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CHOLINERGIC MECHANISMS IN CANINE NARCOLEPSY—II. ACETYLCHOLINE RELEASE IN THE PONTINE RETICULAR FORMATION IS ENHANCED DURING CATAPLEXY

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Abstract—Cataplexy in the narcoleptic canine has been shown to increase after local administration of carbachol into the pontine reticular formation. Rapid eye movement sleep has also been shown to increase after local administration of carbachol in the pontine reticular formation, and furthermore, acetylcholine release in the pontine tegmentum was found to increase during rapid eye movement sleep in rats. Therefore, in the present study we have investigated acetycholine release in the pontine reticular formation during cataplexy in narcoleptic canines. Extracellular acetylcholine levels were measured in the pontine reticular formation of freely moving narcoleptic and control Doberman pinschers using *in vivo* microdialy-sis probes. Cataplexy was induced by the Food-Elicited Cataplexy Test and monitored using recordings of electroencephalogram, electrooculogram and electromyogram. Basal levels of acetylcholine in the microdialysis perfusates were approximately 0.5pmol/10min in both control and narcoleptic canines. Local perfusion with tetrodotoxin (10^{-5} M) or artificial cerebrospinal fluid without Ca^{2+} produced a decrease, while intravenous injections of physostigmine (0.05 mg/kg) produced an increase in acetylcholine levels, indicating that the levels of acetylcholine levels measured are derived from neuronal release. During cataplexy induced by the Food-Elicited Cataplexy Test, acetylcholine levels increased by approximately 50% after four consecutive tests in narcoleptic canines, but did not change after four consecutive tests in control canines. Motor activity and feeding behavior, similar to that occurring during a Food-Elicited Cataplexy Test, had no effect on acetycholine levels in the narcoleptic canines.

These findings show that acetylcholine release in the pontine reticular formation is enhanced during Food-Elicited Cataplexy Tests in narcoleptic canines, and that this increase is specifically linked to the incidence of cataplectic attacks. Thus, cataplexy in the narcoleptic canine is associated with an increase in acetylcholine release in the pontine reticular formation. Furthermore, this study provides evidence supporting the hypothesis that narcolepsy is a disorder of rapid eye movement sleep regulation.

Narcolepsy is an incurable sleep disorder characterized by excessive daytime sleepiness, sudden attacks of muscle atonia called cataplexy, fragmented night-time sleep, sleep paralysis and hypnagogic hallucinations; see Ref. 10. It has been suggested that narcolepsy is a disorder of rapid eye movement (REM) sleep regulation.[10,34] Thus, narcoleptic subjects show unusual sleep onset REM periods, while cataplexy and sleep paralysis represent abnormal triggering of the muscle tone suppression associated with REM sleep.[10,12] Furthermore, pharmacological studies have shown that all therapeutic agents for the treatment of narcolepsy are also strong REM sleep suppressants."

Canine narcolepsy is a genetically determined, autosomal recessive, animal model of the human disease that presents several clinical similarities to the human condition, including cataplexy, fragmented sleep, and daytime sleepiness (see Refs 2, 29). Consistent with the theory that narcolepsy is a REM sleep disorder, narcoleptic canines show a tendency for sleep onset REM periods[24,27,31] and cataplexy.[31] Furthermore, several lines of evidence suggest that canine cataplexy is closely associated with REM sleep mechanisms. Thus, canine cataplexy is associated with a desynchronized electroencephalogram (EEG) and reduction in electromyogram (EMG) activity,[19,24,27] is reduced by monoaminergic REM sleep suppressant agents such as amphetamine and antidepressants,[28] and is increased by cholinergic REM sleep enhancers such as physostigmine.[6]

Whereas monoaminergic modulation of REM sleep is poorly understood, a great deal is known

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Abbreviations: CSF, cerebrospinal fluid; EEG, electroencephalogram; EMG, electromyogram; EOG, electrooculogram; FECT, food-elicited cataplexy test; LDT, laterodorsal tegmental nucleus; PPT, pedunculopontine nucleus; PRF, pontine reticular formation; REM rapid eye movement; RPC, nucleus reticularis pontis caudalis; RPO, nucleus reticularis pontis oralis; TTX, tetrodotoxin.

about cholinergic neuronal systems which are involved in the regulation of REM sleep. Several studies have shown that cholinergic innervation of the brainstem is critical for the regulation of REM sleep. Lesions in the dorsolateral pons, affecting cholinergic projections to the pontine reticular formation (PRF), greatly reduce REM sleep[16,45] and REM sleep muscle atonia.[18] In addition, acetylcholine release in the dorsomedial pontine tegmentum[22] and the nucleus paramedianus[23] is enhanced during REM sleep. These findings suggest that cholinergic projections to the brainstem reticular formation, including the PRF, are activated during REM sleep.

