Pharmacology Biochemistry & Behavior, Vol. 38, pp. 115–124. © Pergamon Press plc, 1991. Printed in the U.S.A. 0091-3057/91 $3.00 + .00

Muscarinic Receptor Subtypes and Sexual Behavior in Female Rats

GARY P. DOHANICH, DAVID M. McMULLAN, DEBRA A. CADA AND KIMBERLY A. MANGUM

Department of Psychology and Neuroscience Program
Tulane University, New Orleans, LA 70118

Received 6 April 1990

DOHANICH, G. P., D. M. McMULLAN, D. A. CADA AND K. A. MANGUM. *Muscarinic receptor subtypes and sexual behavior in female rats.* PHARMACOL BIOCHEM BEHAV **38**(1) 115–124, 1991.—Cholinergic muscarinic systems are involved in the regulation of female sexual behavior in rats and hamsters. This series of experiments was designed to determine whether sexual behavior in female rats is controlled preferentially by one of the traditional muscarinic receptor subtypes. Intraventricular infusion of the muscarinic antagonist scopolamine (10 μg bilaterally) which binds with high affinity to both M1 and M2 subtypes inhibited sexual behavior, as indicated by the incidence of lordosis, in ovariectomized rats treated with estrogen and progesterone. In contrast, the M1-selective antagonist pirenzepine failed to reduce the incidence of lordosis following intraventricular infusion (10 to 80 μg bilaterally). Biochemical analyses revealed that intraventricular infusion of scopolamine (10 μg bilaterally) inhibited both M1 and M2 binding in brain tissues while intraventricular infusion of pirenzepine (10 μg bilaterally) completely inhibited M1 binding without affecting M2 binding. Intraventricular infusions of the acetylcholinesterase inhibitor physostigmine (10 μg bilaterally), the cholinergic agonist carbachol (1 μg bilaterally), and the muscarinic agonist oxotremorine-M (0.1 μg bilaterally) activated lordosis in ovariectomized females primed with low doses of estrogen. In contrast, the putative M1 agonist McN-A-343 failed to significantly increase lordosis following intraventicular infusions (1, 10, 20 μg bilaterally). According to biochemical results, the ability of these agents to activate lordosis in female rats was related to their affinities for M2 binding sites not M1 binding sites. In a final experiment, estrogen treatment of ovariectomized rats did not alter muscarinic subtype binding in several brain areas as measured by the M1-selective ligand [^{3}H] pirenzepine and the M2-selective ligand [^{3}H] oxotremorine-M. The results of these experiments confirm that muscarinic systems contribute to the regulation of lordosis in female rats and indicate that M2 binding sites rather than M1 binding sites may be a critical component of this regulation.

Sexual behavior Acetylcholine Muscarinic subtypes Scopolamine Pirenzepine Physostigmine Carbachol Oxotremorine-M McN-A-343

AGENTS that act at cholinergic synapses have been found to alter sexual behaviors displayed by female rats and hamsters. These compounds are particularly effective in activating or inhibiting the lordosis response, a reflexive arching of the spine that female rodents exhibit during sexual receptivity. Following intracerebral administration, agonists including carbachol, bethanechol, oxotremorine, acetylcholine, and physostigmine reliably activate lordosis in ovariectomized rats primed with low doses of estrogen (5, 7–9). The facilitative effects of cholinergic agonists on lordosis are mediated by muscarinic receptors since the activation of lordosis by these agents is blocked completely by pretreatment with muscarinic receptor blockers such as atropine and scopolamine (5, 8, 9). In addition, compounds that antagonize cholinergic activity, such as the receptor blockers atropine and scopolamine or the acetylcholine synthesis blocker hemicholinium-3, inhibit sexual behaviors in ovariectomized rats and hamsters treated with estrogen and progesterone (5, 6, 10, 23) and in intact female rats (26).

