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Neuropharmacological Characterization of Basal Forebrain Cholinergic Stimulated Cataplexy in Narcoleptic Canines

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Basal forebrain (BF) cholinergic regulation of cataplexy was investigated in narcoleptic canines. Specific cholinergic agonists and antagonists, and excitatory or inhibitory amino acid neurotransmitter receptor agonists, were perfused through microdialysis probes implanted bilaterally in the BF of narcoleptic canines. Cataplexy was monitored using the food-elicited cataplexy test (FECT) and recordings of electroencephalogram, electrooculogram, and electromyogram. In narcoleptic canines, carbachol and oxotremorine (10^{-5}–10^{-3} M), but not McN-A-343 or nicotine (10^{-4}–10^{-3} M), produced a dose-dependent increase in cataplexy. In addition, *N*-methyl-D-aspartate (10^{-4}–10^{-3} M) and kainic acid (10^{-5}–10^{-4} M) did not have any effects, while muscimol (10^{-3} M) produced a weak ($P < 0.10$) increase in cataplexy. In control canines, carbachol (10^{-5}–10^{-3} M), but not oxotremorine (10^{-4}–10^{-3} M), produced muscle atonia after the highest concentration in one of three animals. Carbachol (10^{-3} M)-induced cataplexy in narcoleptic canines was blocked by equimolar perfusion with the muscarinic antagonists atropine, gallamine, and 4-DAMP but not pirenzepine. These findings indicate that carbachol-stimulated cataplexy in the BF of narcoleptic canines is mediated by M2, and perhaps M3, muscarinic receptors. The release of acetylcholine in the BF was also examined during FECT and non-FECT behavioral stimulation in narcoleptic and control canines. A significant increase in acetylcholine release was found in both narcoleptic and control BF during FECT stimulation. In contrast, simple motor activity and feeding, approximating that which occurs during an FECT, did not affect acetylcholine release in the BF of narcoleptic canines. These findings indicate that BF acetylcholine release is enhanced during learned emotion/reward associated behaviors in canines. © 1998 Academic Press

Key Words: **narcolepsy; cataplexy; carbachol; acetylcholine; basal forebrain.**

INTRODUCTION

Narcolepsy is an incurable neurological disorder occurring in approximately 0.05% of the population which is characterized by excessive daytime sleepiness, fragmented nighttime sleep, sleep onset periods of rapid eye movement (REM), and sudden periods of muscle atonia called cataplexy (6). Canine narcolepsy is an animal model of the human disorder with many symptoms paralleling those found in humans, including excessive daytime sleepiness, fragmented nighttime sleep, and cataplexy (5, 35). Cataplexy in the narcoleptic canine can be elicited by the emotional stimulation produced by food, and consequently cataplexy may be reliably induced and quantified using a food-elicited cataplexy test (FECT) (5).

Several lines of evidence suggest that pontine cholinergic systems play an important role in the regulation of cataplexy in narcoleptic canines. Local perfusion in the pontine reticular formation (PRF) with cholinergic agonists carbachol (41) or oxotremorine (43) stimulated cataplexy while local perfusion with the cholinergic antagonist atropine blocked physostigmine (iv)-induced cataplexy (41) in narcoleptic canines. The region explored in these studies extended from the ventral border of the nucleus reticularis pontis caudalis to an area immediately ventral to the dorsal pontine tegmentum (43). Cataplexy in narcoleptic canines is potently modulated by M2 muscarinic receptors. The stimulation of cataplexy by local perfusion with carbachol in the PRF is completely reversed by the nonselective muscarinic antagonist atropine and the M2-selective muscarinic antagonist gallamine, partially reversed by the M1/M3-selective muscarinic antagonist 4-DAMP, and unaffected by the M1 muscarinic antagonist pirenzepine (43). There are many similarities between the above-described regulation of cataplexy and the neuropharmacological regulation of REM sleep. For example, REM sleep and muscle atonia can be elicited by local PRF injections of muscarinic agonists such as carba-

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chol (2, 48), oxotremorine (16, 53), or bethanecol (19) and coadministration of various M2 antagonists in the PRF or pontine tegmentum reduces the REM sleep enhancement produced by the local injection of carbachol (11, 54, 55). However, perhaps the most convincing evidence that cataplexy and REM sleep are regulated by similar cholinergic mechanisms are findings of increased acetylcholine release in the dorsomedial pontine tegmentum (26) and in the pontine reticular formation (including regions of the pontine tegmentum) (42) during REM sleep and cataplexy, respectively. These findings suggest that enhanced acetylcholine release and the subsequent stimulation of M2 (and possibly M3) muscarinic receptors in the PRF is involved in the cholinergic regulation of REM sleep and cataplexy.

Recently, we found evidence for basal forebrain (BF) involvement in the cholinergic regulation of cataplexy in narcoleptic canines (37). In this study microinjections of carbachol (2–10 nmol) or physostigmine (50 nmol) into the magnocellular region of the BF (magnocellular preoptic area (POA)) and the diagonal band of Broca, an area where cholinergic neurons are distributed, potently stimulated cataplexy in narcoleptic canines. This was surprising since there is little previous evidence for this brain region in the regulation of REM sleep. In fact, others have shown that carbachol injections into the POA of cats produce increased wakefulness (4, 47) and in some cases a reduction in spontaneous and carbachol (in the PRF) induced REM sleep (4). Nevertheless, consistent with this earlier work, we did find that the increase in cataplexy was accompanied by a decrease in slow wave sleep in narcoleptic dogs (as well as control dogs). Interestingly, it was also found that very high doses of carbachol (50 nmol), when given bilaterally into the BF, could also produce muscle atonia in control animals which led to the suggestion that the pathophysiology of narcolepsy may involve cholinergic hypersensitivity in this brain region. Because this area sends widespread cholinergic projections throughout the limbic system, thalamus, and much of the cerebral cortex it was proposed that the local carbachol injections result in a global activation of cholinergic networks involved in the regulation of narcolepsy. Furthermore, it was suggested that enhanced acetylcholine release in the BF, induced by emotional triggers, is the initiator of the cataplectic response in narcoleptic canines. Therefore, in the present study we have investigated the release of acetylcholine in the BF, using *in vivo* microdialysis, during FECT-stimulated cataplexy in narcoleptic canines. Various behavioral stimuli, aimed at distinguishing the emotive from nonemotive content of FECT stimulation, were tested. Furthermore, the cholinergic receptors involved in mediating the carbachol effect were characterized by local perfusion with numerous selective cholinergic agonists and antagonists.

MATERIALS AND METHODS

All studies were performed on adult Doberman pinschers, which included five narcoleptic (three males and two females) and three control (males) canines. All animals were between 1 and 5 years of age, weighed 22–28 kg, and were bred at the Stanford University narcoleptic dog colony. Because of the great value of all implanted narcoleptic canines, they had been used in previous experiments (see 41–43). If so, each animal was given a "washout" period of at least 20 days before further use. The animals were kept under a 12:12-h light–dark schedule with food and water available *ad libitum*.

SURGERY

Guide cannulae and recording electrodes were surgically implanted on all canines using sterile procedures according to the protocol of Reid and colleagues (for complete description see 41). Briefly, the animals were anesthetized using isofluorane (2% mixture in air administered via a tracheal tube) and placed on a Kopf stereotaxic device. For cataplexy recording, they were implanted with screw electrodes in the skull over lateral parietal and the midfrontal cortex for recording of electroencephalogram (EEG) and in the orbit of the frontal bone for the recording of electrooculogram (EOG). Stranded stainless-steel wires were inserted into the dorsal neck musculature for recording the electromyogram (EMG). The guide cannulae consisted of two 20-mm stainless-steel cannulae separate by 2 mm and soldered to a nut stack and were lowered into position so that the ventral tips touched the surface of the cortical dura. The guide cannula bundles were positioned over the BF bilaterally (AP, 29.0–31.0; ML, 5.0, according to 30), such that two guide cannulae were positioned over the rostrocaudal extent of the BF, both at the same laterality. The recording electrodes, electrical plug and guide cannulae were cemented to the skull using dental acrylic and the skin around the head stage was sutured close. During recovery from surgery all animals were under 24-h surveillance at the Stanford University Department of Comparative Medicine Intensive Care Unit and postsurgical treatment included analgesics for the first 12–48 h and a regimen of daily antibiotic treatment for up to 5 days. The animals were allowed at least 3 weeks to recover from surgery.