Previously, we reported an increase in muscarinic binding in the PRF of narcoleptic canines,[5,21] indicating that canine narcolepsy is associated with a cholinergic imbalance in the PRF. In the accompanying paper we showed that local perfusion with carbachol in the PRF enhanced cataplexy, and that local perfusion with atropine in the PRF reduced intravenous physostigmine stimulated cataplexy in the narcoleptic canine.[35] Consequently, it may be suggested that cataplexy in narcoleptic canines is associated with the activation of cholinergic mechanisms in the PRF, similar to the cholinergic activation which occurs during REM sleep. In the present study we further explored this hypothesis by investigating acetylcholine levels in the PRF of narcoleptic, control and heterozygous narcoleptic canines during periods of normal waking, motor activity, feeding and cataplexy. This was achieved using *in vivo* microdialysis probes implanted bilaterally into the PRF of freely moving Doberman pinschers.

EXPERIMENTAL PROCEDURES

Adult Doberman pinschers, consisting of five narcoleptic (three male and two female), two control (male), and one heterozygous (female), were used in all studies. All animals were bred at the Stanford University narcoleptic canine colony. Heterozygous canines contain one copy of the narcolepsy transmitting gene locus, *canarc*-l, but do not show symptoms of narcolepsy.[29,30] The animals were kept under a 12/12 h light dark schedule with food and water available *ad libitum.*

Surgery

A detailed description of the surgical techniques may be found in the accompanying paper (see Ref. 35). Briefly, all canines were operated on under isofluorane anaesthesia using a Kopf stereotaxic frame. For sleep/cataplexy recording they were implanted with screw electrodes in the skull over the medial frontal and lateral parietal cortex for recording of EEG, in the orbit of the frontal bone for the recording of electrooculogram (EOG), and stranded stainless-steel wires in the dorsal neck musculature for the recording of EMG. Custom-made guide cannula bundles were positioned over the cortical dura. Four guides, each separated by 2 mm along the rostrocaudal axis, were positioned over the PRF bilaterally (L: 3.2 and A: 1.0-7.0, according to Lim *et al.*[25]) (L = lateral, A = anterior) such that a row of four cannulae were positioned over the rostrocaudal extent of the PRF, all at the same laterality. In the present study the guide cannulae over A 3.0 and A 5.0 were used. The animals were allowed at least three weeks to recover from surgery.

One day prior to experimentation the animals were anaesthetized with isofluorane, and microdialysis probes (70mm shaft, 5mm membrane, CMA 10, CMA Microdialysis, Stockholm, Sweden) were lowered bilaterally into the PRF (L: 3.2, A 3.0 or 5.0, V: 39.0 according to Lim *et al.*[25]) (V = ventral) (for diagram see Ref. 35) and anchored in place with a small screw. The left and right probes were always implanted at the same coordinate on the anterior-posterior axis. The probes used in this study had *in vitro* recovery rates for acetylcholine of 25 + 2% *(n* = 10). After completing the experiment the probes were removed and the animals returned to their cages. Histological verification of the probe placements were performed on seven of eight canines used in this study (see Ref. 35).

Testing procedure

All canines were given approximately five days of habituation to the experimental chamber before testing. During testing, 3-m inlet lines wrapped around a tether were connected to the dialysis probes, perfused at 2.0 /ul/min with artificial cerebrospinal fluid (CSF) (125 mM NaCl, 0.5 mM NaH_2PO_4, 2.5mM Na_2HPO_4, 2mM $CaCl_2$, 1 mM $MgCl_2$, pH7.4) and samples were collected every l0min in *200-/ul* Eppendorf tubes attached to the side of the headstage. The perfusion medium contained 1 mM of the cholinestrase inhibitor neostigmine in order to obtain measurable amounts of acetylcholine. This level of neostigmine was determined to have no effect on cataplexy of its own.[35] Cataplexy was measured using the Food-Elicited Cataplexy Test (FECT; see Ref. 5) combined with recordings of EEG, EOG and EMG. In each FECT the animal ate ten 5-g portions of wet dog food which were lined up on the floor of the chamber in a semi-circle over a distance of approximately 5m. Cataplectic attacks were recorded when the animal stopped forward motion and its hind quarters were lowered towards the floor.