While available data strongly implicate cholinergic muscarinic receptors in the regulation of sexual behavior in female rats and hamsters, these receptors now are known to exist in multiple types throughout the body. Although as many as five independent species of a protein that corresponds to the muscarinic binding site have been identified and cloned (3), two primary subtypes, denoted M1 and M2 (18,22), have been studied extensively with traditional methodology. Evidence indicates that these two muscarinic receptor subtypes exhibit different drug affinities, anatomical distributions, and functional properties. According to most interpretations, the M1 subtype or 'pirenzepine-sensitive' site displays a higher affinity for the antagonist pirenzepine and concentrates in telencephalic structures such as hippocampus and cortex (21, 25, 27, 33, 38). The M2 subtype or 'pirenzepine-insensitive' site displays a higher affinity for agonists such as carbachol and oxotremorine-M and predominates in diencephalic and mesencephalic structures including the hypothalamus and central gray (2, 25, 27, 33). Although controversial, various reports have concluded that M1 and M2 receptor subtypes also differ in cellular locations (24), electrophysiological actions (13), and membrane effector mechanisms (15). To date, few studies have examined the role of muscarinic receptor subtypes in the regulation of mammalian behaviors (19).

The objective of the present study was to determine if sexual behavior in female rats, specifically lordosis behavior, is regulated preferentially by a major cholinergic receptor subtype. In a series of behavioral and biochemical experiments, the ability of various agents to interact with muscarinic receptor subtypes was

compared to the ability of these compounds to alter lordosis in female rats.

GENERAL METHOD

Subjects

Long-Evans hooded female rats purchased from Harlan Sprague-Dawley Inc. (Indianapolis, IN) were housed individually with free access to food and water throughout the study. A reversed light-dark cycle was maintained in the temperature-controlled vivarium (12-h light:12-h dark, lights off at 1100 h). Females were between 100–200 days of age during the course of the experiments.

Muscarinic Binding Assays

For determination of muscarinic receptor binding, tissues were homogenized in 0.32 M sucrose or Krebs-Ringer buffer and stored at −60°C prior to assay. Homogenates were incubated in the appropriate buffer (pH 7.4) containing the nonselective muscarinic antagonist [^{3}H] quinuclidinyl benzilate (42 Ci/mmol), the M1-selective antagonist [^{3}H] pirenzepine (74.4 Ci/mmol), or the M2-selective agonist [^{3}H] oxotremorine-M (87 Ci/mmol). Incubation time and temperature varied with experiment. Nonspecific binding was determined in parallel incubations in the presence of 1 μM atropine sulfate. Following incubation, binding reactions were terminated by rapid filtration under vacuum through glass fiber filters (No. 32, Schleicher and Schuell, Keene, NH). In experiments with [^{3}H] pirenzepine or [^{3}H] oxotremorine-M, filters were presoaked in 0.1% polyethylenimine to reduce nonspecific binding. All filters were washed 4 times with 5 ml of 10 mM sodium-potassium phosphate buffer, transferred to scintillation vials, and dried overnight. Samples were counted in 3 or 5 ml of Ecoscint scintillation fluid (National Diagnostics, Manville, NJ) at 38% efficiency using a Beckman LS1701 scintillation counter. Specific binding expressed as fmol/mg protein was defined as total binding in the absence of atropine minus nonspecific binding in the presence of atropine. Protein determinations were made using the method of Bradford (4).

Surgical Procedures

Rats were anesthetized with ketamine (100 mg/kg, Bristol Laboratories, Syracuse, NY) and xylazine (7.4 mg/kg, Miles Laboratories, Shawnee, KS) during all surgical manipulations. Females were bilaterally ovariectomized at approximately 100 days of age. All ovariectomized females displayed high levels of sexual behavior in response to exogenous hormone treatments administered two weeks after ovariectomy (0.5 μg estradiol benzoate for 3 days followed by 500 μg progesterone 4–5 h before testing). Females were implanted stereotaxically with double-barrel cannulae that consisted of a chronic 23-gauge outer barrel and a 28-gauge inner barrel that was removed only during intracerebral infusions. The outer barrel was anchored to the skull with machine screws and dental acrylic. The inner barrel extended 1 mm beyond the tip of the outer barrel into the lateral ventricle. All cannula implants were placed bilaterally at the level of bregma 1.25 mm lateral to the midline.

Testing and Treatment Procedures

Behavioral tests were conducted in glass aquaria (50 × 25 × 30 cm) occupied by a Long-Evans stimulus male rat. Each female was mounted by the male a total of 10 times during any test. Females failing to receive 10 mounts within 5 min were transferred to another arena where the test was completed with a different male. The number of lordotic responses observed per 10 mounts was recorded and a lordosis quotient was calculated for each test: lordosis quotient = (number of lordotic responses/10 mounts) × 100.