One day prior to experimentation microdialysis probes (70-mm shaft with 5-mm membrane, CMA/10, CMA/Microdialysis, Stockholm, Sweden) were lowered bilaterally through the posterior BF guide cannulae (previously shown to be the most active site of carbachol stimulated cataplexy (38)) into the BF (AP, 29.0; ML, 5.0; V, 46.0 from dura, according to 30) and anchored in place while the animals were under isofluorane anesthesia. Each probe was tested for *in vitro* recovery of 10^{-7} M acetylcholine before implantation and the relative recovery found was

29 ± 5%, $n = 20$. After completing the experiment the probes were removed and the animals returned to their cages. Histological verification of the probe placements was performed on all animals in this study using 2% neutral red which was injected (1.0 µl) through the same guide cannulae and at the same depth coordinate as the dialysis probes. All sections were Nissl stained with cresyl violet for gross anatomy and some sections were also stained for ChAT immunoreactivity (see 37).

TESTING PROCEDURE

All canines were given at least 3 days of habituation to the experimental chamber before testing. A complete description of the experimental chamber has been made previously (41). The probes were perfused via a 3-m inlet line at 2.0 µl/min with artificial cerebrospinal fluid (CSF) (125 mM NaCl, 0.5 mM NaH_2PO_4, 2.5 mM Na_2HPO_4, 2 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.4) using a Harvard pump and samples were collected 10 min in 200-µl Eppendorf tubes attached to the side of the headstage. The perfusion medium contained 1 mM neostigmine in order to obtain measurable amounts of acetylcholine. This level of neostigmine was found to have no effect on cataplexy of its own. Behavioral state and cataplexy were measured using the FECT (see 5), combined with recordings of EEG, EOG, and EMG. Recordings were scored in 30-s epochs for wake, drowsy, light sleep, deep sleep, REM sleep, and cataplexy (for narcoleptic canines) according to the criteria described by Reid and colleagues (44) with the addition of active wake which included low-voltage mixed frequency cortical tracing with an uninhibited EMG that showed occasional high-voltage (>100 µV) episodes (at least one per 30 s epoch) that were often accompanied by movement artifact. In each FECT, the subject ate 10 small bites of wet dog food which were lined up on the floor in a semicircle over a distance of approximately 3 m and cataplectic attacks were scored when the animal stopped forward motion and the hind quarters were lowered toward the floor, thus initiating either a partial or complete attack or a clear loss of muscle tone as noted by the EMG was observed. Time required to eat all 10 bites of food and successfully move away was also recorded.

Experiments on acetylcholine release were performed on days 1–3 during the first 130 min of each microdialysis perfusion session. Each session began approximately 18 h after microdialysis probe implantation between the hours of circadian time (CT) 3 and CT 8. Acetylcholine control tests were always performed on day 1. Three different control tests on the extracellular levels of acetylcholine were performed: (i) baseline levels were collected for 130 min, (ii) the perfusion medium was switched from CSF to CSF without Ca^{2+} after 90 min, or (iii) the perfusion medium was switched from CSF to CSF with 10^{-5} M tetrodotoxin (TTX) after 90 min. The behavioral tests were performed on days 2 and 3. The behavioral testing protocol consisted of baseline sample collection for the first 90 min, followed by a behavioral stimulus condition for the next 20 min and then another 20 min of poststimulus sample collection. There were three different behavioral stimuli: (i) four consecutive FECT trials; (ii) four consecutive motor activity trials in which the animal was leashed as in a FECT and then walked the distance it takes to eat the food in a FECT, and (iii) four consecutive feeding periods in which the animal was given the same amount of food as in a FECT while at rest. The behavioral stimuli were administered over a 20-min period (two stimuli per 10-min perfusate sample) and were administered in a randomized order. Conditions (ii) and (iii) were discounted if any emotional excitement or cataplexy occurred during the 20-min stimulation period. After completion of the behavioral testing protocols on days 1–3 all animals were tested for the effects of locally perfused drugs on FECT induced cataplexy. During these procedures no perfusate samples were collected.

Pharmacological experiments were performed on days 1–5. During days 1–3 baseline levels of cataplexy were obtained either during or following completion of the behavioral testing protocols. During days 4–5 baseline levels of cataplexy were obtained following 60 min of CSF perfusion. Baseline levels of cataplexy were measured using four consecutive FECT trials performed over a period of 20 min. After completion of the four FECT trials, the perfusion medium was switched from CSF to CSF plus drug using a manual liquid switch

TABLE 1

Basal Levels of FECT-Induced Cataplexy in Narcoleptic Canines

Time (min)	−20	−10	20	50	80	110	140	170
Attacks ($n = 5$)	2.2 ± 0.3	2.4 ± 0.5	2.1 ± 0.4	2.0 ± 0.4	2.4 ± 0.6	2.5 ± 0.4	2.1 ± 0.5	1.9 ± 0.8
Elapsed time (s)	56 ± 11	59 ± 8	52 ± 10	55 ± 9	64 ± 7	58 ± 10	51 ± 5	47 ± 12

Baseline cataplexy in five narcoleptic canines perfused bilaterally with cerebrospinal fluid in the basal forebrain. Cataplexy was measured using the same schedule as drug perfused animals, on two separate days for each animal. The mean number of cataplectic attacks and elapsed time for two food-elicited cataplexy tests (FECT) per test period is shown. During each FECT the subject ate 10 bites of wet dog food lined up along the floor (3 m) and cataplectic attacks were scored when the animal stopped forward motion and the hind limbs were lowered toward the floor. The total time required to eat all 10 bites of food was also recorded.