Experiments were performed on days 1-3, beginning approximately 18 h after microdialysis probe implantation, between the hours of circadian time 3 and 8. Acetylcholine control tests were always performed on day 1. Four different control tests on the extracellular levels of acetylcholine were performed: (1) baseline levels were collected for the first l00min; (2) an intravenous injection of physostigmine (0.05 mg/kg) was made after 60 min; (3) the perfusion medium was switched from CSF to CSF plus tetrodotoxin (TTX) (10^{-5} M, pH 7) after 60 min; or (4) the perfusion medium was switched from CSF to CSF without Ca^{2+} after 60 min. The behavioral tests were performed on days 2 and 3. The behavioral testing protocol consisted of baseline sample collection for the first 60 min, followed by a behavioral stimulus condition for the next 20 min, and then another 20-min post-stimulus sample collection. After another 20-min baseline period another behavioral stimulus condition was sometimes tested. There were three different behavioral stimuli: (1) four consecutive FECT trials; (2) 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 (3) 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 20 min (two stimuli per 10-min perfusate sample) and were administered in a randomized order. Conditions 2 and 3 were discounted if any cataplexy occurred during the 20-min stimulation period. After completion of the behavioral testing protocols on day 2, carbachol (10^{-5} to 10^{-3} M) was introduced into the CSF perfusion medium bilaterally for the following 3 h and cataplexy was measured at 30-min intervals (see Ref. 35). An increase in cataplexy indicated proper placement of the dialysis probes in the PRF.

High-performance liquid chromatography. Perfusates were analysed for acetylcholine content using reverse phase chromatography with an enzymatic converter post-column.

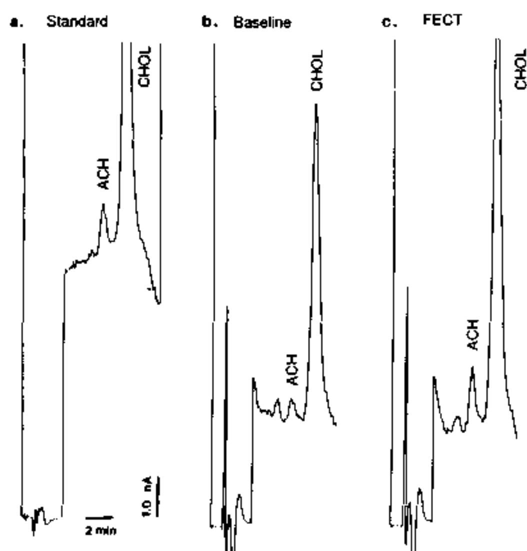

Fig. 1. Typical chromatograms of (a) a standard containing l.0pmol acetylcholine (ACH) and l0.0pmol choline (CHOL), (b) a 10-min perfusion sample collected from the pontine reticular formation of a narcoleptic canine during baseline conditions and (c) a 10-min perfusion sample collected from the pontine reticular formation of a narcoleptic canine after four consecutive FECTs (second of two consecutive 10-min FECT stimulus periods), b and c were taken from the same animal on the same day.

The analytical column (BAS, West LaFayette, IN), pre-packed with polymer-based C18 5-/um particulate, and the enzymatic column (BAS), pre-packed with immobilized acetylcholinesterase and choline oxidase, were perfused at 0.3ml/min with a 30 mM Na_2HPO_4, pH 8.5 buffer. After passing the enzymatic column, the breakdown product of acetylcholine, H_2O_2, was measured electrochemically with a platinum electrode set at 0.500 V and connected to an amperometric detector (LC-4A, BAS). With this configur-ation choline levels could also be detected. The limit of detection for acetylcholine was 0.2pmol. A typical chro-matogram of a 1.0 pmol acetylcholine and 10.0 pmol choline standard is shown in Fig. la.

Drugs

TTX and neostigmine (Sigma, St. Louis, MO) were dissolved directly into CSF and tested to be pH 7.0 before local administration. Physostigmine (Calbiochem) was dis-solved in 0.9% NaCl and administered intravenously in a 1-mg/ml volume.