During behavioral sessions, each female was tested for sexual behavior prior to intracerebral treatment (pretest) and then bilaterally infused with the appropriate cholinergic agent or Krebs-Ringer vehicle. Krebs-Ringer buffer (pH 7.4) consisted of 123 mM NaCl, 4.8 mM KCl, 0.8 mM $CaCl_2$, 1.2 mM $MgSO_4$, 12 mM NaH_2PO_4, and 10 mM glucose. Since glucose has been reported to reduce the effects of muscarinic antagonists (35), the sugar was omitted from buffers used for antagonists. Solutions were delivered in 0.5-μl volumes over a 30-s period through a 28-gauge infusion cannula connected to a Hamilton microsyringe with PE 20 polyethylene tubing. The effects of intracerebral treatment on sexual behavior always were tested 15 min after infusion.

Chemicals

Estradiol-17β benzoate and progesterone were purchased from Sigma Chemical Co. (St. Louis, MO). Pirenzepine dihydrochloride, oxotremorine methiodide (oxotremorine-M), and McN-A-343 were purchased from Research Biochemicals Inc. (Natick, MA). Atropine sulfate, carbamylcholine chloride (carbachol), scopolamine hydrochloride, physostigmine hemisulfate, amitriptyline, and polyethylenimine were purchased from Sigma Chemical Co. [^{3}H] Quinuclidinyl benzilate was purchased from Amersham Corp. (Arlington Heights, IL). [^{3}H] Pirenzepine and [^{3}H] oxotremorine-M were purchased from DuPont Co. (Wilmington, DE).

EXPERIMENT 1

In the first experiment, the affinities of three muscarinic antagonists for M1 and M2 binding sites were evaluated. Muscarinic binding sites corresponding to the putative M1 subtype are confined largely to telencephalic regions such as the hippocampus, while diencephalic and mesencephalic regions (referred to here as brain stem) are enriched in the putative M2 subtype (25, 27, 33, 38). To determine M1 and M2 binding affinities, competition analyses of the antagonists scopolamine, pirenzepine, and amitriptyline were conducted using hippocampal binding sites labelled by the M1-selective antagonist [^{3}H] pirenzepine (34,38) and brain stem binding sites labelled by the M2-selective agonist [^{3}H] oxotremorine-M (16,38). Previous competition experiments indicate that scopolamine has at least a 10-fold greater affinity for M1 sites and at least a 100-fold greater affinity for M2 sites than pirenzepine (14). In addition, pirenzepine normally displays a 50-fold greater affinity for M1 binding sites compared to M2 sites. Amitriptyline, a tricyclic antidepressant, has been reported to inhibit cortical acetylcholine release regulated by M2 receptors which has been interpreted as indicative of M2 selectivity (17). In Experiment 1, the subtype affinities of these three antagonists were assessed in vitro in brain tissues obtained from ovariectomized rats treated with estrogen and progesterone and in a Krebs-Ringer buffer used in later behavioral experiments for intraventricular infusions.

METHOD

Three ovariectomized rats were treated with 1 μg/kg estradiol benzoate at 72, 48, and 24 h before sacrifice and 500 μg progesterone at 5 h before sacrifice. In 3 independent experiments, the

hippocampus and brain stem were dissected and homogenized in 6 ml of Krebs-Ringer buffer (pH 7.4). The entire hippocampus was removed from both halves of the hemisected brain. Brain stem then was removed from the ventral aspect of the brain that extends from the caudal border of the preoptic area to the rostral border of the cerebellum. To determine the binding affinities of scopolamine, pirenzepine, or amitriptyline for M1 sites approximately 100 μg of hippocampal protein were added to Krebs-Ringer buffer containing 1 nM of [^{3}H] pirenzepine and increasing concentrations of competing antagonist in a final volume of 500 μl. To determine the binding affinities of the three antagonists for M2 sites approximately 125 μg of brain stem protein were added to Krebs-Ringer buffer containing 1 nM of [^{3}H] oxotremorine-M and increasing concentrations of competing antagonist in a final volume of 500 μl. All preparations were incubated for 1 h at room temperature. Competing antagonist concentrations ranged from 10^{-11} to 10^{-4} M. In all experiments, the concentrations of M1 hippocampal binding sites and M2 brain stem binding sites were equal. Means of percent specific binding at each concentration of antagonist obtained from 3 separate experiments were computed. IC_{50} values were estimated from linear regression analyses of mean competition data transformed to log/logit plots (logit = ln [P/100 − P] where P = percent specific binding at each concentration of competitor).

