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Cholinergic Regulation of Cataplexy in Canine Narcolepsy in the Pontine Reticular Formation is Mediated by M2 Muscarinic Receptors

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Summary: Both rapid eye movement sleep and cataplexy in the narcoleptic canine have been shown to increase after both systemic and local administration of cholinergic agonists in the pontine reticular formation. Furthermore, binding studies indicate an increase in the number of M2 muscarinic receptors in the pontine reticular formation of narcoleptic canines. In the present study we have investigated the receptor subtypes involved in mediating the cholinergic stimulation of cataplexy, as defined by brief periods of hypotonia induced by emotions, within the pontine reticular formation of narcoleptic canines. Specific cholinergic and monoaminergic agonists and antagonists, and excitatory or inhibitory amino-acid neurotransmitter receptor agonists, were perfused through microdialysis probes implanted bilaterally in the pontine reticular formation of narcoleptic canines, and cataplexy was monitored using the Food-Elicited Cataplexy Test and recordings of electroencephalogram, electrooculogram and electromyogram. In narcoleptic canines, bilateral perfusion with oxotremorine (M2 muscarinic) (10^{-5}–10^{-3} M) in the pontine reticular formation produced a dose-dependent increase in cataplexy, which reached complete muscle atonia (status cataplecticus) during the highest concentration. In control canines bilateral perfusion with oxotremorine (10^{-5}–10^{-3} M) did not produce any cataplectic attacks, but did produce muscle atonia after the highest concentration. Bilateral perfusion with either McN-A-343 (M1 muscarinic) or nicotine (both 10^{-5}–10^{-3} M) did not have any effect on cataplexy in either narcoleptic or control canines. The increase in cataplexy in narcoleptic canines produced by local perfusion with carbachol (10^{-4} M) followed by equimolar perfusion with a muscarinic antagonist was rapidly reversed by atropine (muscarinic) and gallamine (M2 muscarinic), partially reversed by 4-DAMP (M3/M1 muscarinic) and completely unaffected by pirenzepine (M1 muscarinic). Bilateral perfusion with excitatory, glutamatergic receptor agonists *N*-methyl-D-aspartate, AMPA (both at 10^{-4}–10^{-3} M) and kainic acid (10^{-5}–10^{-4} M) did not have any effect on cataplexy, whereas bilateral perfusion with the inhibitory GABAergic receptor agonist muscimol (10^{-4}–10^{-3} M) produced a moderate increase in cataplexy in the narcoleptic canines. Bilateral perfusion with numerous monoaminergic compounds, BHT-920 (alpha-2 agonist), yohimbine (alpha-2 antagonist), propranolol (beta antagonist) and prazosin (alpha-1 antagonist), did not have any effect on cataplexy. These findings demonstrate that cholinergic regulation of cataplexy in the narcoleptic canine at the level of the pontine reticular formation is mediated by M2, and possibly M3, muscarinic receptors. The effects of muscimol indicate that the stimulation of cataplexy might be elicited by local neuronal inhibition. **Key Words:** Narcolepsy—Cataplexy—Oxotremorine—Muscimol.

It is well documented that pontine cholinergic systems play an important role in the regulation of rapid eye movement (REM) sleep and muscle tone (for review and references see 1). Considerable evidence has shown that REM sleep and muscle atonia can be elicited by local injection of cholinergic agonists, such as carbachol (2,3), oxotremorine (4,5), and bethanecol (6), in the pontine reticular formation (PRF). Furthermore, cholinergic antagonists such as atropine (7,8) and gallamine (9) can reduce REM sleep and block cholinergically stimulated REM sleep after local injection in the PRF. These findings suggest that muscarinic receptors are involved in mediating the cholinergic regulation of REM sleep within the PRF.

Studies with narcoleptic canines have indicated that pontine cholinergic mechanisms may also be involved in the regulation of cataplexy. Thus, muscarinic receptor binding is upregulated in the brainstem reticular formation (10,11), and local administration of car-

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bachol in the PRF produces an increase in cataplexy (12) in narcoleptic canines. These findings indicate that both REM sleep and cataplexy are regulated by similar cholinergic mechanisms in the brainstem reticular formation. Indeed, perhaps the most compelling evidence supporting this hypothesis are the findings that acetylcholine release in the dorsomedial pontine tegmentum and the PRF of cats is enhanced during REM sleep (13) and carbachol-induced REM sleep (14), respectively, and that acetylcholine release in the PRF of the narcoleptic canine is enhanced during cataplexy (15).

Muscarinic cholinergic receptors are pharmacologically classified into four major subtypes, M1, M2, M3 and M4, based upon their relative specificity for cholinergic antagonists (16–18). These four muscarinic subtypes have been characterized with antagonists, which have only relatively selective affinity for their respective receptor subtypes. However, they have also been identified, along with a fifth M5 receptor, by molecular cloning. In studies using in situ hybridization techniques it has been shown that the rat pons contains mainly M2 receptor mRNA (19,20) and low levels of M3 receptor mRNA (19). Consistent with these findings, competition binding studies have shown that M2 and M3 antagonists bind most potently in both the rat and cat pons (21–24), and immunoprecipitation studies with muscarinic receptor subtype antibodies have shown that M2 receptors are greater than 10-fold more abundant than all other muscarinic receptor subtypes in the rat brainstem (25).

Functional studies have shown that REM sleep is most potently modulated by M2 selective muscarinic compounds. Thus, REM sleep is reduced by the M2 muscarinic antagonist methoctramine after intracerebroventricular administration (26) and is enhanced by the M2 agonist oxotremorine after local injection in the PRF (5). In addition, coadministration of various M2 antagonists in the PRF or pontine tegmentum reduces the REM sleep enhancement produced by the local injection of carbachol (9,27,28). However, similar studies in the PRF have also reported that the M1 antagonist pirenzepine and nicotine (28,29), as well as the alpha-2 adrenergic agonist clonidine (30) and antagonist idazoxan (31), the beta adrenergic antagonist propranolol (32) and non-*N*-methyl-D-aspartic acid (NMDA) receptor agonists (in the subcoeruleus) (33) also have some REM sleep and muscle tone modulatory effects. Because of the similarity in pontine cholinergic mechanisms in REM sleep and cataplexy, these findings indicate the possibility that cataplexy in the narcoleptic canine may be modulated by a variety of cholinergic (most likely M2 muscarinic) and possibly noncholinergic compounds when administered in the PRF. Therefore, in the present study we have perfused several different muscarinic receptor-selective agonists and antagonists, as well as nicotine, into the PRF of narcoleptic canines in order to pharmacologically characterize the cholinergic stimulation of cataplexy within the PRF. Monoaminergic compounds and amino-acid neurotransmitter receptor agonists, which have been shown to modulate cataplexy when given intravenously to narcoleptic canines or to modulate REM sleep and muscle atonia when administered in the PRF of cats, were also tested in the PRF of narcoleptic canines. This was accomplished by perfusing specific concentrations of selected drugs through microdialysis probes implanted bilaterally into the PRF of narcoleptic canines.