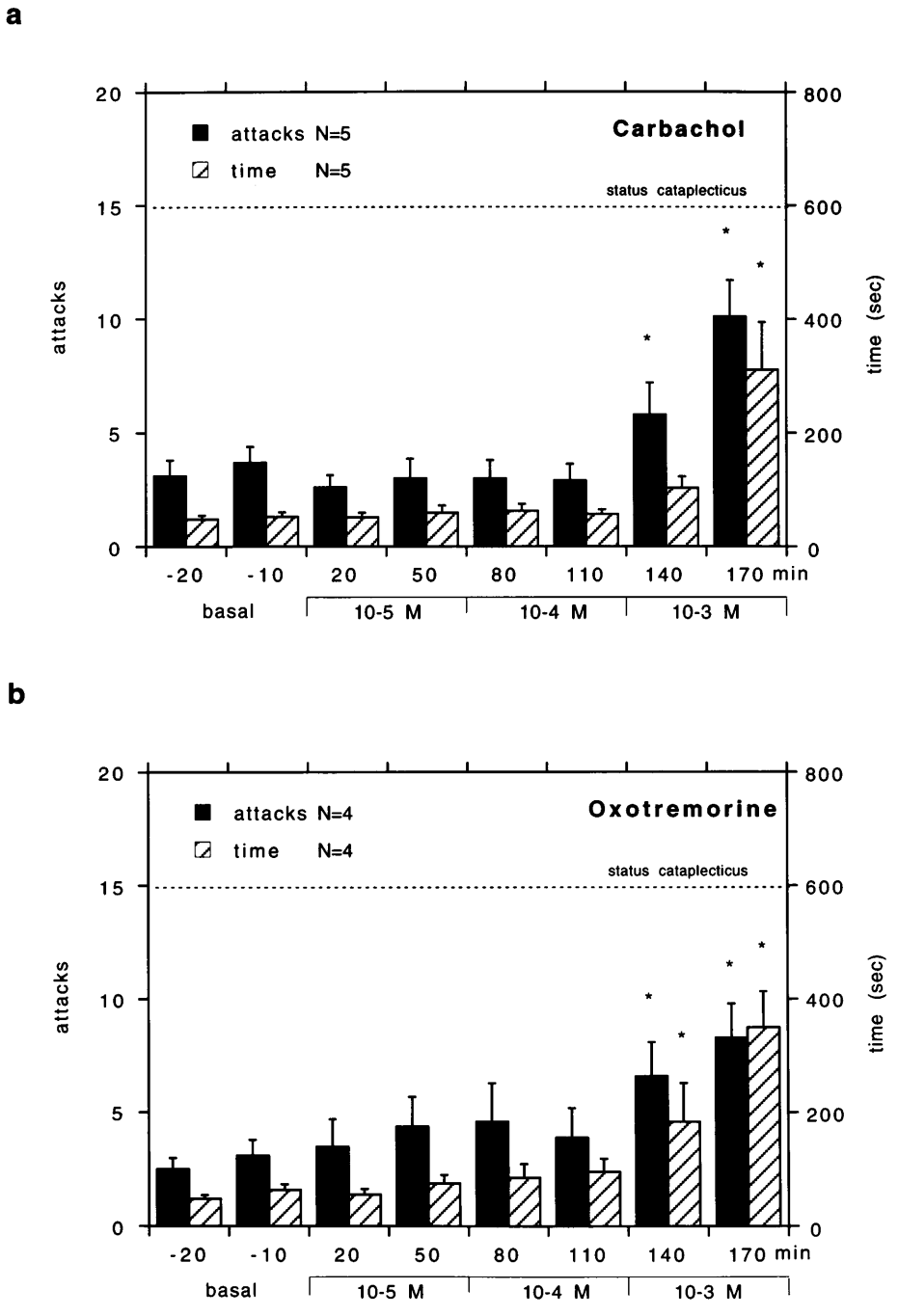


FIG. 1. Effect of local perfusion of various cholinergic agonists in the basal forebrain on cataplexy in the narcoleptic canine. Local bilateral perfusion with (a) carbachol (10^{-5}–10^{-3} M), (b) oxotremorine (10^{-5}–10^{-3} M), (c) McN-A-343 (10^{-4}–10^{-3} M), (d) nicotine (10^{-4}–10^{-3} M), (e) atropine (10^{-3}–10^{-2} M), and (f) muscimol (10^{-3} M) in narcoleptic canines is shown. Drugs were mixed into artificial cerebrospinal fluid and perfused through microdialysis probes in increasing concentrations over the course of a 2- to 4-hour experiment in a stepwise fashion, eg, none during the first hour, 10^{-5} M during the second hour, 10^{-4} M during the third hour, and 10^{-3} M during the fourth hour. The mean number of cataplectic attacks and elapsed time for two food-elicited cataplexy tests per test period is shown. For the purpose of figure presentation, *status cataplecticus* (carbachol atonia) was arbitrarily designated as 15 attacks elapsed over 600 s. Each drug perfusion time point prior to *status cataplecticus* was compared with the basal time points using a Fisher PLSD *post hoc* test. $*P < 0.05$ satisfactory for comparison with either basal time point.

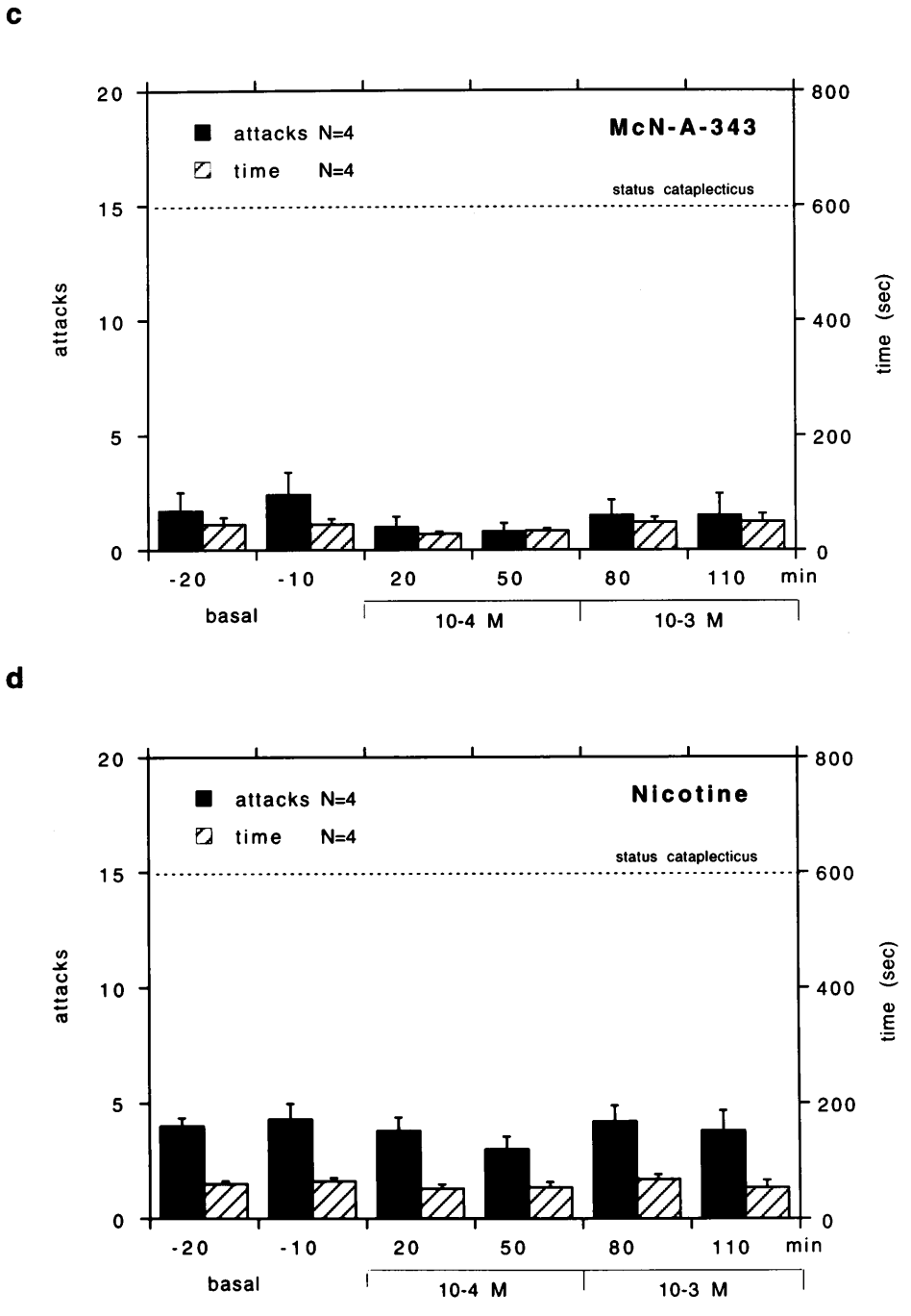


FIG. 1—*Continued*

(CMA/110, CMA/Microdialysis). All drugs were tested bilaterally at various concentrations in the perfusion medium: carbachol (10^{-5}–10^{-3} M), oxotremorine (10^{-5}–10^{-3} M), McN-A-343 (10^{-4}–10^{-3} M), nicotine (10^{-4}–10^{-3} M), *N*-methyl-D-aspartate (NMDA) (10^{-4}–10^{-3} M), kainic acid (10^{-5}–10^{-4} M), muscimol (10^{-3} M), atropine (10^{-3}–10^{-2} M), gallamine (10^{-3} M), pirenzepine (10^{-3} M), and 4-diphenylacetoxy-*N*-methylpiperidine (4-DAMP) (10^{-3} M). All drug solutions were pH 7.0. Drugs which were tested at more than one concentration were done so by increasing the concentration of the drug over the course of an experiment; e.g., 10^{-5} M was perfused for the first hour, 10^{-4} M, for the second hour, and 10^{-3} M, for the third hour. Drug-modulated cataplexy was measured by two consecutive FECT trials during min 20–30 and min 50–60 of each 1-h drug concentration period. Control levels of cataplexy were tested under the same schedule in narcoleptic canines receiving no