RESULTS

As indicated in Fig. 1, scopolamine displayed the highest affinity for M1 binding sites in hippocampal tissue with IC_{50} values (0.3 nM) much lower than IC_{50} values for pirenzepine (13.7 nM) or amitriptyline (14 nM). Scopolamine also displayed the highest affinity for M2 binding sites in brain stem tissue with IC_{50} values (2.9 nM) substantially lower than pirenzepine (635.8 nM) or amitriptyline (50.5 nM). All three antagonists displayed a higher affinity for M1 rather than M2 binding sites. Although scopolamine and amitriptyline had moderately higher affinities for M1 compared to M2 binding sites the affinity of pirenzepine for M1 sites was almost 50-fold greater than its affinity for M2 sites.

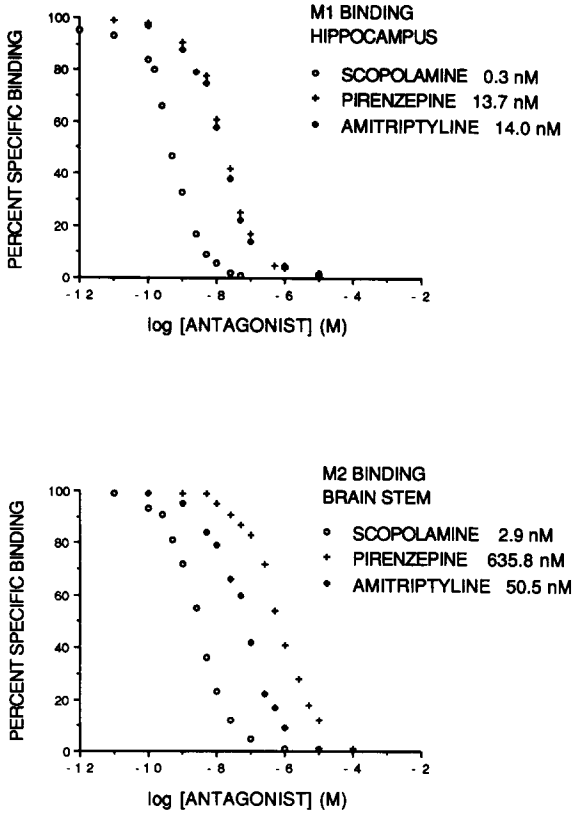

FIG. 1. Competition of antagonists for muscarinic subtype binding in rat brain. Various concentrations of scopolamine, pirenzepine, or amitriptyline were competed against (upper) the M1-selective ligand [^{3}H] pirenzepine (1 nM) in hippocampal homogenates or (lower) the M2-selective ligand [^{3}H] oxotremorine-M (1 nM) in brain stem homogenates. Duplicate samples were incubated for 1 h at room temperature in 500-μl final volumes. Values represent means from 3 separate experiments for scopolamine and pirenzepine and 2 separate experiments for amitriptyline. IC_{50} values were calculated on mean data by linear regression following log/logit transformations.

EXPERIMENT 2

The results of Experiment 1 confirm previous in vitro experiments that indicate high but unequal affinities of scopolamine for M1 and M2 binding sites (14, 34, 37). In further agreement with earlier in vitro work, pirenzepine displayed a moderately high affinity for M1 sites but a relatively weak affinity for M2 sites (34,37). Unfortunately, few experiments have addressed the binding of these antagonists with muscarinic binding sites in vivo. Experiment 2 was conducted to assess the in vivo binding of scopolamine and pirenzepine with muscarinic binding sites in various brain regions following intraventricular infusions. Specifically, several brain areas were dissected within 20 min after intraventricular infusions of scopolamine (10 μg/cannula) or pirenzepine (10 μg/cannula) and muscarinic receptor binding was measured in homogenates with the muscarinic antagonist [^{3}H] quinuclidinyl benzilate. Although the brain regions at which cholinergic compounds act to alter female sexual behavior are unknown, several putative loci were examined including the septum, hypothalamus, hippocampus, midbrain central gray, and peripeduncular nucleus. In addition, in order to determine if intraventricular infusions of scopolamine or pirenzepine bind to M1 and M2 sites in brain regions enriched in these subtypes, muscarinic subtype binding in hippocampal and brain stem regions was measured in homogenates using the M1-selective antagonist [^{3}H] pirenzepine and the M2-selective agonist [^{3}H] oxotremorine-M.

