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Muscarinic Cholinergic Receptors and the Canine Model of Narcolepsy

Thomas S. Kilduff, S. Scott Bowersox, Kenneth I. Kaitin, Theodore L. Baker, Roland D. Ciaranello, and William C. Dement

Department of Psychiatry and Behavioral Sciences, Stanford University School of Medicine, Stanford, California, U.S.A.

Summary: The role of the muscarinic cholinergic receptor in narcolepsy was examined using radioligand binding to various brain regions of normal and genetically narcoleptic Doberman pinschers. In this multi-litter study, a previous report of a proliferation of muscarinic cholinergic receptors in the brainstem was confirmed, and the concentration of the M_2 receptor subtype, in particular, was elevated. This up-regulation of brainstem cholinergic receptors suggests a problem with release of acetylcholine, which, together with previous reports of an impairment of dopamine release, may be indicative of a fundamental membrane problem in narcolepsy. **Key Words:** Narcolepsy—Cataplexy—Sleep—Acetylcholine—Muscarinic receptor—Aminal model—Heredity.

Cataplexy, the loss of muscle tonus due to emotional stimulation, is the pathognomic symptom of narcolepsy in humans and dogs. In the canine model of narcolepsy (1–3), the food-elicited cataplexy test (FECT) has been established as a standardized tool for testing the efficacy of various pharmacological compounds on cataplexy (4). Earlier studies utilizing the FECT demonstrated that cholinergic compounds had marked effects on both the incidence and the severity of cataplectic episodes (5). These effects appear to be central in nature, since the anticholinesterase physostigmine facilitates cataplexy, whereas neostigmine, which does not penetrate the blood–brain barrier, has no effect. The muscarinic cholinergic receptor, in particular, is implicated, since the muscarinic agonist arecoline hydrochloride facilitates cataplexy, whereas nicotine and nicotinic antagonists are ineffective. This hypothesis is supported by the observation of a proliferation of the number of muscarinic cholinergic receptors in the brainstem and a decrement in receptor number in several forebrain and diencephalic regions of the genetically narcoleptic dog (6). In this previous study, however, the control and experimental groups each comprised only a single litter of dogs, which raises the possibility that the results were due to variation between litters rather than true differences between narcoleptic and normal animals.

Displacement studies of [^{3}H] muscarinic antagonists by muscarinic agonists suggest the possibility of multiple muscarinic agonist binding sites (7). These agonist sites are indis-

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The present address of Dr. Baker is Sleep Disorders Center, Department of Psychiatry, Health Sciences Center, State University of New York, Stony Brook, NY 11794, U.S.A.

Address correspondence and reprint requests to Dr. T. S. Kilduff at Sleep Disorders Center, Department of Psychiatry TD-114, Stanford University School of Medicine, Stanford, CA 94305, U.S.A.

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tinguishable by classical antagonists but can be discriminated by the atypical muscarinic antagonist pirenzepine (8). Recent studies (9,10) have shown that there are at least two distinct populations of muscarinic receptors in brain tissue. The purposes of the present study were to confirm the earlier report of an alteration of the number of muscarinic cholinergic receptors utilizing experimental and control groups comprising multiple litters and to examine the relative involvement of the two major subtypes of the muscarinic cholinergic receptor, M_1 and M_2.

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METHODS

Five normal (3 male, 2 female) and five narcoleptic (2 male, 3 female) Doberman pinschers, previously entrained to a 12L:12D light cycle with lights off at 2000 h, were killed with an overdose of sodium thiopental between 1500 and 1730 h. Narcoleptic animals were derived from three separate litters; the normal dogs were from two litters. Brains were frozen in 2-methylbutane/dry ice and subsequently slightly thawed and dissected into subregions. The regions of interest were homogenized in 6 ml of 50m*M* NaK-phosphate buffer (pH 7.4), and an aliquot containing ~100 mg tissue was spun at 14,000 *g* for 10 min. Other aliquots were set aside for benzodiazepine and dopamine receptor assays (11). The supernatant was discarded and the pellet washed in 6 ml buffer and respun. The resultant pellet was resuspended in 6 ml buffer and frozen at − 80°C until assayed within 48 h. At the time of assay, the frozen homogenates were thawed and respun again at 14,000 *g* and the pellet brought to appropriate volume for the assay.