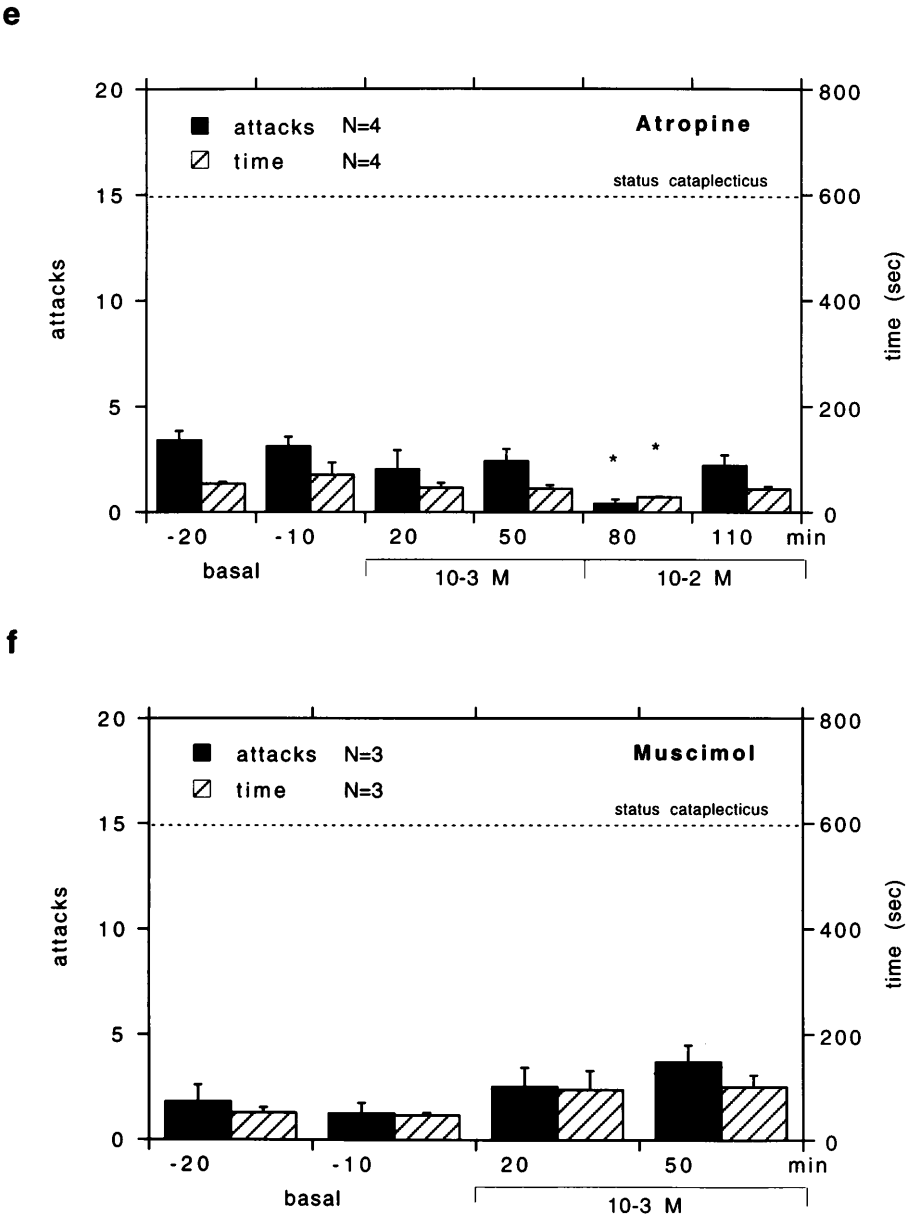


FIG. 1—*Continued*

drug infusion. In some tests carbachol (10^{-3} M) was coperfused with muscarinic antagonists atropine, gallamine, pirenzepine, and 4-DAMP (all antagonists at 10^{-3} M) for 60 min and cataplexy was measured by two consecutive FECT trials during min 20–30 and min 50–60 of drug perfusion. The animals were awake by both behavioral and EEG criteria at the start of all FECT trials.

DRUGS

Carbamylcholine HCl (carbachol) (cholinergic agonist), nicotine (nicotinic agonist), atropine sulfate (muscarinic antagonist) (Calbiochem, San Diego, CA), oxotremorine M (M2 muscarinic agonist), McN-A-343 (M1 muscarinic agonist), pirenzepine HCl (M1 muscarinic antagonist), gallamine triethiodide (M2 muscarinic antagonist), muscimol (GABA A receptor agonist), NMDA (NMDA receptor glutamatergic agonist), kainic acid (kainate/AMPA receptor glutamatergic agonist), and 4-DAMP methiodide (M1/M3 muscarinic antagonist) (Research Biochemicals Incorporated, Natick, MA) were dissolved directly into artificial CSF and tested for pH before local administration.

STATISTICS

Baseline levels of acetylcholine were analyzed with one-way ANOVA using the raw data. Due to interindividual and interday variability, changes in acetylcho-

line levels after drug (TTX or CSF no Ca^{2+}) or behavioral stimulation acetylcholine levels were expressed as the percentage of a control value, which was defined as the final baseline perfusion sample prior to treatment. Drug and behavioral treatment effects were analyzed within subject group using one-way ANOVA followed by *post hoc* Fisher PLSD tests. For comparison between drug treatment groups or behavioral treatment groups a two-way repeated ANOVA was used. All cataplexy results are presented as mean ± SEM. FECT scores for cataplectic attacks and elapsed time during control and drug treatments were analyzed within group using one-way ANOVA followed by *post hoc* Fisher PLSD tests. For comparison between drug treatment groups, as well as between narcoleptic and control groups, a two-way repeated ANOVA was used.

RESULTS

Basal FECT-induced cataplectic attacks ranged from partial, in which the animal could remain upright though a clear loss of muscle tone was indicated by the drop in EMG signal, to complete, in which the animal would go down on all four limbs and remain atonic for up to 2 min. During cataplexy the EEG signal remained desynchronized and the EOG was mostly quiet (for illustration see 37). While some variability in the levels of cataplexy existed, between different animals and within individuals between different experiment days (particularly when separated by 1 month or greater), the FECT scores were relatively stable on any given day over a 4-h control period with no drug treatment (Table 1), averaging between two and three cataplectic attacks per FECT ($F(7,34) = 0.605$, not significant (ns)) and elapsing over a time of 40 to 60 s ($F(7,34) = 0.904$, ns).

The effects of carbachol, oxotremorine, McN-A-343, nicotine, and atropine perfusion in the BF on cataplexy in the narcoleptic canines are shown in Fig. 1a and 1c–f, Bilateral carbachol and oxotremorine perfusion (10^{-5}–10^{-3} M) produced a dose-related increase in FECT-induced cataplexy, which was evident in both the number of attacks (carbachol, $F(7,34) = 7.443$, $P < 0.01$; oxotremorine, $F(7,27) = 4.378$, $P < 0.01$) and the elapsed FECT time (carbachol, $F(7,34) = 10.711$, $P < 0.01$; oxotremorine, $F(7,27) = 13.433$, $P < 0.01$) (Figs. 1a and 1b). Carbachol- and oxotremorine-stimulated cataplectic attacks were characterized by significant periods of muscle atonia accompanied by low amplitude, generally desynchronized EEG, and little EOG activity similar to baseline cataplexy. At the highest concentration (10^{-3} M) two of five animals receiving carbachol and two of four animals receiving oxotremorine became completely atonic after the full hour of perfusion, which was defined as *status cataplecticus*. These properties were similar to those which occurred after local injections of the same drugs into the BF of narcoleptic canines (see 37). Bilateral perfusion with high concentrations of McN-A-343 (10^{-4}–10^{-3} M) or nicotine (10^{-4}–10^{-3} M) perfusion did not have any effects on cataplexy, either on the number of cataplectic attacks (McN-A-343, $F(5,19) = 0.123$, ns; nicotine, $F(5,19) = 1.208$, ns) or elapsed time (McN-A-343, $F(5,19) = 0.208$, ns; nicotine, $F(5,19) = 0.829$, ns), and did not produce any notable change in basal muscle tone in all animals tested (Figs. 1c and 1d). Bilateral perfusion with atropine (10^{-3}–10^{-2} M) produced a mild reduction in the number of cataplectic attacks ($F(5,19) = 4.974$, $P < 0.01$) and elapsed time ($F(5,19) = 3.350$, $P < 0.05$) that was evident at the highest concentration (10^{-2} M) (Fig. 1e). Bilateral perfusion with the excitatory amino acids NMDA (10^{-4}–10^{-3} M) or kainic acid (10^{-5}–10^{-4} M) had no significant effects on cataplexy, evident in the number of attacks (NMDA, $F(5,19) = 1.509$, ns; kainic acid, ($F(5,19) = 0.905$, ns) (data not shown). Kainic acid was not perfused at 10^{-3} M in order to avoid possible local lesion effects. It was noted, however, that four of four animals displayed an increase in active wakefulness during perfusion with the highest concentration of each respective excitatory amino acid and were reluctant to perform the FECT protocol. Bilateral perfusion with the inhibitory GABAergic agonist muscimol (10^{-3} M) also had no significant effects on cataplexy, evident in the number of attacks ($F(5,14) = 2.588$, ns) and elapsed time ($F(5,14) = 2.389$, ns), though a trend toward more attacks ($P = 0.098$) and a longer elapsed time ($P = 0.116$) was noted (Fig. 1f).

The control canines were tested with the higher concentrations of carbachol and oxotremorine (10^{-4}–10^{-3} M) (data not shown) since only these levels produced any appreciable enhancement in cataplexy in narcoleptics. Bilateral perfusion with carbachol (10^{-4}–10^{-3} M) did not induce any cataplectic attacks though complete muscle atonia, *status cataplecticus*, was observed in one of three control animals after 1 full h of perfusion at the highest concentration (10^{-3} M). In addition, a significant increase in elapsed time to complete an FECT was found ($F(5,14) = 2.841$, $P < 0.05$). Bilateral perfusion with oxotremorine (10^{-4}–10^{-3} M) did not induce any cataplectic attacks nor was muscle atonia observed in any of the control animals. A significant increase in elapsed time to complete an FECT was found ($F(5,14) = 3.273$, $P < 0.05$). Other than the one animal which experienced *status cataplecticus* after carbachol perfusion no significant decrease in basal muscle tone, as noted by behavioral observation and EMG analysis, was noted with either carbachol or oxotremorine perfusion in the BF of the control canines.