Statistics

All results are presented as mean + S.E.M. Baseline acetylcholine levels were analysed with a one-way ANOVA using the raw data. Due to inter-individual and inter-day variability, changes in acetylcholine levels after drug or behavioral treatment were expressed as the percentage of a control value, which was defined as the final perfusion sample prior to drug or behavioral treatment. Drug and behavioral treatments were analysed using one-way ANOVA, with *post hoc* Fisher PLSD tests. Comparison between behavioral, drug treatment or animal groups across time was tested using two-way ANOVA.

RESULTS

From the five narcoleptic canines studied, a total of 14 bilateral *in vivo* microdialysis sessions were per-formed, and from the control group of two control and one heterozygous canines that were studied, a total of nine bilateral *in vivo* microdialysis sessions were performed. No significant differences were found between the heterozygous and control canines in their basal levels of acetylcholine and in their responses to FECT treatment (see below); therefore, they are presented together as the control group. At the beginning of each session the animals were connected to the CSF perfusion line and acetylcholine levels were allowed to equilibrate for l0min before collecting perfusion samples. Acetylcholine levels in the perfusate were essentially stable 30 min after the beginning of perfusion and remained stable over the following 90 min (Narcoleptic: $F = 0.092$, d.f. = 9, $P = 0.997$; control: $F = 0.056$, d.f. = 6, $P = 0.992$) (see Table 1). Choline levels were relatively stable, but did not show any significant response to pharmaco-logical or behavioral treatment. Therefore, only acetylcholine levels are presented in this report. Basal levels of extracellular acetylcholine in the PRF were taken between 60 and 70 min after beginning per-fusion, immediately prior to drug or behavioral stimulation. Basal levels of acetylcholine in the nar-coleptic (0.510 ± 0.060 pmol/10 min, $n = 28$) and control (0.502 ± 0.065 pmol/10 min, $n = 17$) animals were not significantly different ($t = 0.749$, d.f. = 43, $P = 0.459$). Within the control group, basal levels of acetylcholine in the control (0.537 + 0.079, $n = 12$) and heterozygous (0.419 ± 0.083, $n = 5$) canines were also not significantly different ($t = 0.876$, d.f. = 15, $P = 0.395$). Basal and cataplexy-induced levels of acetylcholine showed no differences between right and left sides (F = 0.166, d.f. = 1, $P = 0.969$) or between probe locations at A 3.0 and A 5.0 ($F = 3.084$, d.f. = 1, $P = 0.082$). In addition, slightly

Table 1. Basal acetylcholine levels in pontine reticular formation (pmol/10 min)

	30	40	50	60	70	80	90 min
Narcoleptic ($n = 10$)	0.39 ±0.09	0.38 ±0.06	0.35 ±0.06	0.39 ±0.07	0.38 + 0.06	0.36 ±0.05	0.40 ±0.08
Control ($n = 6$)	0.47 ±0.07	0.50 ±0.05	0.46 ±0.07	0.50 ±0.06	0.47 ±0.08	0.48 ±0.06	0.47 ±0.05

Basal levels of extracellular acetylcholine collected by *in vivo* microdialysis from the pontine reticular formation of freely moving, narcoleptic, and control Doberman pinschers. The narcoleptic values represent the mean of five narcoleptic canines tested bilaterally and the control values represent the mean of two control and one heterozygous canines tested bilaterally. Means and S.E.M. are presented.

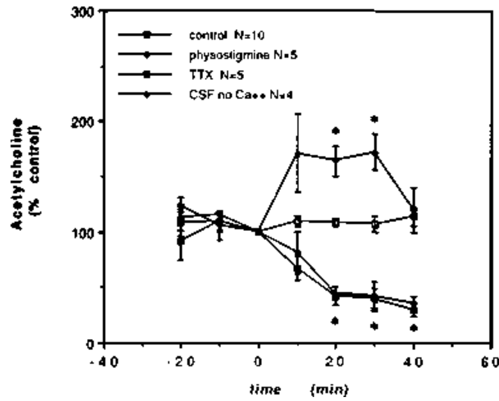

Fig. 2. Control tests on the extracellular levels of acetylcholine in the pontine reticular formation of Doberman pinschers measured by *in vivo* microdialysis. The effects of local perfusion with TTX or CSF without Ca^{2+}, and an intravenous injection of physostigmine are shown. TTX was perfused at 10^{-5} M in the perfusion medium for 40 min and the TTX group represents five unilateral microdialysis sessions in one control and four narcoleptic canines. CSF without Ca^{2+} was perfused for 40 min and the CSF without Ca^{2+} represents two bilateral microdialysis sessions in two control canines. An intravenous injection of physostigmine (0.05 mg/kg) was given at min 0 and the physostigmine group represents three bilateral microdialysis sessions in one control and two narcoleptic canines (one group of samples was omitted because acetylcholine levels could not be detected). The control group was perfused with CSF but did not receive any drug treatment and the control group represents five bilateral microdialysis sessions in one control and four narcoleptic canines. Within each group the post drug time points were compared with the basal time points using a Fisher PLSD *post hoc* test; $^*P < 0.05$ satisfactory for comparison with either basal time-point.

lower levels of acetylcholine were collected on the third day of testing, presumably due to gliotic processes around the dialysis membrane.