MATERIALS AND METHODS

All studies were performed on adult Doberman pinschers, which included five narcoleptic (three males and two females), two control (both males) and one heterozygous narcoleptic (female) canines. All animals were between 1 and 5 years of age, weighed 20–25 kg and were bred at the Stanford University narcoleptic dog colony. Heterozygous narcoleptic canines contain one copy of canarc-1, the narcolepsy transmitting gene locus, but do not express the symptoms of narcolepsy (34,35). Because the heterozygous canine showed no differences from the control canines in the present study, it was included in the control group. Because of the great value of all implanted narcoleptic canines, most (seven of the eight used in this study) have been used in previous experiments (see references 12 and 15). If so, each animal was given a "wash-out" period of at least 10 days before further use. The animals were kept under a 12:12 hour 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 reference 12). Briefly, the animals were anesthetized using isofluorane (2% mixture in air administered via a trachial 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 separated by 2 mm and soldered to a nut stack and were lowered

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into position so that the ventral tips touched the surface of the cortical dura. The guide cannulae bundles were positioned over the PRF bilaterally [lateral (L): 3.2 and anterior (A): 3.0–5.0 from stereotaxic zero, according to 36], such that two guide cannulae were positioned over the rostrocaudal extent of the PRF, both at the same laterality. The recording electrodes, electrical plug and guide cannulae were cemented to the skull using dental acrylic, and the skull was sutured closed. During recovery from surgery all animals were under 24-hour surveillance at the Stanford University Department of Comparative Medicine Intensive Care Unit. Postsurgical treatment included analgesics for the first 12–48 hours 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 the microdialysis probes (70-mm shaft with 5-mm membrane, CMA/10, CMA/Microdialysis, Stockholm, Sweden) were lowered bilaterally into the PRF [L: 3.2, A: 3.0, ventral (V): 39.0 from stereotaxic zero, according to 36] and anchored in place while the animals were under isofluorane anesthesia. The left and right probes were always implanted at the same coordinate on the anterior–posterior axis. Each probe was tested for in vitro recovery of 10^{-7} M acetylcholine before implantation; the relative recovery found was 27 + 2%, n = 12. After the experiment was completed, the probes were removed and the animals returned to their cages. Histological verification of the probe placements was performed on six of the eight animals in this study (three narcoleptics, three controls), using 2% neutral red injected (1.0 μl) through the same guide cannulae and at the same depth coordinate as the dialysis probes.

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 published previously (12). The probes were perfused via a 3-m inlet line at 2.0 μl/minute 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. Cataplexy was measured using the Food-Elicited Cataplexy Test (FECT) (37), combined with recordings of EEG, EOG and EMG. 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. Cataplectic attacks were scored when the animal stopped forward motion and the hind quarters were lowered towards 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 were performed on days 1–5, beginning approximately 18 hours after microdialysis probe implantation, between the hours of circadian time (CT) 3 and CT 8. Each animal was connected to the dialysis inlet lines and perfused for 60 minutes with CSF before testing for cataplexy. At the end of this 60-minute control period, baseline levels of cataplexy were measured using four consecutive FECT trials performed over a period of 20 minutes. After completion of the four FECT trials, the perfusion medium was switched from CSF to CSF plus drug using a manual liquid switch (CMA/110, CMA Microdialysis). All of the following drugs were tested bilaterally at various concentrations in the perfusion medium: oxotremorine (10^{-5}–10^{-3} M), McN-A-343 (10^{-4}–10^{-3} M), carbachol (10^{-4} M), nicotine (10^{-4}–10^{-3} M), atropine 10^{-4} M), gallamine (10^{-4} M), pirenzepine (10^{-4} M), 4-diphenylacetoxy-*N*-methylpiperidine (4-DAMP) (10^{-4} M), alpha-amino-3-hydroxy-5-methylisoxazole-4-proprionic acid (AMPA) (10^{-4}–10^{-3} M), kainic acid (10^{-5}–10^{-4} M), NMDA (10^{-4}–10^{-3} M), muscimol (10^{-4}–10^{-3} M), BHT-920 (10^{-3} M), yohimbine (10^{-3} M) and prazosin (10^{-3} M). All drug solutions were pH 7.0. Drugs that 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 1st hour, 10^{-4} M for the 2nd hour and 10^{-3} M for the 3rd hour. Cataplexy was measured by two consecutive FECT trials during minutes 20–30 and minutes 50–60 of each 1-hour drug concentration period. Control levels of cataplexy were tested under the same schedule in narcoleptic canines receiving no drug infusion. The animals were awake by both behavioral and EEG criteria at the start of all FECT trials. The cataplexy stimulatory effects of carbachol in the PRF, and the ability of antagonists to reverse its effects, were also tested. Briefly, carbachol (10^{-4} M) was perfused for 1 hour, followed by an antagonist (10^{-4} M) for 1 hour, and basal cataplexy was measured as described above, while drug-induced cataplexy was measured during minutes 20–30 and minutes 50–60 of each drug-perfusion period. The cholinergic antagonists tested were atropine, gallamine, pirenzepine and 4-DAMP.