The increase in cataplexy during local perfusion with carbachol in the BF of narcoleptic canines was com-

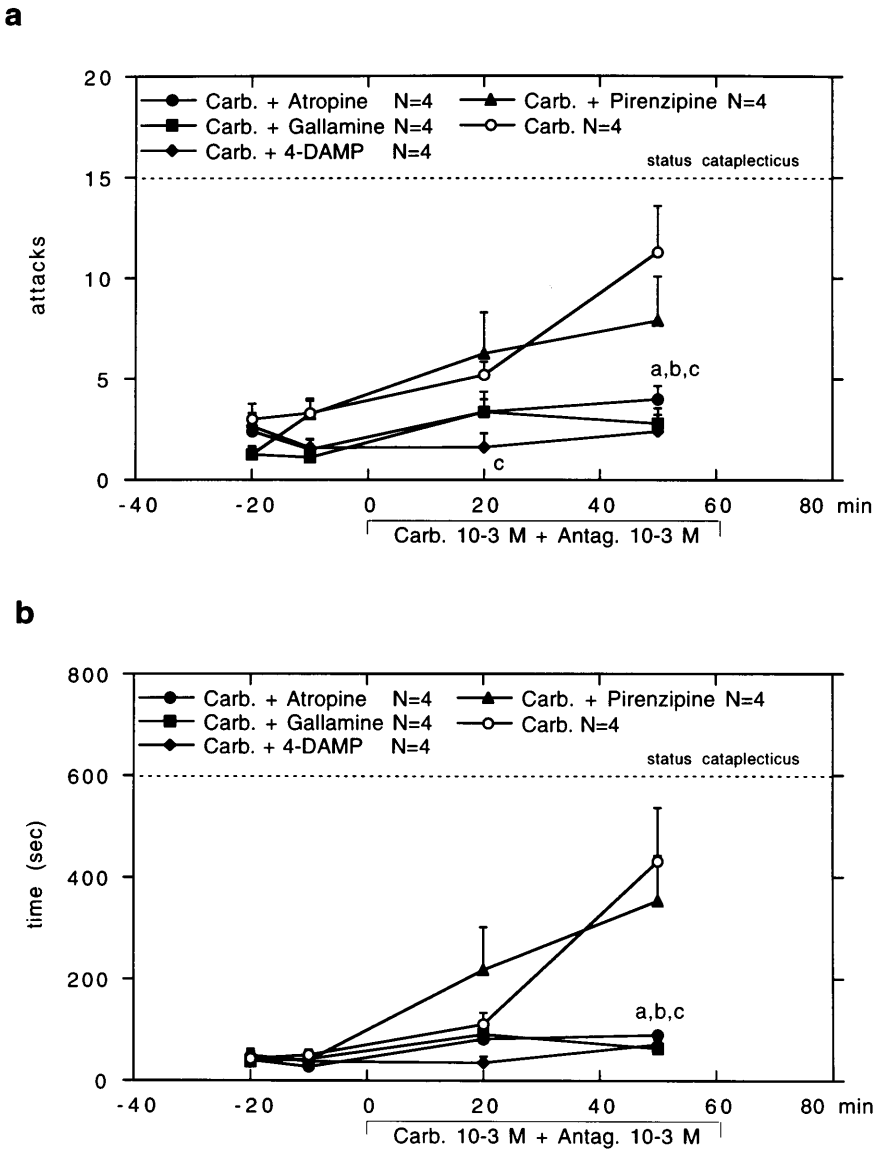


FIG. 2. Effect of perfusion in the basal forebrain of narcoleptic canines with bilateral carbachol (Carb.) (10^{-3} M) for 60 min alone or combined with an equal concentration (10^{-3} M) of atropine, gallamine, 4-DAMP, or pirenzepine (each at 10^{-4} M) on cataplexy. The mean number of cataplectic attacks (a) and elapsed time (b) for two food-elicited cataplexy tests per test period is shown. For the purpose of figure presentation, *status cataplecticus* (carbachol atonia) was arbitrarily designated as 15 attacks elapsed over 600 s. Each carbachol plus antagonist time point was compared with the corresponding carbachol alone time point using a Neuman-Kewls *post hoc* test; a, b, and c indicate $P < 0.05$ satisfactory for comparison of control with the atropine, gallamine, and 4-DAMP groups, respectively.

pletely blocked when coadministered with atropine, gallamine, or 4-DAMP, but not pirenzepine (Figs. 2a and 2b). Bilateral carbachol (10^{-3} M) alone produced a strong increase in cataplexy which reached *status cataplecticus* in one of four animals tested with this regimen. This increase in cataplexy was reversed when non-M1 receptor muscarinic antagonists were coadministered with carbachol at the same concentration (10^{-3} M), evident in the reduction of the number of attacks (atropine, $F(3,18) = 6.245$, $P < 0.01$; gallamine, $F(3,18) = 7.483$, $df = 1$, $P < 0.01$; 4-DAMP, $F(3,18) = 8.866$, $P < 0.01$) and elapsed time (atropine, $F(3,18) = 9.001$, $P < 0.01$; gallamine, $F(3,18) = 11.684$, $df = 1$, $P < 0.01$; 4-DAMP, $F(3,18) = 9.551$, $P < 0.01$). Coadministration of the M1 receptor muscarinic antagonist pirenzepine (10^{-3} M) with carbachol (10^{-3} M) did not significantly reduce carbachol-enhanced cataplexy, evident both in number of attacks ($F(3,18) = 0.674$, ns) and in elapsed time ($F(3,18) = 0.746$, ns).

From the five narcoleptic canines studied in the BF, perfusion samples were collected from a total of 12 bilateral *in vivo* microdialysis sessions (three animals tested on 2 consecutive days, two animals tested on 3 consecutive days, 24 total observations). From the

three control canines studied in the BF, perfusion samples were obtained from six bilateral *in vivo* microdialysis sessions (three animals tested on 2 consecutive days, 12 total observations). Extracellular acetylcholine levels in both narcoleptic and control animals were essentially stable after 60 min of perfusion and baseline levels were obtained from the 80- to 90-min sample (the last 10-min perfusion period prior to FECT stimulation). Baseline acetylcholine levels in the narcoleptic and control BF (narcoleptic, 0.665 ± 0.281 pmol/10 min, $n = 24$; control, 0.467 ± 0.104 pmol/10 min, $n = 12$) were not significantly different ($t(34) = 1.794$, ns) though a trend toward higher levels in narcoleptic animals was noted ($P = 0.086$). In control studies on the acetylcholine levels in the BF of both narcoleptic and control canines (Fig. 3) switching the perfusion medium from CSF to CSF without Ca^{2+} produced a moderate reduction in acetylcholine levels ($F(6,59) = 3.758$, $P < 0.01$) which reached a maximal decrease (36 ± 12%) after 20 min. Introducing TTX (10^{-5} M) into the perfusion medium also produced a significant drop in acetylcholine levels ($F(6,47) = 15.471$, $P < 0.01$) which reached a maximal decrease (67 ± 9%) after 30 min. The effects of FECT stimulation in narcoleptic and control canines on acetylcholine levels in the BF are shown in Fig. 4a. During FECT-induced cataplexy in narcoleptics acetylcholine levels increased approximately 27%, which was significant by univariate analysis ($F(6,143) = 2.713$, $P < 0.05$). During FECT stimulation in controls acetylcholine levels increased approximately 40%, which was also significant by univariate analysis ($F(6,71) = 3.202$, $P < 0.01$). Repeated measures analysis comparing narcoleptic and control acetylcholine levels during FECT stimulation revealed no significant difference between groups ($F(6,204) = 0.807$, ns). Behavioral tests in narcoleptic canines on acetylcholine levels in the BF are shown in Fig. 4b. During locomotor stimulation without cataplexy ($F(6,83) = 0.654$, ns) and feeding without cataplexy ($F(6,59) = 0.251$, ns) there was no change in acetylcholine levels. Repeated measures analysis comparing each behavioral control test with FECT-stimulated cataplexy in narcoleptic canines did not reveal any significant differences (FECT vs motor, $F(6,216) = 1.685$, ns; FECT vs. feeding, $F(6,192) = 0.823$, ns).

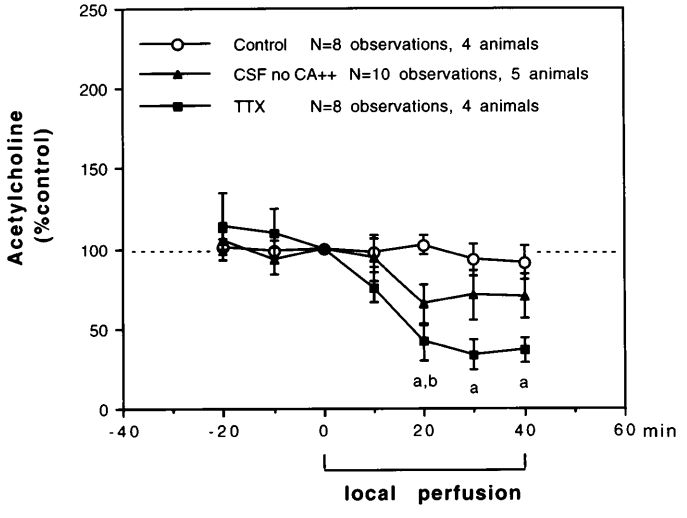


FIG. 3. Control tests on the extracellular levels of acetylcholine in the BF of narcoleptic and control canines measured by *in vivo* microdialysis. The effects of local perfusion with tetrodotoxine (TTX) (10^{-5} M) or artificial cerebrospinal fluid (CSF) without Ca^{2+} are shown. Following a 60-min baseline perfusion period with normal CSF the perfusion medium was switched to one containing TTX or no Ca^{2+} for 40 min and samples were collected every 10 min. The control group was perfused with normal CSF for the entire testing period. The control group represents four bilateral sessions in four animals (two control, two narcoleptic), the CSF no Ca^{2+} group represent five bilateral sessions in five animals (two control, three narcoleptic) and the TTX group represents four bilateral sessions from four animals (two control, two narcoleptic). Experimental and control groups were compared at each time point using Neuman-Kewls *post hoc* tests; a and b indicate $P < 0.05$ satisfactory for comparison of control with the TTX and CSF no Ca^{2+} groups, respectively.