Control studies on the responses of extracellular PRF acetylcholine levels to peripheral and local drug treatment are shown in Fig. 2. Physostigmine and TTX were tested in both narcoleptic and control animals while CSF without Ca^{2+} was tested in control animals only. An intravenous injection of physostigmine (0.05 mg/kg) produced a rapid increase in acetylcholine levels ($F = 3.688$, d.f. = 6, $P = 0.008$), which reached a maximum (71 + 35%, n = 5) in the first 10 min and decreased thereafter. Introduction of TTX (10^{-5}M) into the perfusion medium produced a strong decrease in acetylcholine levels ($F = 8.637$, d.f. = 6, $P = 0.0001$), which reached a maximal decrease (70 + 7%, n = 5) after 40 min. Introduction of CSF without Ca^{2+} into the perfusion medium produced a similar decrease in acetylcholine levels ($F = 7.108$, d.f. = 6, $P = 0.0003$) which reached a maximal decrease (64 + 6%, n =4) after 40 min.

Behavioral studies on the response of extracellular PRF acetylcholine levels to FECT trials, motor activity and feeding behavior stimulation in the narcoleptic canines and the control group are shown in Fig. 3a,b. FECT stimulation induced cataplectic attacks in the narcoleptic canines which ranged from moderate, in which the animal could remain upright while a clear loss of muscle tone was indicated by the EMG, to severe, in which the animal would go down on all four limbs and remain atonic for up to 1 min. During episodes of cataplexy the EEG was desynchronized and both EOG and EMG activity was greatly reduced. Polygraphic data representing the characteristics of a typical FECT-induced cataplectic attack are shown in the accompanying paper.[42] FECT-induced cataplexy was accompanied by a sub-

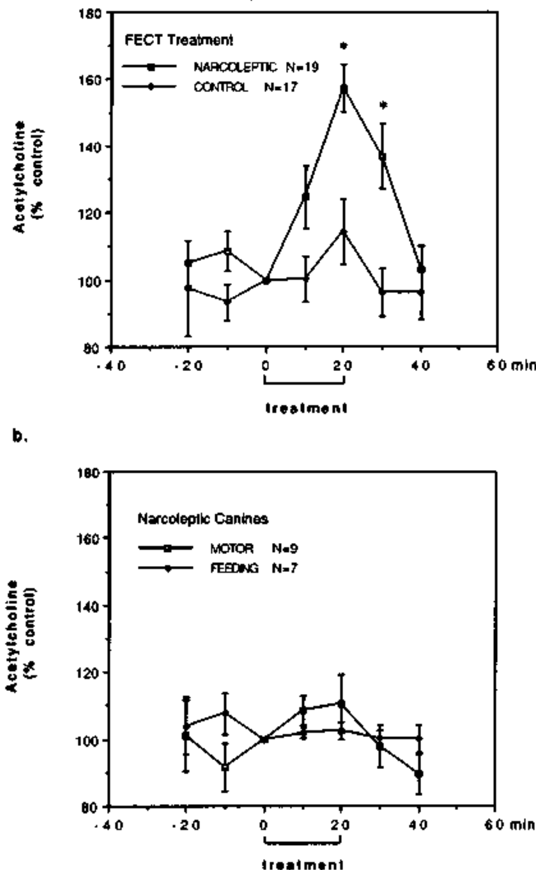

Fig. 3. Behavioral tests on the extracellular levels of acetylcholine in the pontine reticular formation of narcoleptic and control canines measured by *in vivo* microdialysis. FECT treatment (a) represents data from 10 bilateral microdialysis sessions on five narcoleptic canines and nine bilateral microdialysis sessions on three control animals (two control and one heterozygous narcoleptic canine). During min 0-10 of FECT treatment the narcoleptic canines had 8.3 + 0.9 cataplectic attacks per FECT, and during min 10-20 of FECT treatment they had 6.6 ± 0.7 cataplectic attacks per FECT. Motor and feeding treatment (b) represents data from five bilateral microdialysis sessions on five narcoleptic canines (motor) and four bilateral microdialysis sessions on four narcoleptic canines (feeding). Because acetylcholine levels were not always detectable some sample runs were omited (one per each treatment group). Within each group the post drug time-points were compared with the basal time-points using a Fisher PLSD*post hoc* test; $^*P < 0.05$ satisfactory for comparison with either basal time-point.