To measure the total population (M_1 and M_2) of muscarinic receptors, homogenates from each brain were incubated in [^{3}H]quinuclidinyl benzilate (QNB; spec. act. 31.9 Ci/mmol) with and without 1 μ*M* atropine (12). Tissues were incubated in a final reaction volume of 1.0 ml at 30°C for 60 min. To measure the M_1 receptor selectively, tissue homogenates were incubated in [^{3}H]pirenzepine (82.0 Ci/mmol) with and without 1 μ*M* atropine in a volume of 0.5 ml at 30°C for 60 min. Reactions were terminated using a rapid filtration assay onto glass fiber filters (Micro Filtration Systems, Dublin, CA, U.S.A.) that had been immersed in 0.1% polyethylenimine (Sigma Chemical Co., St. Louis, MO, U.S.A.) for at least 10 min before use to prevent polar binding of compounds to the filters. Triplicate measurements were made of total binding at each concentration; nonspecific binding was measured in duplicate. All tritiated ligands were obtained from New England Nuclear (Boston, MA, U.S.A.). Binding data were analyzed by Scatchard analysis using the LIGAND program (13) modified for use on an IBM PC-XT.

RESULTS

Preliminary studies revealed that [^{3}H]QNB binding to dog frontal cortex was linear in the concentration range of 0.04–0.35 mg protein per milliliter and [^{3}H]pirenzipine binding was linear between 0.05 and 0.47 mg protein per milliliter. To minimize tissue requirements for each assay, a concentration of 0.05 mg/ml was utilized for [^{3}H]QNB assays and 0.28 mg/ml for [^{3}H]pirenzepine assays. Varying the time of incubation revealed that optimal specific binding for both ligands was achieved at 60 min.

A typical saturation isotherm and Scatchard plot for QNB binding is illustrated in Fig. 1. The affinity (K_d) did not differ between groups for QNB or pirenzepine in any of the five regions studied. The K_d was consistently higher for QNB (25–50 p*M*) than for pirenzepine binding (4–10 n*M*) in all regions examined in both groups. However, binding for

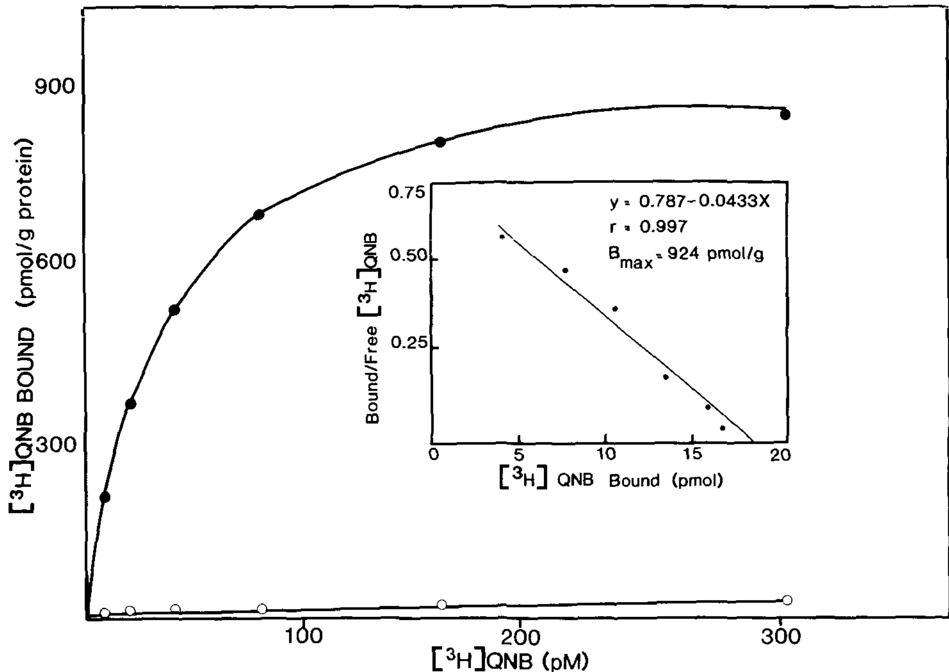

FIG. 1. Saturation isotherm for [^{3}H]quinuclidinyl benzilate (QNB) binding to olfactory lobe from a control Doberman pinscher. Filled circles, specific binding; open circles, nonspecific binding; inset, Scatchard plot computed from these data. B_{max}, binding sites.

pirenzepine was nonspecific and nonsaturable in the two brainstem regions examined (nucleus pontis oralis and nucleus reticularis gigantocellularis).