Drugs

Carbamylcholine HCl (carbachol) (cholinergic agonist), nicotine (nicotinic agonist), kainic acid (kainate receptor glutamatergic agonist), NMDA (NMDA receptor glutamatergic agonist), yohimbine HCl (alpha-2 antagonist) (Sigma, St. Louis, MO, U.S.A.), atropine sulfate (muscarinic antagonist) (Calbiochem, San Di-

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TABLE 1. *Basal levels of FECT-induced cataplexy in narcoleptic canines*[a]

	Time (minutes)							
	−20	−10	20	50	80	110	140	170
Attacks	2.9 ± 0.4	3.2 ± 0.5	2.6 ± 0.4	2.2 ± 0.5	2.7 ± 0.5	2.4 ± 0.5	2.8 ± 0.6	2.8 ± 0.7
Elapsed time (seconds)	55 ± 9	53 ± 9	46 ± 7	42 ± 6	58 ± 11	51 ± 7	60 ± 6	53 ± 5

[a] Baseline cataplexy in four narcoleptic canines perfused bilaterally with cerebrospinal fluid in the pontine reticular formation. Cataplexy was measured using the same schedule as drug-perfused animals, on two separate days for each animal (n = 8). The mean number of cataplectic attacks and elapsed time for two Food-Elicited Cataplexy Tests (FECT) per test period are 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 towards the floor. The total time required to eat all 10 bites of food was also recorded.

ego, CA, U.S.A.), oxotremorine M (M2 muscarinic agonist), McN-A-343 (M1 muscarinic agonist), pirenzepine HCl (M1 muscarinic antagonist), gallamine triethiodide (M2 muscarinic antagonist), 4-DAMP methiodide (M1/M3 muscarinic antagonist), AMPA HBr (quisqualate receptor glutamatergic agonist), muscimol HBr [γ-amino-butyric acidergic (GABAergic agonist], prazosin HCl (alpha-1 antagonist) (Research Biochemicals Incorporated, Natick, MA, U.S.A.) and BHT-920 (alpha-2 and D2 dopaminergic agonist) (Forsch, Germany) were dissolved directly into CSF and tested for pH before local administration.

Statistics

All results are presented as mean ± SEM FECT scores for cataplectic attacks. Elapsed time during control and drug treatments was analyzed with one-way ANOVA, followed by post hoc Fisher Permutated Least Square Difference (PLSD) tests. For comparison between drug treatment groups, as well as between narcoleptic and control groups, a two-way ANOVA was used.

RESULTS

Basal FECT-induced cataplectic attacks ranged from partial, in which the animal could remain upright although a clear loss of muscle tone was indicated by a drop in EMG signal, to complete, in which the animal would go down on all four limbs and remain atonic for up to 1 minute. During cataplexy the EEG signal remained desynchronized and the EOG was mostly quiet (for illustration see reference 12). Although some variability in the levels of cataplexy existed between different animals and within individuals between different experiment days (particularly when separated by ≥1 month), the FECT scores were relatively stable on any given day over a 4-hour control period with no drug treatment (Table 1), averaging between two and three cataplectic attacks per FECT ($F = 0.394$, df = 7, p = 0.904) and elapsing over a time of 40–60 seconds ($F = 0.469$, df = 7, p = 0.855).

The effects of oxotremorine, McN-A-343 and nicotine perfusion in the PRF on cataplexy in the narcoleptic canines are shown in Fig. 1a, c and d. Bilateral oxotremorine perfusion (10^{-5}–10^{-3} M) produced a dose-dependent increase in FECT-induced cataplexy, which was evident in both the number of attacks ($F = 18.327$, df = 7, p = 0.0001) and elapsed FECT time ($F = 25.917$, df = 7, p = 0.0001) (Fig. 1a). During oxotremorine-stimulated cataplectic attacks, the EEG was desynchronized, the EMG was greatly reduced and the EOG signal was mostly quiet, similar to baseline cataplexy. At the lower concentrations (10^{-5}–10^{-4} M) the increase was significant during the 2nd hour of perfusion, in which complete cataplectic attacks became more prevalent and would last longer than during the baseline period. At these levels of oxotremorine there was no significant decrease in basal muscle tone in the narcoleptic canines, as noted by the EMG. At the highest concentration (10^{-3} M), all animals became completely atonic after a full hour of perfusion, which was defined as status cataplecticus. Bilateral McN-A-343 (10^{-4}–10^{-3} M) and bilateral nicotine (10^{-4}–10^{-3} M) perfusion did not have any effects on cataplexy, either on the number of cataplectic attacks (McN-A-343: $F = 0.447$, df = 4, p = 0.7743; nicotine: $F = 0.115$, df = 4, p = 0.977) or elapsed time (McN-A-343: $F = 0.166$, df = 4, p = 0.954; nicotine: $F = 0.311$, df = 4, p = 0.869), and did not produce any notable change in basal muscle tone in all animals tested.

The control and heterozygous canines all responded similarly to oxotremorine and are therefore presented together as the control group. The effects of oxotremorine perfusion in the PRF of the control group are shown in Fig. 1b. At low concentrations, bilateral oxotremorine (10^{-5}–10^{-4} M) did not induce cataplexy in the control group, though a significant increase in elapsed time occurred after 10^{-4} M oxotremorine administration ($F = 3.457$, df = 4, p = 0.019). No significant decrease in basal muscle tone, as noted by behavioral observation and EMG analysis, was noted at these concentrations of oxotremorine. At the highest concentration, oxotremorine (10^{-3} M) produced complete muscle tone suppression in the control canines, similar to status cataplecticus in the narcoleptic canines. As with the narcoleptic canines, bilateral McN-A-343 (10^{-4}–10^{-3} M) and bilateral nicotine (10^{-4}–10^{-3}