stantial increase in acetylcholine levels (F = 9.649, d.f. = 6, P = 0.0001), which was evident after the first 10-min stimulation period (25 ± 9%, n = 19) and reached a maximum after the second 10-min stimulation period (57 ± 7%, n = 19). The elevated levels of acetylcholine returned to basal levels within two 10-min samples following FECT stimulation. Scores for the number of cataplectic attacks per FECT during each 10-min stimulation period are indicated in the legend of Fig. 3a,b. Figure 1 illustrates a typical chromatogram of (i) a l.0pmol acetylcholine and l0.0pmol choline standard, (ii) a sample obtained from a narcoleptic canine during basal conditions, and (iii) a sample obtained from the same narcoleptic canine during FECT induced cataplexy. Though it appears that the choline levels increased during FECT induced cataplexy in Fig. 1c, this was not consistent and statistical analysis revealed that choline levels were not significantly changed during FECT-induced cataplexy in all narcoleptic canines (F = 0.647, d.f. = 6, P = 0.665). FECT stimulation did not induce cataplexy in the control group, and no changes in PRF acetylcholine levels were observed (F= 1.807, d.f. =6, P =0.104). Within the control group, PRF acetylcholine levels in the control and heterozygous canines displayed a similar lack of change after FECT stimulation (F = 0.558, d.f. = 6, P = 0.973). Comparison between narcoleptic and control animals revealed a significant difference in the response of acetylcholine levels to FECT stimulation (F = 2.632, d.f. = 6, P = 0.017). Motor activity without cataplexy in the narcoleptic canines did not significantly alter acetycholine levels (F — 1.025, d.f. = 6, P = 0.419). Eating behavior without cataplexy in the narcoleptic canines also did not alter acetylcholine levels (F = 0.586, d.f. = 6, P = 0.738). Comparison between the three behavioral stimulus conditions applied to the narcoleptic canines revealed that the increase in acetylcholine levels after FECT-induced cataplexy was significantly different than changes in acetylcholine levels after motor activity (F = 2.260, d.f. = 6, P = 0.039) and eating behavior (F = 3.319, d.f. = 6, P = 0.004). Furthermore, the motor activity and eating behavior treatment groups were not significantly different from one another (F = 0.699, d.f. = 6, P = 0.651).

Histological analysis revealed that the microdialysis probes were correctly located within the PRF (see Ref. 35). When implanted at A 5.0, the ventral and central portions of the dialysis membrane were located in the nucleus reticularis pontis oralis (RPO), near to its border with the nucleus reticularis pontis caudalis (RPC). When implanted at A 3.0 the ventral and central portions of the dialysis membrane were located in the RPC. The most dorsal portions of the membrane had contact with the dorsal pontine tegmentum.

DISCUSSION

The present study demonstrates that extracellular acetylcholine levels may be monitored in the PRF of awake, freely moving Doberman pinschers with sub-chronically implanted microdialysis probes. The levels of acetylcholine were responsive to pharmacological treatment either locally or systemically, and were enhanced during specific behavioral events in the narcoleptic canines. Thus, extracellular acetylcholine levels in the PRF of narcoleptic canines increased during episodes of FECT-induced cataplexy, which indicates that acetylcholine release in the PRF is enhanced during cataplexy in the narcoleptic canine.

Neurochemical and functional properties of the perfusion site

The recovery of a neurotransmitter from the surounding brain tissue by *in vivo* microdialysis is regulated by tissue tortuosity and interstitial clearance processes such as active re-uptake, metabolism and microvasculature.[3,33] In the present study acetylcholine was measured in the PRF using microdialysis probes containing 10^{-6}M neostigmine, which was included in the perfusion medium in order to obtain measurable levels of acetylcholine.[13] Since the retrieval acetylcholine is mediated via the re-uptake of its metabolite, choline, and since the metabolism of acetylcholine was inhibited by neostigmine, it is likely that the acetylcholine levels measured by microdialysis in this study were mainly under the influence of tissue tortuosity and microvasculature. Studies on the recovery of labeled compounds (Ca^{2+}, sucrose and dopamine) under similar conditions have shown that the concentrations of these compounds begin to decrease at about 1.0-1.5 mm, and are less than 50% of normal extracellular at 0.5 mm from the surface of the dialysis membrane.[3,4,26] Based on these findings, it may be suggested that the extracellular acetylcholine levels measured in the present study were sampled from a restricted area of the PRF within 1.0-1.5 mm of the microdialysis probe.