METHOD

Eighteen ovariectomized Long-Evans females with indwelling ventricular cannulae were injected intramuscularly with 1 μg/kg estradiol benzoate for 3 days and 500 μg progesterone 4–5 h before infusion. Females were infused bilaterally with Krebs-Ringer buffer (0.5 μl/cannula, n = 6), scopolamine (10 μg/cannula, n = 6), or pirenzepine (10 μg/cannula, n = 6) and sacrificed 10 min after treatment. The brain was removed and five regions were dissected as described previously (11,12) within 20 min after infusion: septum, medial basal hypothalamus, dorsal hippocampus, central gray, and peripeduncular area. Tissues were homogenized in 200 μl of 0.32 M sucrose in plastic microfuge tubes with a fitted teflon pestle and frozen at −60°C prior to assay. For determination of muscarinic receptor binding, 40 μl of homogenate were incubated in 10 mM sodium-potassium phosphate buffer (pH 7.4) containing 5 nM [^{3}H] quinuclidinyl benzilate in final volumes of 50 μl for 2 h at room temperature. For determination of muscarinic subtype binding, 15 ovariectomized rats were treated with estradiol benzoate and progesterone and infused intraventric-

ularly as described above (n = 5 per treatment). The ventral hippocampus and brain stem were dissected and M1 binding was measured in hippocampal homogenates with [^{3}H] pirenzepine while M2 binding was measured in brain stem homogenates with [^{3}H] oxotremorine-M. Binding was determined in Krebs-Ringer buffer containing 5 nM [^{3}H] ligand in final volumes of 50 μl for 1 h at room temperature.

RESULTS

As indicated in Fig. 2, [^{3}H] quinuclidinyl benzilate binding in tissues from females infused with scopolamine was less than 15% of binding measured in corresponding tissues from control females. Bilateral infusion of scopolamine at a dose that normally reduces lordosis (10 μg/cannula) almost completely inhibited muscarinic binding in all brain areas examined within the time that behavioral effects are observed. Muscarinic binding in areas distant from the infusion site such as the central gray and peripeduncular area was reduced almost to the same degree as binding in areas proximal to the infusion site such as the septum and dorsal hippocampus. These results indicate that at a dose of 10 μg/cannula scopolamine can gain access to a wide extent of the brain and inhibit muscarinic binding in these regions.

In contrast, [^{3}H] quinuclidinyl benzilate binding was reduced to approximately 50% of control values in septum, hypothalamus, and hippocampus following intraventricular infusion of pirenzepine (10 μg/cannula). Pirenzepine failed to alter [^{3}H] quinuclidinyl benzilate binding in central gray and peripeduncular regions. The weaker effects of pirenzepine on [^{3}H] quinuclidinyl benzilate binding compared to scopolamine may be the result of either limited diffusion or lower affinity for muscarinic binding sites, particularly M2 sites concentrated in brain stem areas.

As indicated in Fig. 2, intraventricular infusion of scopolamine strongly inhibited M1 binding in ventral hippocampus and M2 binding in brain stem. In contrast, intraventricular infusion of pirenzepine dramatically inhibited M1 binding in ventral hippocampus but had little effect on M2 binding in brain stem. Since the ventral hippocampal and brain stem regions that were removed in our dissections are at equal distance from the intraventricular infusion site, it is unlikely that differential diffusion of pirenzepine could account for differences in the ability of pirenzepine to inhibit M1 in the hippocampus and M2 binding in brain stem. The lack of inhibition of M2 binding in brain stem regions following intraventricular infusion of pirenzepine is probably the result of the weak affinity of pirenzepine for M2 binding sites concentrated in this area. Consequently, an intraventricular dose of pirenzepine of 10 μg/cannula appeared to block M1 binding without affecting M2 binding.