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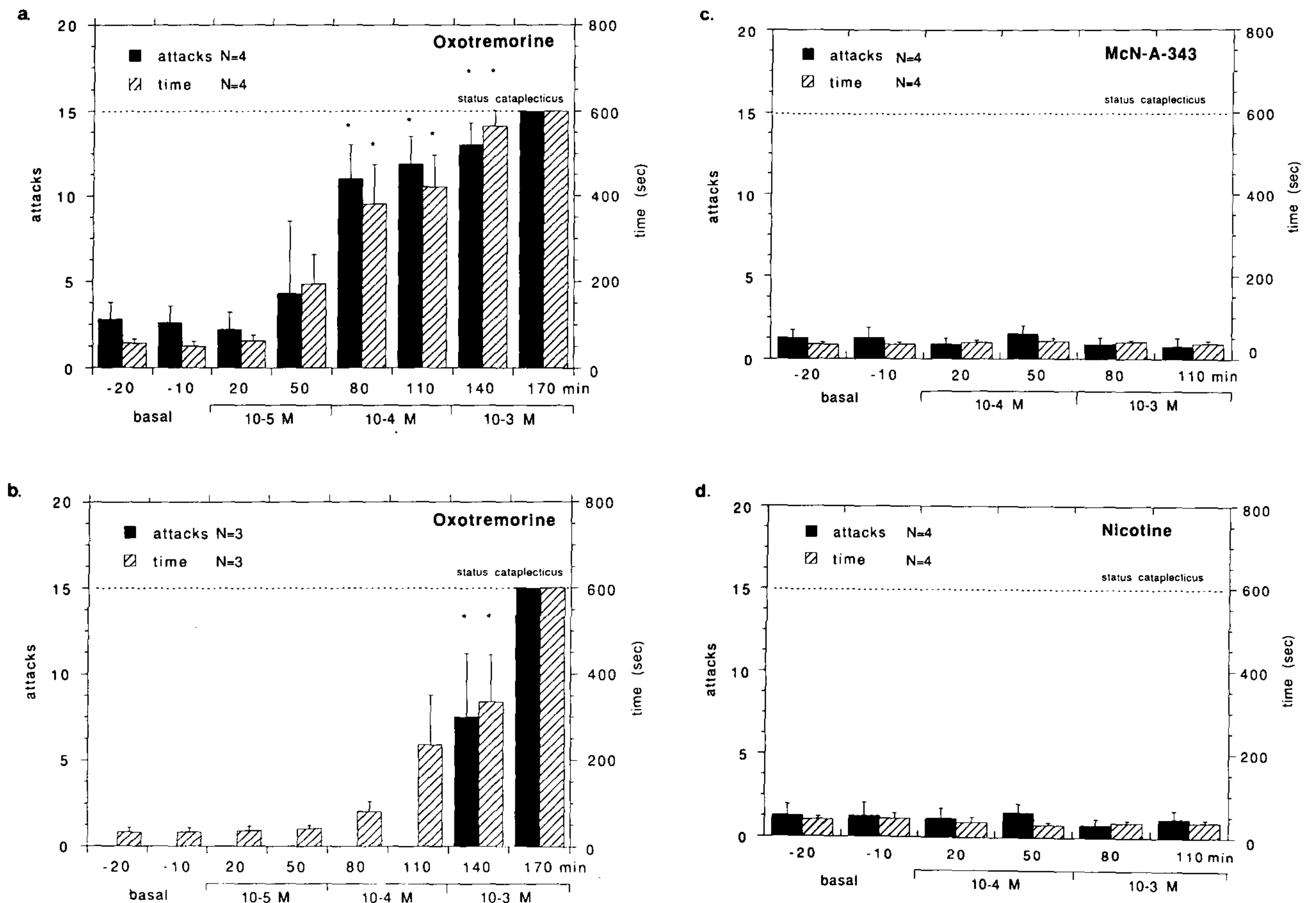

FIG. 1. Effect of local perfusion of various cholinergic agonists in the pontine reticular formation on cataplexy in the narcoleptic canine. Local perfusion with bilateral oxotremorine (10^{-5}–10^{-3} M) in (a) narcoleptic and (b) control canines, bilateral McN-A-343 (10^{-4}–10^{-3} M) (c) in narcoleptic canines and bilateral nicotine (10^{-4}–10^{-3} M) (d) in narcoleptic canines is shown. Drugs were mixed into artificial cerebrospinal fluid and perfused through microdialysis probes at the indicated concentrations over the course of a 4-hour experiment: none during the 1st hour, 10^{-5} M during the 2nd hour, 10^{-4} M during the 3rd hour and 10^{-3} M during the 4th hour. Four narcoleptic canines (narcoleptics), two controls and one heterozygous canine (control group) were tested. The mean number of cataplectic attacks and elapsed time for two Food-Elicited Cataplexy Tests per test period are shown. For the purpose of figure presentation, status cataplecticus (carbachol atonia) was arbitrarily designated as 15 attacks elapsed over 600 seconds; thus, for these levels of cataplexy there was no statistical variability in the score. Each drug perfusion time point prior to status cataplecticus was compared with the basal time points using a Fisher PLSD post hoc test; * indicates $p < 0.05$, satisfactory for comparison with either basal time point.

M) perfusion did not produce cataplexy or an increase in elapsed FECT time (McN-A-343 = 2.161, df = 4, p = 0.101; nicotine: $F = 0.750$, df = 4, p = 0.566), and did not produce any notable change in basal muscle tone in all animals tested.

The increase in cataplexy after local perfusion with carbachol in the PRF of narcoleptic canines was rapidly reversed when followed with atropine or gallamine, but not pirenzepine, and only partially reversed when followed with 4-DAMP (Fig. 2a and b). Bilateral carbachol (10^{-4} M) produced a strong increase in cataplexy, which reached status cataplecticus in nearly all drug trials (12 out of 14). This high level of cataplexy was maintained for over 1 hour after switching the bilateral perfusion medium to one containing pirenzepine (10^{-4} M) or CSF without drug, but was rapidly reversed to basal levels after switching to a bilateral perfusion medium containing gallamine (10^{-4} M) or atropine (10^{-4} M). The reverse of the cataplectic effects did not fall below the baseline levels. This reversal to basal levels of cataplexy was evident in the number of attacks (atropine: $F = 1.121$, df = 1, p = 0.160; gallamine: $F = 2.889$, df = 1, p = 0.099) and elapsed time (atropine: $F = 1.012$, df = 1, p = 0.211; gallamine: $F = 1.685$, df = 1, p = 0.208). Switching to a bilateral perfusion medium containing 4-DAMP (10^{-4} M), after carbachol, produced only a partial reversal in the increase in cataplexy, which was still significantly higher than basal levels, both in number of attacks ($F = 12.751$, df = 1, p = 0.0013) and elapsed time ($F = 14.374$, df = 1, p = 0.0007), after the full hour of 4-DAMP perfusion (only one of four animals returned to basal levels of cataplexy).

The effects of local perfusion with muscimol, kainic acid, AMPA and NMDA in the PRF of narcoleptic canines are shown in Fig. 3a–d. Bilateral perfusion with