Histological analysis revealed that all the microdialysis probes were located within the BF. The tracts produced by injection cannulae at the same stereotaxic coordinates as the microdialysis probes were seen near the anterior preoptic area and within the magnocellular region of the BF. Accordingly, the tips of the microdialysis membranes were located near the ventral border of the BF and the membranes extended 5 mm dorsally to dorsal portions of the BF close to the lateral ventricle. The positions of the cannula tracts varied along the rostrocaudal extent of the BF by approximately 4.0 mm and along the mediolateral extent of the BF by approximately 2.0 mm. Comparison of the bilateral implantations showed that they were close (within 0.5 mm) to the same transectional plane. In Figs. 5A–5C typical tracts after cannulation of the left hemisphere BF with dummy cannulae at the same coordinates as the microdialysis probes are shown (in Figs. 5A and 5B the coronal section with the ventral most 3–4 mm of each tract are shown (black arrows)). In Fig. 5A (approximately AP, 25 according to 30) the tract represents the most caudal implantation site found in the BF close to the level of the preoptic area, in Fig. 5B (at approximately AP, 29 according to 30) the tract represents a medial implantation site in the vertical limb of the diagonal band of Broca and in Fig. 5C (approximately AP, 27 according to 30) evidence of old tracts from previous implantations (small arrows) as well as a more dorsal portion (4–5 mm) of a dummy cannula implantation tract (large arrows) in the horizontal limb of the diagonal band of Broca are shown. All of narcoleptic canines presented in Figs. 5A–5C showed significant carbachol enhanced cataplexy. For further analysis of BF implantation sites in the same animals see Nishino and colleagues (37).

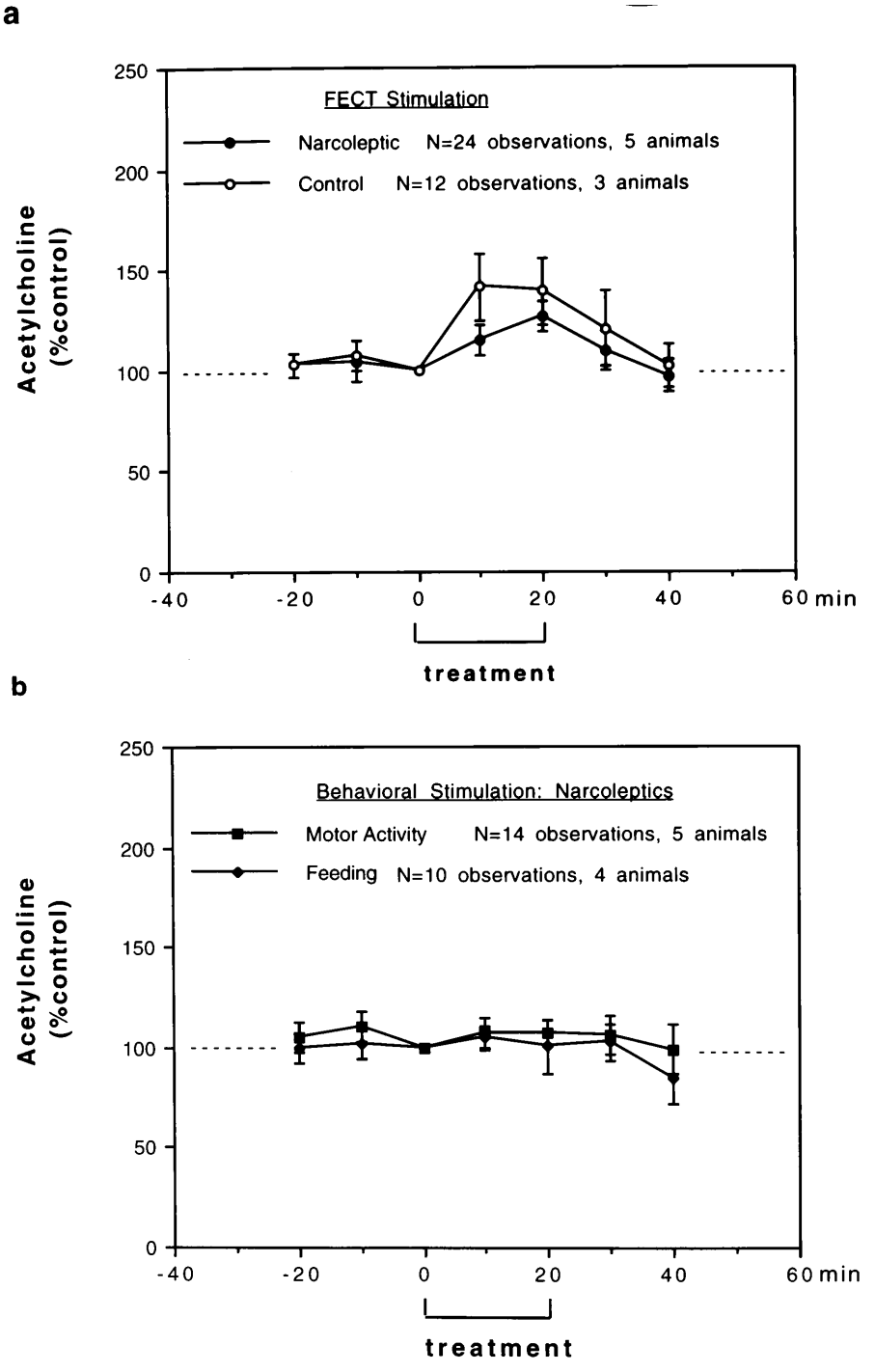


FIG. 4. Effects of FECT and control behavioral tests on the extracellular levels of acetylcholine in the basal forebrain of narcoleptic and control canines measured by *in vivo* microdialysis. In (a) FECT stimulation in narcoleptic canines represents three bilateral sessions in two animals and two bilateral sessions in three animals and in control animals represents two bilateral sessions in three animals. In (b) behavioral stimulation studies were studied on narcoleptic canines only. Motor activity tests represent two bilateral sessions in two animals and one bilateral session in three animals and feeding tests represent two bilateral sessions in one animal and one bilateral session in three animals. During FECT treatment each animal performed two FECT trials per 10 min, during motor activity treatment each animal performed two motor activity trials without having cataplexy per 10 min and during feeding treatment each animal consumed 10 small bites of wet dog food without having cataplexy per 10 min.