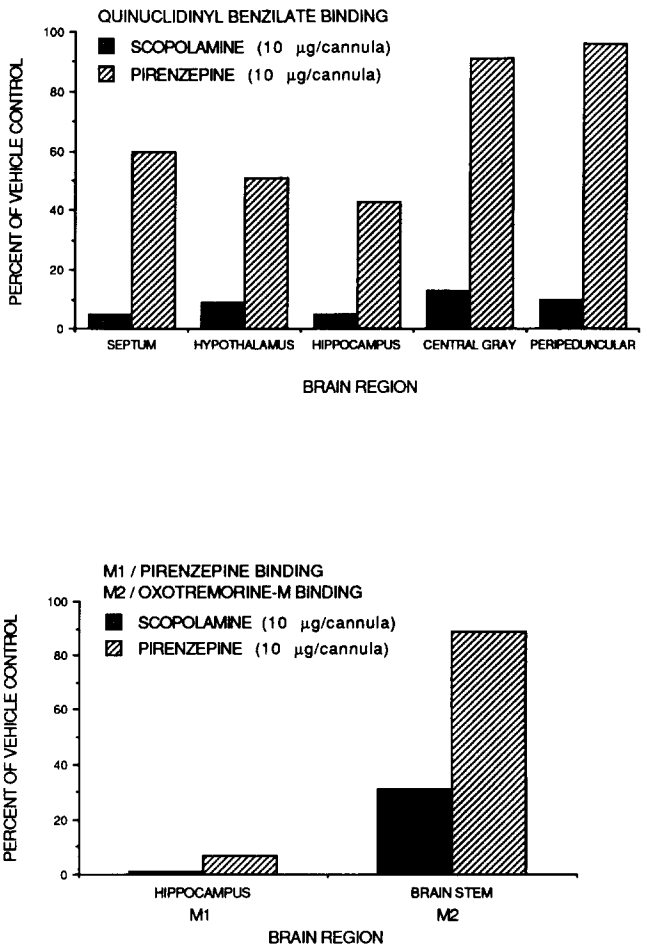

FIG. 2. Effects of intraventricular infusion of muscarinic antagonists on muscarinic binding in rat brain. Ovariectomized rats primed with estrogen and progesterone were infused bilaterally in the lateral ventricles with Krebs-Ringer vehicle (0.5 μl/cannula), scopolamine (10 μg/cannula), or pirenzepine (10 μg/cannula). Animals were sacrificed 10 min after infusion and various brain regions were dissected within 20 min after infusion. Tissues were homogenized in 200 μl of sucrose and duplicate 40 μl aliquots were incubated in final volumes of 50 μl with (upper) [^{3}H] quinuclidinyl benzilate (5 nM) for 2 h at room temperature or (lower) [^{3}H] pirenzepine (5 nM) or [^{3}H] oxotremorine-M (5 nM) for 1 h at room temperature. Values represent percent of specific binding compared to rats infused with Krebs-Ringer vehicle.

EXPERIMENT 3

In Experiment 3, the sexual behavioral effects of the M1-selective antagonist pirenzepine, the tricyclic antidepressant amitriptyline, and the muscarinic antagonist scopolamine were compared. Intraventricular doses of scopolamine of 5 to 20 μg/cannula significantly inhibit lordosis in ovariectomized Sherman albino rats primed with estrogen and progesterone [(5), unpublished data)]. Experiment 2 indicated that an intraventricular pirenzepine dose of 10 μg/cannula inhibits M1 binding with minimal effect on M2 binding. Consequently, the effects of pirenzepine on sexual behavior were evaluated over a dose range extending from 5 to 80 μg/cannula. A previous report indicated that bilateral crystalline implants of pirenzepine in the ventromedial hypothalamus failed to inhibit lordosis in ovariectomized Long-Evans rats treated with estrogen and progesterone (23). Amitriptyline was studied over a limited range of doses since infusion of amitriptyline at doses above 20 μg/cannula induced an aggressiveness that made animals untestable.