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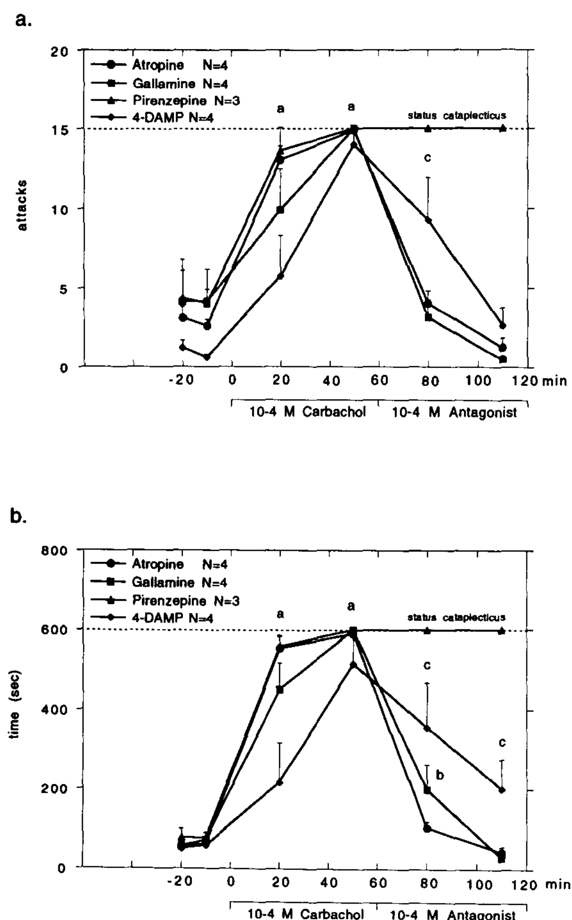

FIG. 2. Effect of perfusion in the pontine reticular formation on cataplexy in narcoleptic canines with bilateral carbachol (10^{-4} M) administration for 60 minutes, followed by bilateral atropine, gallamine, pirenzepine or 4-DAMP (each at 10^{-4} M) for 60 minutes. The mean number of cataplectic attacks (a) and elapsed time (b) for two Food-Elicited Cataplexy Tests per test period are shown. For the purpose of figure presentation, status cataplecticus (carbachol atonia) was arbitrarily designated as 15 attacks elapsed over 600 seconds. Each drug time point was compared with the basal time points using a Fisher PLSD post hoc test; **a** indicates $p < 0.05$, satisfactory for comparison of all four drug groups with either of their respective basal time points; **b** indicates $p < 0.05$, satisfactory for comparison of the gallamine group with either of its basal time points; and **c** indicates $p < 0.05$, satisfactory for comparison of the 4-DAMP group with either of its basal time points.

muscimol (10^{-4}–10^{-3} M) produced a moderate increase in FECT-induced cataplexy, which was evident both in number of attacks (F = 7.027, df = 4, p = 0.0001) and elapsed time (F = 4.234, df = 4, p = 0.0044) (Fig. 3a). A higher concentration of muscimol (10^{-2} M) was tested to ascertain whether status cataplecticus could be obtained. The one animal tested showed a further increase in cataplexy; however, because of detrimental side effects, such as decreased respiration and heart rate, this level of muscimol was no longer tested. Bilateral perfusion with the non-NMDA agonists kainic acid (10^{-5}–10^{-4} M) and AMPA (10^{-4}–10^{-3} M) did not produce any significant effects on FECT-induced cataplexy in either number of attacks (kainic acid: F = 0.746, df = 4, p = 0.593; AMPA: F = 0.528, df = 4, p = 0.715) or elapsed time (kainic acid: F = 0.450, df = 4, p = 0.811; AMPA: F = 0.871, df = 4, p = 0.489) (Fig. 3b and c). Two animals were also tested at a higher concentration of kainic acid (10^{-3} M) and did not show any change in cataplexy at this level either; however, this level of kainic acid was discontinued because of adverse side effects. Bilateral perfusion with NMDA (10^{-4}–10^{-3} M) did not produce any significant effects on FECT-induced cataplexy in either the number of attacks (NMDA: F = 1.075, df = 4, p = 0.381) or elapsed time (NMDA: F = 0.271, df = 4, p = 0.895) (Fig. 3d).

Some monoaminergic compounds were also perfused locally in the PRF of narcoleptic canines, but none of them had any effects on FECT-induced cataplexy. The drugs tested, BHT 920 (10^{-3} M) (n = 4), yohimbine (10^{-3} M) (n = 3), propranolol (10^{-3} M) (n = 2) and prazosin (10^{-3} M) (n = 2) were perfused bilaterally for 1 hour, and none had any effect on the number of cataplectic attacks or elapsed FECT time (data not shown).

Histological analysis revealed that all the microdialysis probes were located within the PRF. The tracts produced by injection cannulae at the same stereotaxic coordinates as the microdialysis probes were seen in the central portions of the nucleus reticularis pontis caudalis (RPC). Accordingly, the tips of the microdialysis membranes were located near the ventral border of the RPC, and the membranes extended 5 mm dorsally to ventral portions of the dorsal pontine tegmentum, just above the RPC. The positions of the cannula tracts varied along the rostrocaudal extent of the RPC by about 1.5 mm and along the mediolateral extent of the tract by about 2.5 mm. Comparison of the bilateral implacements showed that they were close (within 0.5 mm) to the same transectional plane. In Fig. 4 the left and right tracts after bilateral cannulation of the PRF with dye injection cannulae implanted at the same coordinates as the microdialysis probes are shown. In this coronal transection the ventralmost 4 mm of the tract on the left side and a dorsal portion of the tract on the right side, penetrating the laterodorsal tegmental nucleus (LDT) are shown (black arrows). The tracts were the most medial of six animals analyzed, and in subsequent sections (approximately 100 μm caudally) the most ventral tip of the tract on the right side was at the same depth as that shown on the left side.

DISCUSSION

These results show that cataplexy in the narcoleptic canine may be differentially modulated by selective