Histological analysis of the probe implantation sites revealed that the dialysis membranes were located within the PRF, in the RPO when implanted at A 5.0 and in the RPC when implanted at A 3.0. These two structures comprise a relatively large component of the PRF, and thus, it is likely that the acetylcholine levels measured in the present study were primarily derived from this area. Importantly, no differences were found in the basal and cataplexy stimulated levels of acetylcholine in the RPO and RPC. The RPO and RPC are heavilly innervated by cholinergic projections from the laterodorsal tegmental nucleus (LDT) and the pedunculopontine nucleus (PPT).[9,16,17,32,42] Indeed, it has been suggested that this cholinergic innervation represent collaterals of a much larger projection that innervates the entire brainstem reticular formation.[15] Thus, the acetylcholine levels measured in the present study were most likely derived from cholinergic projection neurons originating in the LDT and PPT, and the modulation of these levels might represent similar

modulations of acetylcholine release within the entire brainstem reticular formation. It was also noted that dorsal regions of the dialysis membranes were in contact with the dorsal pontine tegmentum, an area which contains the cholinergic cell bodies of the LDT and PPT.[9,17,32,42] Thus, it is possible that neurotrans-mitter released by the proximal axons and/or dendrite varicosities of the LDT and PPT neurons[36] may have also contributed to the acetylcholine levels measured in this study.

Neurons in the LDT and PPT contribute to the regulation of REM sleep (see Refs 15, 37). Studies on cats have shown that unit activity in the LDT/PPT complex is increased during REM sleep[7,14,41] and that lesions of this area can reduce REM sleep[16,45] and REM sleep atonia.[18] Indeed, the reduction in REM sleep has been correlated with the loss of cholinergic cells in the LDT/PPT complex.[45] Lesions ventral to these cholinergic nuclei, in the RPO, have also been shown to reduce REM sleep.[8,38] More recently Kodama and colleagues have shown that acetylcholine levels in the dorsomedial pontine tegmentum[22] and the nucleus paramedianus[23] are enhanced during REM sleep in cats, indicating that cholinergic projections to the brainstem reticular formation are activated during REM sleep. This would suggest that there is an increase in acetylcholine release in the PRF during REM sleep. Thus, several lines of evidence indicate that the activation of cholinergic projections to the PRF is critically involved in the generation of REM sleep.

Characterization of acetylcholine levels measured in the pontine reticular formation

The basal levels of acetylcholine in the PRF of the narcoleptic, control and heterozygous narcoleptic canines measured by *in vivo* microdialysis were approximately 0.5 pmol/l0min perfusate sample and remained stable throughout the course of the 1.5-h experimental procedure. Based on the *in vitro* recovery rate of 25% for our dialysis probes, and on the suggestion that *in vivo* recovery rates are approximately one half of *in vitro* recovery rates,[1,3,26,43] it was estimated that the extracellular concentration of acetylcholine in the PRF, under our basal conditions, was 0.3 nM. This level is similar to the basal level of acetylcholine estimated in the dorsomedial pontine tegmentum of cats during wake (0.7 nM), as reported by Kodama and colleagues in a study using 2-mm dialysis probes with a perfusion medium containing 10^{-4}M physostigmine.[22] Interestingly, we found no differences in the basal levels of acetylcholine in the PRF of control and narcoleptic canines. This was a bit of a surprise since previous studies have shown that muscarinic binding in the PRF is elevated in narcoleptic canines,[5,21] a finding which might suggest receptor supersensitivity due to chronically reduced levels of acetylcholine neurotransmission. The data on basal levels should be taken with a note of caution, however, because of the limited number of control animals tested. Perhaps the increased level of muscarinic receptors in the PRF of narcoleptic canines represents a fundamental abnormality in narcoleptic canines which contributes directly to the incidence of cataplexy.