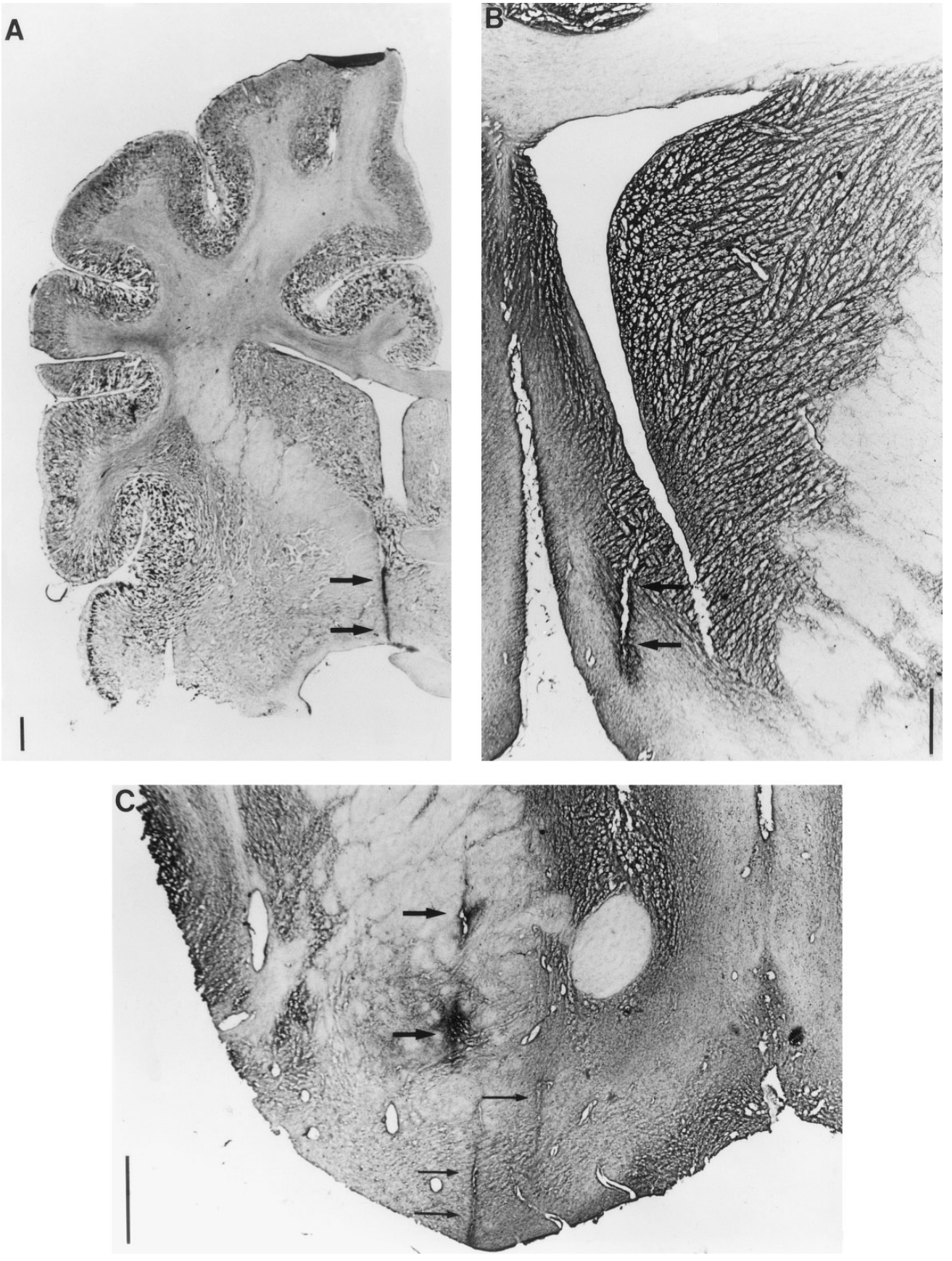
DISCUSSION

These results show that cataplexy in the narcoleptic canine may be differentially modulated by selective stimulation or blockade of M2, and possibly M3, muscarinic receptors in the BF. These findings are similar to earlier studies on the cholinergic regulation of cataplexy in the PRF (see 43), indicating that cataplexy in narcoleptic canines is regulated by similar cholinergic mechanisms in the PRF and BF. Acetylcholine release in the BF was enhanced during FECT-induced cataplexy in narcoleptic animals, similar to the PRF (see 42); however, acetylcholine release was also enhanced during FECT stimulation without cataplexy in control animals. This increase in BF acetylcholine release was specific to the FECT behavioral paradigm, as no change in acetylcholine release was observed when the basic components of an FECT (motor activity and feeding) were tested separately.

The role of the BF in the cholinergic regulation of cataplexy in narcoleptic canines has been studied previously in our laboratory, in which local microinjections of carbachol-stimulated cataplexy in narcoleptic canines at moderate doses (10 nmol/1 µl, unilaterally) and muscle atonia in control canines at high doses (50 nmol/l µl, bilaterally) (37). It was suggested that this cholinergic hypersensitivity in the BF of narcoleptic animals could represent a critical pathophysiology of the disorder. The results from the present study examining locally perfused carbachol, via *in vivo* microdialysis probes, were similar to this previous study in terms of both enhanced cataplexy in narcoleptics and evidence for carbachol-induced atonia in control animals (in one of three of controls tested). This indicates that the results obtained by local microdialysis perfusion are comparable with those obtained by local microinjection. The only notable difference was that the level of cataplexy obtained with bilateral carbachol perfusion was slightly lower than that obtained by local injections (in terms of amount of *status cataplecticus* observed). This is likely due to differences in drug delivery and pharmacokinetics between local injection and *in vivo* microdialysis experimental protocols. However, because microdialysis probes can be used for numerous drug delivery tests (without the need for reimplantation) over 3–5 days after implantation this provides an advantage over local drug injections for the purpose of pharmacological characterization of the effect. Therefore, the cholinergic receptors involved in mediating the cataplexy-enhancing effects of carbachol, as well as the potential role of noncholinergic BF neurotransmitters in regulating cataplexy, were examined using bilaterally implanted microdialysis probes.

In addition to carbachol, cholinergic agonists selective for both muscarinic and nicotinic receptors were tested in the BF of narcoleptic canines. Bilateral oxotremorine perfusion produced an increase in cataplexy in the narcoleptic canines, in a similar dose range as carbachol, but did not produce muscle atonia in control canines. The strong effects on cataplexy are consistent with autoradiographic studies showing a high level of [^{3}H]oxotremorine binding sites in the BF of rats (49, 56). Bilateral McN-A-343 and nicotine perfusion did not have any effects on cataplexy in the narcoleptic canines which is consistent with the lack of [^{3}H]pirenzepine and [^{3}H]nicotine binding in the rat (12, 49, 56) and monkey (10) BF. Based on the putative selectivity of these compounds for cholinergic receptor subtypes, McN-A-343 at M1 muscarinic, oxotremorine at M2 muscarinic, and nicotine at nicotinic, these results indicate that the cholinergic stimulation of cataplexy in the BF is selectively mediated by M2 muscarinic receptors. This is consistent with studies showing high levels of M2 receptors (29) and mRNA for M2, M3, M4, and M5, but not M1, receptors (57) in the rat BF. However, the specificity of McN-A-343 and oxotremorine is based on the relative degree of binding sensitivity to these respective muscarinic receptor subtypes (7, 17, 58), and it has been shown that McN-A-343 can also bind at M2 receptors at high concentrations (58) and that oxotremorine can bind at both M4 and M2 receptors with similar affinity in the rat brain (56). In light of these receptor selectivity studies and the inability of McN-A-343 and nicotine to stimulate cataplexy, the results from the cholinergic agonist studies are most conclusive in ruling out the role of M1 muscarinic and nicotinic receptors in the cholinergic stimulation of cataplexy in the PRF. The finding that oxotremorine stimulates cataplexy suggests, but does not prove, that cholinergic stimulation of cataplexy in the BF is mediated by M2 muscarinic receptors alone.

In the muscarinic antagonist studies the increase in cataplexy produced by local carbachol perfusion in the BF was blocked when coadministered with atropine, gallamine, or 4-DAMP. In contrast, pirenzepine did not affect the carbachol-induced increase in cataplexy. The ability of atropine and gallamine, but not pirenzepine, to block the effects of carbachol is consistent with our previous study in the pontine reticular formation in which both drugs could rapidly reverse carbachol-stimulated cataplexy (43) and indicate that muscarinic receptors mediate the effect of carbachol in the BF. The inability of pirenzepine, a well-known M1 muscarinic receptor-selective antagonist (18, 58), to reduce carbachol stimulated cataplexy shows that M1 muscarinic receptors are not involved in mediating the effects of carbachol. This finding is not surprising considering the paucity of M1 receptors shown in the BF of rats (9, 28) and the lack of effect of McN-A-343 perfusion in the BF on cataplexy in the narcoleptic canines. The ability of gallamine, a muscarinic antagonist with high M2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and low M1 muscarinic receptor selectivity (31, 34) to block the effects of carbachol suggests that the carbachol-induced cataplexy in the BF is mediated by M2 muscarinic receptors and is consistent with the high level of M2 muscarinic receptors shown in the BF in the diagonal band of Broca and magnocellular area (9, 28, 29, 56) and the cataplexy-stimulatory effects of oxotremorine in the BF. Interestingly, 4-DAMP was also able to block the effects of carbachol in a manner which was much more potent than the moderate reduction in carbachol-induced cataplexy seen in the pontine reticular formation (43). 4-DAMP has been characterized as a muscarinic antagonist with high affinity for both the M1 and the M3 and low affinity for the M2 muscarinic receptor subtypes (33, 36). Thus, the strong effects of 4-DAMP may be attributed to antagonism of M3 muscarinic receptors in the BF, which have been demonstrated at levels just slightly less than M2 muscarinic receptors in the diagonal band of Broca and the magnocellular area of the rat BF (9, 57). This would suggest that carbachol stimulation of cataplexy could be mediated by M3, as well as M2, muscarinic receptors in the BF.