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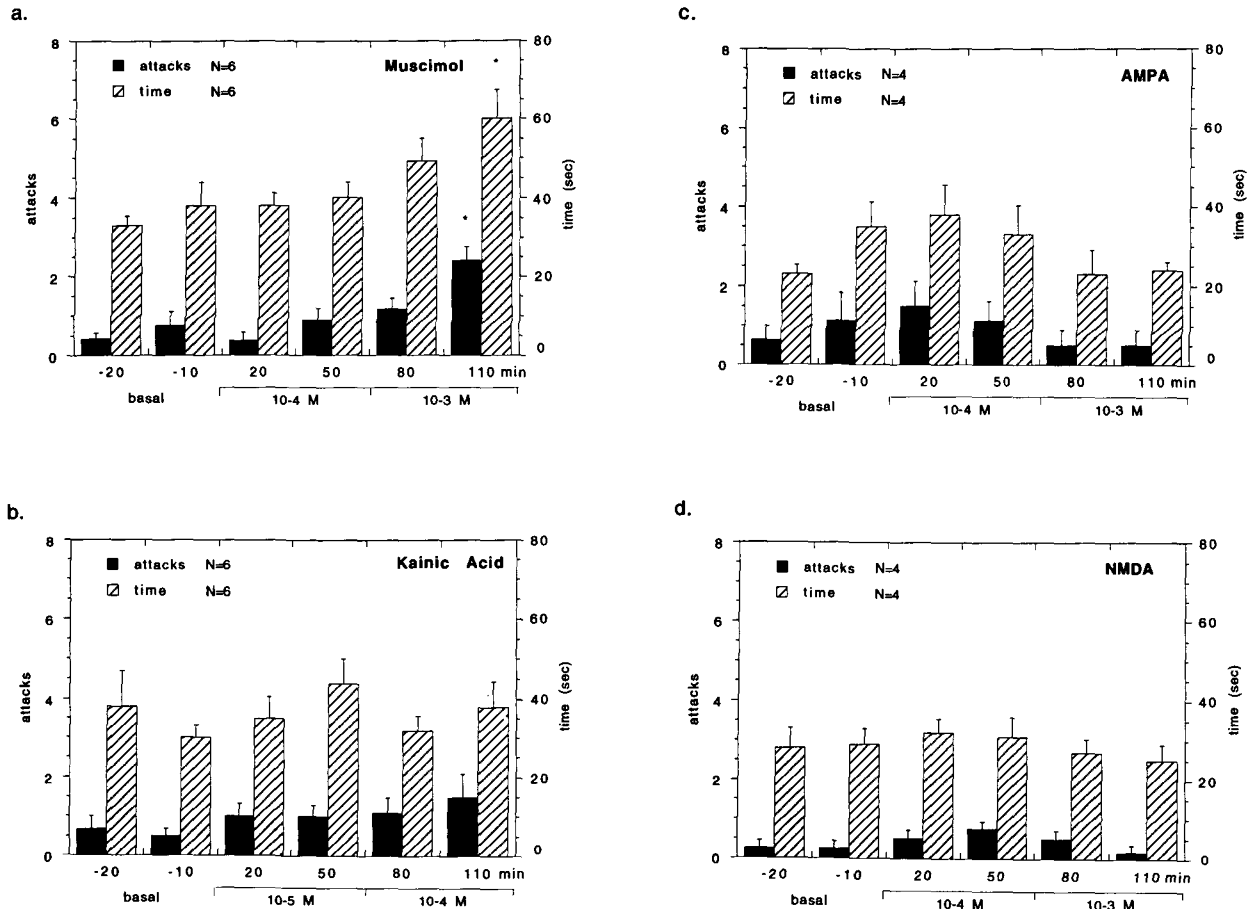

FIG. 3. Effect of local perfusion of various amino-acid neurotransmitter receptor agonists in the pontine reticular formation on cataplexy in the narcoleptic canine. Local perfusion with bilateral (a) muscimol (10^{-4}–10^{-3} M), (b) kainic acid (10^{-5}–10^{-4} M), (c) alpha-amino-3-hydroxy-5-methylisoxazole-4-proprionic acid (AMPA) (10^{-4}–10^{-3} M) and (d) *N*-methyl-D-aspartate (NMDA) (10^{-4}–10^{-3} M) in narcoleptic canines was performed. The number of cataplectic attacks and elapsed time for two Food-Elicited Cataplexy Tests per test period are shown. Each drug perfusion time point was compared with the basal time points using a Fisher PLSD post hoc test; * indicates $p < 0.05$, satisfactory for comparison with either basal time point.

stimulation or blockade of M2 muscarinic receptors in the PRF. These findings are similar to earlier studies on the cholinergic regulation of REM sleep in the PRF, indicating that cataplexy and REM sleep are regulated by similar mechanisms in the brainstem reticular formation. In addition, the results with muscimol and the excitatory amino acids suggest that the stimulation of cataplexy in the PRF may be mediated by a cellular inhibitory mechanism.

The role of the PRF in the cholinergic regulation of cataplexy in narcoleptic canines has been studied previously, with the findings indicating this structure is a critical component in mediating the cholinergic stimulation of cataplexy (12). Carbachol (10^{-5}–10^{-3} M) perfusion in the PRF very potently stimulated cataplexy and muscle atonia in the narcoleptic canines, both bilaterally and unilaterally, at concentrations which were 1 and 2 orders of magnitude lower than those that produced muscle atonia in the control canines, respectively. In the present study oxotremorine (10^{-5}–10^{-3} M) perfusion in the same site also produced an increase in cataplexy and muscle atonia in the narcoleptic canines, in a similar dose range as carbachol. Also, when compared with control canines, it was apparent that oxotremorine was 10 times more sensitive in producing muscle atonia in narcoleptic canines. These strong effects on cataplexy are consistent with autoradiographic studies showing a high density of [^{3}H]oxotremorine binding sites in the pons of rats (20,38). Furthermore, McN-A-343 and nicotine (10^{-4}–10^{-3} M) perfusion in the PRF did not have any effects on cataplexy in the narcoleptic canines. 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 PRF is selectively mediated by M2 muscarinic receptors. This is consistent with previous

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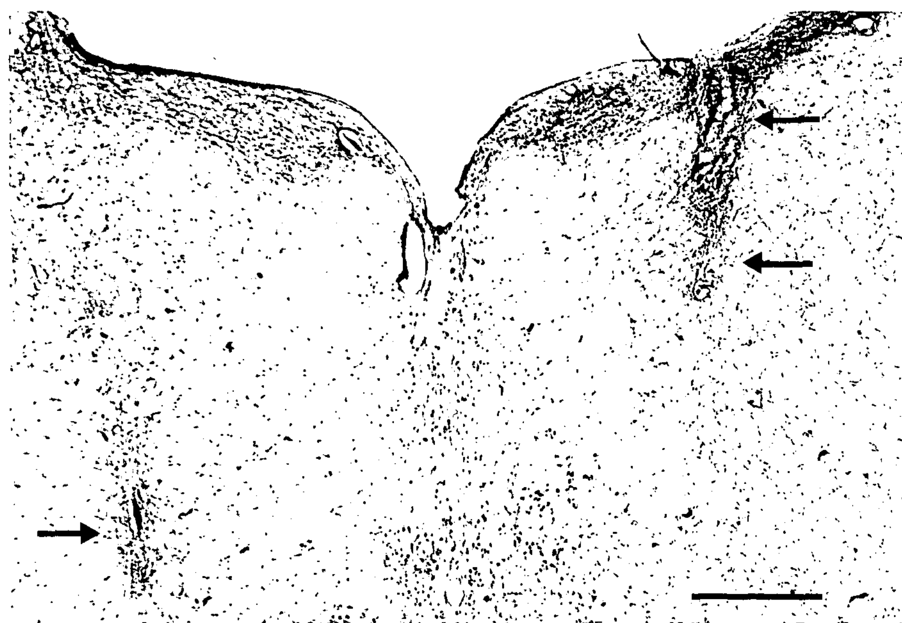