The number of pirenzepine binding sites (B_{max}) did not differ in any of the three regions in which specific binding was measurable (olfactory lobe, amygdala, and medial caudate nucleus). On the other hand, for QNB binding, there was a nonsignificant tendency for an increased B_{max} in the caudate nucleus and a significantly higher level in the nucleus reticularis gigantocellularis (Fig. 2).

In the three forebrain regions examined, >90% of sites labeled with [^{3}H]QNB were also labeled with [^{3}H]pirenzepine and, therefore, were presumably of the M_1 subtype. The absence of specific binding with [^{3}H]pirenzepine in the brainstem is suggestive of an M_2 receptor distribution in this region.

DISCUSSION

The current study, utilizing experimental and control groups derived from multiple litters, supports the previous claim (6) of a proliferation of the muscarinic cholinergic receptor in brainstem regions. However, the report of a decrement in receptor number in the amygdala and medial caudate nucleus (6) was not verified; the current study found a tendency for an increase in B_{max} in the medial caudate nucleus rather than a significant decrease. The differing results in these two studies can be attributed in part to a single versus multiple litter design in each experimental group. Other factors that may influence receptor number include the time of day of death (14), differing sex distribution in the two studies, and a smaller number of animals in the control group in the present study. However, given that

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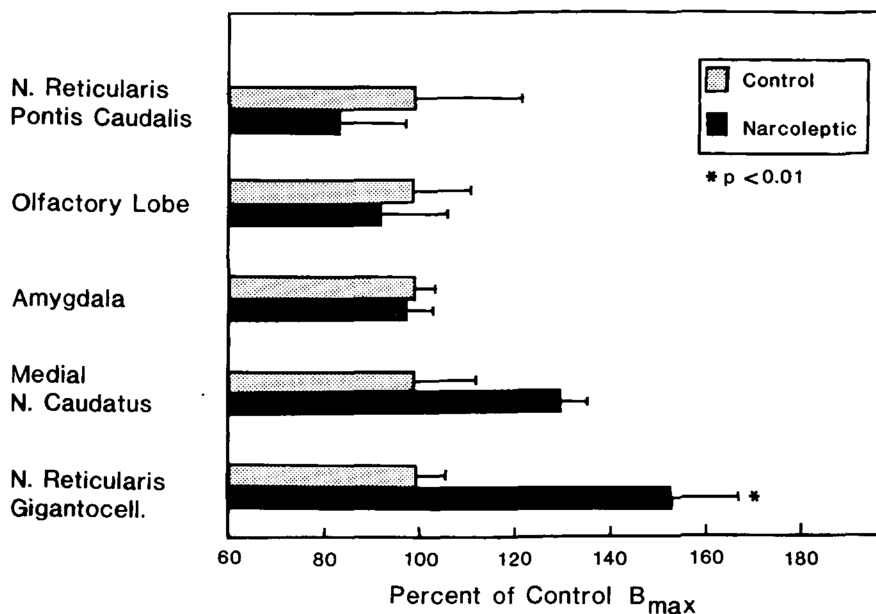

FIG. 2. Normalized distribution of the number of muscarinic cholinergic receptors (B_{max}) in five regions of Doberman pinscher brain.

a greater B_{max} was found in the medial caudate as measured with both [^{3}H]QNB and [^{3}H]pirenzepine, we have a high degree of confidence in the current results.

Proliferation of muscarinic cholinergic receptor in the nucleus reticularis gigantocellularis verified in the current study is probably of the M_2 subtype. Although we have not measured the M_2 receptor directly, an increase was detectable with QNB, which measures the total population of muscarinic cholinergic receptors, and no M_1-specific binding was measurable in this region with [^{3}H]pirenzepine. This observation is consistent with the fact that M_1 receptors are generally found in forebrain and M_2 receptors in the brainstem of the rat (10).

Our results support the concept of a hypersensitivity of the cholinergic system in narcolepsy (5,15). A proliferation or up-regulation of receptor number can occur as a compensatory response to a problem with synthesis or release of neurotransmitter. On the basis of neurochemical and metabolite measurements, a deficit in dopamine release has previously been suggested in narcoleptic dogs (16,17). Furthermore, M_2 cholinergic receptors are thought to regulate the release of acetylcholine from cholinergic nerve terminals (18). Taken together, these observations suggest that transmitter release may be a general problem in narcoleptics and may indicate a fundamental molecular membrane deficit.

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