The levels of extracellular acetylcholine measured in the PRF were strongly reduced when tetrodotoxin (10^{-5} M) was introduced into the perfusion medium, which indicates that the acetylcholine levels were predominantly derived from a voltage dependent pool of neurotransmitter.[46] Furthermore, extracellular acetylcholine levels in the PRF were strongly reduced when CSF without Ca^{2+} was introduced into the perfusion medium, suggesting that the acetylcholine levels were derived from Ca^{2+}-dependent neurotransmitter release. Therefore, it is likely that the acetylcholine levels monitored in this study represent neuronally released neurotransmitter of synaptic origin. In addition, we also found that the acetylcholine levels in the PRF were greatly enhanced after intravenous injections of physostigmine. The increase was rapid and the levels remained elevated up to 40min after the injection and returned to baseline shortly thereafter, which is similar to the time-course for the increase in cataplexy which occurs after the administration of physostigmine.[35] This finding is also consistent with the suggestion that the acetylcholine levels monitored in this study represent neuronally released neurotransmitter, and furthermore, this demonstrates that the increase in cataplexy produced by systemic administration of physostigmine is associated with a significant increase in acetylcholine levels in the PRF.

Acetylcholine release in the pontine reticular formation during behavioral stimulation

The levels of acetylcholine collected in the PRF of the narcoleptic canines were greatly increased during periods of FECT-induced cataplexy. This effect was evident after the first two FECT trials and appeared to be cumulative in nature, since the increase was greater after all four FECT trials. This cumulative effect, as well as the gradual return to basal levels over the following two perfusate samples, may be due to the diminished clearance of acetylcholine from the extracellular space because of the presence of an acetylcholinesterase inhibitor (neostigmine). The increase in acetylcholine levels likely reflects an increase in acetylcholine release from afferent cholinergic nerve terminals. This increase in acetylcholine levels is similar to that reported by Kodama and colleagues, who found a 60% increase in acetylcholine levels in the dorsomedial pontine tegmentum[22] and a 30% increase in acetylcholine levels in the nucleus paramedianus[23] during REM sleep. Thus, these findings suggest that cataplexy and REM sleep are regulated by similar cholinergic mechanisms in the brainstem reticular formation. Though these studies have not provided conclusive evidence to describe the neuronal pathways mediating these responses, it is probable

that the cholinergic neurons of the LDT/PPT complex play an important role.

The other behavioral tests in this study, motor activity and feeding behavior, failed to produce any significant changes in acetylcholine levels in the PRF of the narcoleptic canines. These findings show that the increase in acetylcholine levels seen during the FECT trials was not due to the motor activity and/or eating behavior which accompanies a FECT trial. This is important to note, since previous reports have shown that unit activity in the medial PRF is similarly increased during both REM sleep and motor activity,[39,40] and that extracellular levels of acetylcholine in the hippocampus[20] and the striatum[44] are similarly increased during REM sleep and motor activity. Furthermore, FECT trials administered to the control group, which exhibited the normal motor and eating behavior of a FECT without the cataplexy, also did not produce a significant change in PRF acetylcholine levels. However, it must be recognized that a tendency, though not statistically significant, for an increase in acetylcholine levels was observed after motor behavior in the narcoleptic animals and after FECT stimulation in the control animals. It is possible that this represents an increase in pontine cellular activity, as was reported to occur during motor activity in cats.[39,40] Nevertheless, it is suggested that the increase in acetylcholine levels seen in the narcoleptic canines in the present study was primarily linked to the cataplectic behavior induced by the FECT trials. Indeed, it is possible that cataplexy is elicited as a result of the enhanced release of acetylcholine in the PRF of narcoleptic canines.

CONCLUSION

The present findings demonstrate that extracellular acetylcholine levels in the PRF of Doberman pinsch-ers may be monitored *in vivo* during specific behavioral events. The levels of acetylcholine decreased after local perfusion of TTX and CSF without Ca^{2+}, and increased after intravenous physostigmine, which indicates that they represent neuronally released neurotransmitter. Acetylcholine levels in the PRF of narcoleptic canines were increased during FECT induced cataplexy, but not during feeding behavior or motor activity. Furthermore, acetylcholine levels in the PRF of control animals did not change during FECT stimulation. Therefore, it is suggested that cataplexy is associated with an increase in acetylcholine release in the PRF. This is similar to the reported increases in acetylcholine release in the dorsomedial pontine tegmentum[22] and the nucleus paramedianus[23] during REM sleep in cats. Thus, the present study provides strong evidence that cataplexy and REM sleep are regulated by similar brainstem cholinergic mechanisms.

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