FIG. 4. Photomicrograph showing the positions of the microdialysis probes in the pontine reticular formation. The pontine sections were stained with thionin after bilateral cannulation through the guide cannula positioned at A 3.0, with injection cannulae for the injection of neutral red at coordinates A 3.0, L +3.2 and −3.2, and V 39.0. The black arrows point to the injection cannula tracts on the right and left sides in the nucleus reticularis pontis caudalis (RPC). The most ventral aspects of the implantation tract on the left, extending approximately 4 mm along the dorsoventral axis in the RPC, and dorsal portions of the implantation tract on the right, extending approximately 2 mm along the dorso-ventral axis through the laterodorsal tegmental nucleus, are indicated. When the microdialysis probes were implanted in the RPC of this canine, at the same coordinates as the dye-injection cannulae, the 5-mm dialysis membranes were located in approximately the same positions as the tracts. The black bar corresponds to 2 mm.

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studies indicating an increase in the number of M2 muscarinic receptors in the PRF of narcoleptic canines (10,11). However, the specificity of McN-A-343 and oxotremorine is based on the relative degree of binding sensitivity to these respective muscarinic receptor subtypes (39–41). It has been shown that McN-A-343 can also bind at M2 receptors at high concentrations (41) and that oxotremorine can bind at both M4 and M2 receptors with similar affinity in the rat brain (20). 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 PRF is mediated by M2 muscarinic receptors alone.

In the muscarinic antagonist studies the increase in cataplexy produced by local carbachol perfusion was rapidly reversed when followed by local atropine or gallamine perfusion in the PRF. In contrast, local pirenzepine perfusion following carbachol did not reverse the increase in cataplexy, as all animals remained in status cataplecticus for over 60 minutes after switching from carbachol, and local 4-DAMP perfusion following carbachol was only able to moderately reduce the increase in cataplexy. The ability of atropine to reverse the effects of carbachol is consistent with our previous study (12) and indicates that muscarinic receptors mediate the effect of carbachol in the PRF. The inability of pirenzepine, a well-known M1 muscarinic receptor-selective antagonist (41,42), to reverse the increase in cataplexy shows that M1 muscarinic receptors are not involved in mediating the effect of carbachol. This finding is not surprising considering the paucity of M1 receptors shown in the brainstem of rats (19,25), the low level of [^{3}H]pirenzepine binding in the rat brainstem (20,38) and the lack of effect of McN-A-343 perfusion in the PRF on cataplexy in the narcoleptic canines. The ability of gallamine, a muscarinic antagonist with high M2 and low M1 muscarinic receptor selectivity (43,44), to reverse the effects of carbachol suggests that the cataplexy stimulatory effects of carbachol are mediated by M2 muscarinic receptors and is consistent with the cataplexy stimulatory effects of oxotremorine in the PRF. Considering the high level of M2 muscarinic receptors shown in the brainstem at the level of the pons (19,20,25), these findings were expected. Interestingly, 4-DAMP was unable to reverse the effects of carbachol, but did reduce the level of cataplexy in three out of four of the dogs tested. 4-DAMP has been characterized as a muscarinic antagonist with high affinity for both the M1 and M3 and

low affinity for the M2 muscarinic receptor subtypes (45,46). The weak effects of 4-DAMP may be attributed to its weak affinity for M2 muscarinic receptors. Alternatively, 4-DAMP antagonism of M3 muscarinic receptors in the PRF, which has been demonstrated at low levels in the brainstem (19), might also mediate its effects on carbachol-stimulated cataplexy. This would suggest that carbachol stimulation of cataplexy is partially mediated by M3 receptors and is consistent with a recent study showing that 4-DAMP is a potent displacer of the nonselective muscarinic receptor agonist [^{3}H]QNB in competition binding assays using cat pontine homogenates (22). In fact, 4-DAMP was a more potent displacer of [^{3}H]QNB than either pirenzepine or AF-DX 116 (M2 muscarinic receptor antagonist) (22).

As with previous studies (12,15), the microdialysis probes were located in the PRF. Histological analysis revealed that the central and ventral portions of the 5-mm dialysis membranes were in direct contact with the RPC, whereas the dorsal (approximately 1-mm) portions of the dialysis membranes were in contact with structures in the pontine tegmentum, LDT, pedunculopontine tegmental nucleus (PPT) and the subcoeruleus (see Fig. 4). The drugs perfused through the membranes most likely crossed over the membrane and into the surrounding tissue at approximately 10–12% of their internal concentration (for reviews and references see 12). Thus, the theoretical total dose of drug infused across the membrane of one probe was calculated to be 0.14 nmol, 1.54 nmol and 16.9 nmol after the completion of each hour of perfusion with 10^{-5} M, 10^{-4} M and 10^{-3} M drug concentrations, respectively. Although it is possible that a cumulative drug effect may have occurred within each 1-hour perfusion period, it is less likely that this occurred over the course of the entire 3-hour procedure, because each ensuing drug dose/concentration is at least 10 times greater than the total preceding drug dose/concentration. The area affected likely encompassed all tissue within 1–1.5 mm of the dialyzing membrane (47,48); consequently, it may be assumed that the drugs infused in this study affected both cholinoceptive areas in the PRF and cholinergic nuclei in the pontine tegmentum. Careful analysis of previous autoradiographic binding studies reveals that [^{3}H]oxotremorine binding in the rat pons is highest in the pontine tegmentum, including the PPT and LDT, and the medial aspects of the PRF (20,38). On the other hand, the in situ hydridization studies showing M2 muscarinic receptor mRNA are much less clear, showing a more homogenous distribution of M2 receptor messenger across the entire pontine transection at the level of the PRF, with an only slightly higher level in the pontine nuclei, LDT and PPT (19, 20). Previous reports on the distribution of M3 muscarinic receptors in the pons have shown moderate levels of M3 receptor binding in the cat PRF, PPT, substantia gelatinosa and central grey (21), and in situ hybridization studies showed a more homogenous, low-level distribution of M3 muscarinic receptor mRNA in the rat PRF, with a slightly higher level in the central grey and pontine nuclei (19). Thus, in the present study, M2 muscarinic stimulation of cataplexy in the pons of narcoleptic canines may have been mediated via receptors located either on noncholinergic neurons in the cholinoceptive region of the PRF or on the cholinergic neurons of the PPT and LDT in the pontine tegmentum. Possible M3 muscarinic stimulation of cataplexy also could have been mediated by receptors in these same areas. However, the stimulation of M2 muscarinic receptors on cholinergic pontine tegmental cells has been reported to hyperpolarize them (49), which would suggest that cholinergic stimulation of cataplexy via these receptors is associated with a reduction in the activity of the cholinergic pontine tegmental neurons. In our previous study we found that acetylcholine release in the PRF was enhanced during cataplexy (15), which suggests that cholinergic pontine tegmental neurons are actually activated during cataplexy. Therefore, it seems more likely that M2 muscarinic stimulation of cataplexy in the pons of narcoleptic canines is mediated via receptors in the cholinoceptive region of the PRF on noncholinergic neurons.

