1993) and postsynaptic nicotinic (Jones et al. 1999) receptors modulate cortical excitability. In immature rat cortical neurons (Kimura and Baughman 1997) and interneurons (Kondo and Kawaguchi 2001) there is evidence for muscarinic receptor modulation of both excitatory and inhibitory synaptic responses. Even nonneuronal mechanisms may contribute to ACh actions on cortical excitability. For example, glial cells have been shown to modulate presynaptic ACh release in cultured molluscan neu-

rons (Smit et al. 2001). Elucidation of the postsynaptic mechanisms activating the EEG in prefrontal cortex could have important implications for treatment of a host of neurodegenerative and psychiatric disorders (reviewed in Groenewegen and Uylings 2000).

The present data support the conclusion that enhanced ACh release in prefrontal cortex—caused by blocking M2 autoreceptors—activates EEG in contralateral prefrontal cortex. EEG is a strongly heritable trait in both humans and mice (Franken et al. 1998), and EEG can be regarded as an intermediate phenotype that is determined, in part, by ACh as a lower level phenotype. Cholinergic neurons are phenotypically defined by the ACh synthetic enzyme ChAT and by the presynaptic vesicular ACh transporter (VAChT). The gene loci for ChAT and VAChT are contiguous, and this contiguity has been hypothesized to have regulatory significance (Erickson et al. 1994). The present finding that presynaptic muscarinic autoreceptors modulate EEG is consistent with evidence that sleep and EEG are altered by inhibiting presynaptic VAChT (Capece et al. 1997). EEG is a phenotypic trait that is known to differ across mouse strains (Huber et al. 2000), and the present results encourage characterizing cholinergic mechanisms regulating EEG in different strains of mice. Neurophysiological studies of mouse provide an essential step in translational research that can attribute phenotypic traits to specific genes and proteins (Behringer 2001; Denny and Justice 2000).

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