Cholinergic neurons represent only a minority of cells in the BF and give rise predominantly to ascending projections whereas a large population of noncholinergic and GABAergic neurons in the region give rise to descending projections. The role of inhibitory and excitatory amino acid neurotransmitters in BF regulation of sleep has been studied more recently, and evidence supporting the involvement of both GABA and glutamate has been reported. In BF microinjection studies, NMDA antagonists blocked the increase in cortical acetylcholine release and AMPA antagonists blocked cortical activation (both of which are suggested to be mediated by a BF cholinergic projection to cortex (32, 45)), following stimulation of the pedunculopontine nucleus in rats (40). This suggests that BF glutamate may play a facilitatory role in arousal and is consistent with the recent report that glutamate release in the preoptic area is highest during wakefulness (3). In studies on GABA function in the BF muscimol injections, or localized lesions, at the level of the diagonal band of Broca produced insomnia while more medially located muscimol injections into the anterior hypothalamus produced sleep (see 47, 51). In the present study glutamatergic stimulation with local NMDA and kainic acid perfusion in the BF produced an increase in active wakefulness while no significant change in FECT-induced cataplexy was observed (though animals became more reluctant to perform an FECT). This might suggest that the arousal and cataplexy regulatory functions in the BF of narcoleptic canines are not related. GABAergic stimulation with local muscimol perfusion in the BF produced a mild increase in cataplexy which, though not statistically significant, indicates that neuronal inhibition in this structure could enhance cataplexy in narcoleptic canines. This observation can only be considered preliminary since only three narcoleptic animals were studied with muscimol in the BF (due to research animal population constraints). Similar findings were also made in the pontine reticular formation, in which NMDA and kainic acid had no effect while muscimol produced a moderate increase in cataplexy (43). In this previous study it was suggested that the effects of muscimol mimicked those of M2 receptor agonist (M2 receptor activation, similar to muscimol, reduces neuronal excitability in the parabrachial nucleus (13)). Consistent with this, unit recording studies in the rat anterior preoptic area have shown a reduced firing rate in a majority of the neurons following the administration of carbachol (8) and the application of muscarine to guinea pig slices of the BF hyperpolarized cholinergic neurons (25). This would suggest that carbachol-induced cataplexy in the BF of narcoleptic canines involves M2 receptor-mediated neuronal inhibition.

In a recent single unit recording study in the BF of narcoleptic canines we found evidence for state-dependent neurons, including wake, deep sleep, and REM sleep/cataplexy active, all of which were modulated by cholinergic drugs (38). Interestingly, the REM sleep/cataplexy active cells were excited by systemic administration of an indirect cholinergic agonist, physostigmine (38). Though these findings should be considered preliminary, they suggest that carbachol-induced cataplexy in the BF of narcoleptic canines may instead be mediated by neuronal excitation. Consistent with this suggestion, others have shown that BF-corticocholinergic neurons are excited by iontophoretically applied carbachol (27). Therefore, we suggest that caution should be taken when interpreting the cholinergic mechanisms, inhibitory or excitatory, that are involved in mediating BF-stimulated cataplexy.

In the present study extracellular BF acetylcholine levels were reduced when CSF without Ca^{2+} or TTX

FIG. 5. Photomicrograph showing the positions of the microdialysis probes in the basal forebrain (BF). The BF sections were stained with cresyl violet after bilateral implantation with dummy cannulae at coordinates: AP 29.0, ML 5.0, and DV 46.0 (from dura) used for the microdialysis probes. The black arrows point to the injection cannulae tracts on the left side of the BF near the anterior preoptic area (A) and in the magnocellular region including the vertical (B) and horizontal (C) limbs of the diagonal band of Broca. In (A) and (B) the most ventral aspects of the implantation tracts on the left, extending approximately 3–4 mm along the dorsoventral axis, are indicated. In (C) the most ventral aspects of older tracts from previous implantations (small arrows) as well as dorsal portions of a dummy cannulae tract (large arrows), both extending approximately 4–5 mm along the dorsoventral axis, are shown. Bars, 2 mm.

was perfused through the microdialysis probes, indicative of voltage-dependent, neuronally released neurotransmitter. While the neuroanatomical origins of these acetylcholine levels were not directly investigated in this study other reports on the neuroanatomy of cholinergic projections in both the cat and the rat indicate that the ascending cholinergic projections of pontine tegmentum provide only a minor source of BF acetylcholine (23, 22). Thus, it is possible that BF acetylcholine levels in the present study represent neurotransmitter release from either local axon collaterals of BF cholinergic projections or axon terminals of the ascending pontine tegmental projections. Comparison of the basal levels of acetylcholine did not reveal any statistically significant difference between narcoleptic and control canines though indications of higher levels in the narcoleptic animals were found. Assuming that basal acetylcholine release was in fact upregulated in the BF of narcoleptics this would be consistent with our earlier hypothesis that an upregulated cholinergic system in the BF is involved in the pathophysiology of canine narcolepsy (see 37). However, the data on the basal levels of acetylcholine should be taken with a note of caution because of the limited number of control animals tested.

Numerous studies on neurotransmitter release in various forebrain structures during the sleep–wakefulness cycle have demonstrated significant wake and REM sleep-related increases in the extracellular levels of acetylcholine. This has been reported in the rat hippocampus (24), cat striatum (14), and cat cortex (21), all of which showed higher levels of acetylcholine release during REM sleep. While there are no studies which monitored acetylcholine release within the BF (to our knowledge) all of these above brain regions are known to receive cholinergic innervation from the BF, and alterations in acetylcholine release in these terminal regions is suggested to reflect the activity of these BF cholinergic neurons (39, 46, and see 45). Thus, it has been suggested that the BF cholinergic projection neurons of the magnocellular area and the diagonal band of Broca are activated during REM sleep and wakefulness (56). In addition to sleep-related changes in acetylcholine release other studies in the cortex and hippocampus have shown enhanced acetylcholine release during periods of behaviorally relevant stimuli presentation (1) and during the anticipatory stage of reward and food consumption (20). These findings suggest that BF cholinergic projection neurons may also play a role in mediating attentional and trained reward-based behavior. In the present study acetylcholine release in the BF of both narcoleptics and control animals was enhanced during the FECT procedure, but not during control tests for the locomotor and feeding components of an FECT. Because the FECT is a learned procedure in which emotions are elicited and reward (food) is obtained (see 5), it can be argued that this behavior resembles the trained, rewarded group in Inglis and colleagues' (20) study on cortical and hippocampal acetylcholine release in rats. Similar to their control studies we found that simple locomotor activity or feeding, taken out of context, did not produce an increase in acetylcholine release. Therefore, we propose that the increase in BF acetylcholine release, in both narcoleptic and control canines, was due to the conditioned, emotionally-relevant response to FECT stimulation. Furthermore, because the BF acetylcholine levels might be derived from axon collaterals of ascending projections it is possible that FECT stimulation also results in enhanced acetylcholine release in terminal regions such as the hippocampus and cortex. Such an explanation could provide a basis for EEG cortical activation during cataplexy in narcoleptics.

An alternative interpretation of the FECT-related increase in acetylcholine release in the BF of narcoleptic and control canines would be that it represents the activation of the ascending, cholinergic, pontine tegmental projection. This would be consistent with our previous report showing that acetylcholine release in the pontine reticular formation is enhanced during cataplexy (42). However, in this study FECT-induced acetylcholine release was not seen in the control canines. Furthermore, neuroanatomical studies suggest that the pontine tegmental projection provides only a minor source of BF cholinergic innervation. Thus, the increase in acetylcholine release in the BF of narcoleptic and control canines following FECT stimulation is less likely to be mediated via the ascending, cholinergic, pontine tegmental projection.

The findings in the present study provide strong evidence that M2 and M3 muscarinic receptors in the BF play an important role in the regulation of cataplexy in the narcoleptic canine. Previous studies on the pontine reticular formation have shown that M2 muscarinic receptors in this brain region are also important in the regulation of cataplexy (41, 43) suggesting that the cholinergic mechanisms regulating cataplexy in narcoleptic canines are similar in both brain regions. One important distinction should be made, however, since acetylcholine release in the BF was associated with the FECT procedure in both animal groups while acetylcholine release in the pontine reticular formation was associated with FECT-induced cataplexy in narcoleptics only. Previously we suggested that cholinergic-mediated cataplexy in the pontine reticular formation of narcoleptic canines represents selective activation of REM sleep muscle atonia mechanisms (43). In contrast, cholinergic-mediated cataplexy in the BF of narcoleptic canines might represent the emotion-activated mechanisms which trigger cataplexy via hypersensitive muscarinic receptors. Furthermore, while the ascending BF cholinergic projections likely mediate cortical EEG

activation a descending projection from the BF, most likely noncholinergic, might mediate/trigger the muscle atonia seen during cataplexy. Indeed, an association between cholinergic BF and pontine REM sleep regulation has been previously shown (4). The ventral tegmental area, which we have recently shown is a site for dopamine (D2 and D3 receptor)-stimulated cataplexy (44), is also a projection target for descending BF projections (52) and is also reciprocally innervated by the pontine tegmental area (15, 50, 59). Thus, with its limbic system interconnections this nucleus could serve as a relay between brain regions involved in mediating emotion-related activity and muscle atonia. Further studies on the relationship between the BF and midbrain structures such as the ventral and pontine tegmental areas are needed to address these issues.

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