The role of excitatory and inhibitory amino-acid neurotransmitters in brainstem regulation of REM sleep and muscle atonia has been studied more recently, and evidence supporting the involvement of both GABA and glutamate has been reported. Microinjections of NMDA agonists into the subcoeruleus of cats produce an increase in muscle tone, whereas non-NMDA agonists produce muscle atonia (33). Furthermore, GABA release in the central grey area has been shown to increase during REM sleep (50). In the present study, neither NMDA nor the non-NMDA agonists had any effect, whereas muscimol produced a moderate increase in cataplexy in the narcoleptic canines when perfused bilaterally in the PRF. As discussed above, the perfusion area included regions of the subcoeruleus, yet we were unable to elicit muscle atonia or enhance cataplexy with the non-NMDA agonists, kainic acid and AMPA. Furthermore, NMDA did not reduce cataplexy or produce any observable increase in muscle tone. These findings would seem to be discrepant with the studies on decerebrate cats (33). A possible explanation for this could be the differences in route of drug administration, because continuous delivery of a drug via a 5-mm membrane lacks the site specificity and high initial concentration of a microinjection. However, the most critical difference in these studies is that

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those in the cat were done with decerebrate animals, which no longer contain intact projections to and from higher brain structures that could mediate muscle control functions opposite to those of the intact descending pathways. Interestingly, we found that muscimol perfusion produced an increase in cataplexy, indicating that neuronal inhibition via GABA receptors in the PRF could enhance cataplexy. This is consistent with a recent report indicating that brainstem GABA plays a role in the regulation of REM sleep (50) and suggests the possibility that REM sleep and cataplexy are regulated by similar GABA mechanisms in the pons. Alternatively, it has been shown that non-M1 muscarinic receptor activation hyperpolarizes peribrachial cells in the lateral pontine tegmentum (49). Furthermore, although the majority of PRF cells have been reported to be depolarized by carbachol in vitro, one-third was found to be hyperpolarized (51) and subsequent studies have shown that this is a K+-dependent hyperpolarization, which is elicited by non-M1 muscarinic receptor activation (52). Both reports suggest that M2 muscarinic receptors mediate this inhibitory effect. Thus, the ability of muscimol perfusion in the PRF to enhance cataplexy in narcoleptic canines might actually be equivalent to M2 muscarinic stimulation of cataplexy in the PRF via neuronal hyperpolarization. Indeed, multiple-unit recording studies have found that most cells in the PRF reduce activity during cataplexy in narcoleptic canines (53). We hypothesize that the perfusion of carbachol and/or oxotremorine into the PRF of narcoleptic canines produces a decrease in the activity of PRF neurons via M2 muscarinic receptors, similar to the reduction in unit activity seen during spontaneous cataplexy (53), and thereby enhances the incidence of cataplectic behavior.

Four different monoaminergic compounds were perfused bilaterally in the PRF of the narcoleptic canines, BHT 920, yohimbine, prazosin and propranolol, and none of them had any effects on cataplexy. Previous studies have shown that intravenous injections of BHT 920, a mixed alpha-2 adrenergic/D2 dopamine receptor agonist, and prazosin, an alpha-1 adrenergic receptor antagonist, produce an increase, whereas yohimbine, an alpha-2 adrenergic receptor antagonist, produces a decrease in cataplexy in the narcoleptic canine (35,54). The present findings suggest that the PRF and/or pontine tegmentum do not play a significant role in mediating these monoaminergic cataplexy modulatory effects. Indeed, these findings suggest that the noradrenergic cell bodies in the locus coeruleus, which lie in the dorsal pontine tegmentum just lateral to the probe tracts and may have been affected by proximal diffusion of locally perfused compounds (see Fig. 4), are also not involved in mediating these monoaminergic effects. Interestingly, pontine injections of the alpha-2 agonist clonidine (30) and the alpha-2 antagonist idazoxan (31) have been reported to have strong REM sleep suppressant and stimulatory effects, respectively, and the beta-adrenergic receptor antagonist propranolol has significant, though weak, REM sleep stimulatory effects in the PRF (32). Thus, the findings in the present study would suggest that pontine monoaminergic regulation of REM sleep has little to do with pontine cataplexy regulatory mechanisms. However, the effects of propranolol on REM sleep are only moderate, and comparison with the effects of clonidine on REM sleep should be made with a note of caution because clonidine is unable to modify cataplexy when given intravenously to narcoleptic canines (55).

The findings in the present study provide strong evidence that M2 muscarinic receptors in the PRF play an important role in mediating the cholinergic stimulation of cataplexy in the narcoleptic canine. Previous studies have shown that M2 muscarinic receptors in the PRF are also important in the regulation of REM sleep (5,9,26–28), which would suggest that cataplexy and REM sleep are regulated by similar cholinergic mechanisms in the PRF. Indeed, the inability of M3 muscarinic antagonists to affect REM sleep (26) and the weak effects of the M3 antagonist 4-DAMP on carbachol-induced cataplexy, which could be attributed to its low affinity at M2 receptors, are also consistent with this hypothesis. It has been proposed that M2 muscarinic receptors in the PRF mediate REM sleep muscle atonia regulation, whereas M1 muscarinic and nicotinic receptors mediate EEG desynchronization and ponto-geniculo-occipital (PGO) waves, respectively (28). Thus, it may be suggested that cataplexy in the narcoleptic canine represents selective activation of REM sleep muscle atonia mechanisms. The effects of nicotinic and M1 agonists on cortical desynchrony and PGO waves in narcoleptic canines could be investigated in future studies.

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