METHOD

Forty-one ovariectomized Long-Evans rats with indwelling cannulae placed bilaterally in the lateral ventricles were injected intramuscularly with 1 μg/kg estradiol benzoate for 3 days and 500 μg progesterone at 4–5 h before behavioral testing. Following a pretest, females were infused bilaterally with Krebs-Ringer buffer without glucose (pH 7.4), pirenzepine (10, 20, 40, or 80 μg/cannula), or amitriptyline (10 or 20 μg/cannula). The highest dose of pirenzepine was delivered in 1 μl instead of 0.5 μl volumes to reduce viscosity. All females received hormone treatment on a second week and were tested again following

administration of a different intracerebral treatment. In order to confirm the effectiveness of scopolamine in Long-Evans rats, random females received hormone treatment on a third week and were tested following bilateral infusion of buffer vehicle or scopolamine (10 μg/cannula).

RESULTS

As indicated in Fig. 3, bilateral infusion of pirenzepine at doses up to 80 μg/cannula produced only a weak and nonsignificant reduction in the incidence of lordosis displayed by female rats. The tricyclic antidepressant amitriptyline was completely ineffective following bilateral infusion. Infusion of scopolamine at a dose of 10 μg/cannula significantly reduced lordosis in Long-Evans females with a magnitude similar to that observed in Sherman albino female rats [(5), unpublished data)].

EXPERIMENT 4

In Experiment 4, the binding affinities of carbachol, oxotremorine-M, and McN-A-343 to M1 and M2 binding sites from subtype-enriched membranes were determined in Krebs-Ringer infusion buffer. Using a procedure similar to Experiment 1, in vitro competition analyses of the three agonists were conducted for hippocampal binding sites labelled by the M1-selective antagonist [^{3}H] pirenzepine and brain stem binding sites labelled by the M2-selective agonist [^{3}H] oxotremorine-M. Previous evidence indicates that carbachol and oxotremorine-M display higher affinities for the M2 binding sites concentrated in the brain stem compared to M1 binding sites concentrated in the hippocampus (22,36). Although McN-A-343 has been proposed as an M1 agonist based upon in vivo activity, the selectivity of this agent as indicated by subtype binding appears to be weak (14).

METHOD

Three ovariectomized female rats were treated with 1 μg/kg estradiol benzoate at 72, 48, and 24 h before sacrifice. In 3 independent experiments, the hippocampus and brain stem were dissected and homogenized in 6 ml of Krebs-Ringer buffer (pH 7.4). To determine the binding affinities of carbachol, oxotremorine-M, and McN-A-343 for M1 sites approximately 100 μg of hippocampal protein were added to Krebs-Ringer buffer containing 1 nM of [^{3}H] pirenzepine and increasing concentrations of competing agonist in a final volume of 500 μl. To determine the binding affinities of the three agonists for M2 sites approximately 125 μg of brain stem protein were added to Krebs-Ringer buffer containing 1 nM of [^{3}H] oxotremorine-M and increasing concentrations of competing agonist in a final volume of 500 μl. All preparations were incubated for 1 h at room temperature. Competing agonist concentrations ranged from 10^{-12} to 10^{-2} M. The concentrations of M1 and M2 binding sites labelled in hippocampal and brain stem preparations were equal. Means of percent specific binding at each concentration of agonist obtained from 3 separate experiments were computed. IC_{50} values were estimated from linear regression analyses of mean competition data transformed to log/logit plots (logit $= \ln [P/100 - P]$ where P = percent specific binding at each concentration of competitor).

RESULTS

As indicated in Fig. 4, oxotremorine-M displayed the highest affinity for M1 binding sites in hippocampal homogenates with IC_{50} values (5 μM) slightly lower than those obtained for McN-A-343 (12 μM) but much lower than IC_{50} values obtained for carbachol (74 μM). Oxotremorine-M also displayed the highest affinity for M2 binding sites in brain stem homogenates (0.002 μM). However, carbachol displayed a higher affinity for M2 sites (0.02 μM) than McN-A-343 (0.75 μM). Consequently, in agreement with previous reports, the order of affinity of these three agonists for M1 binding sites is oxotremorine-M > McN-A-343 > carbachol but for M2 binding sites is oxotremorine-M > carbachol > McN-A-343. In direct contrast to the subtype selectivity of the antagonists in Experiment 1, all three agonists, including

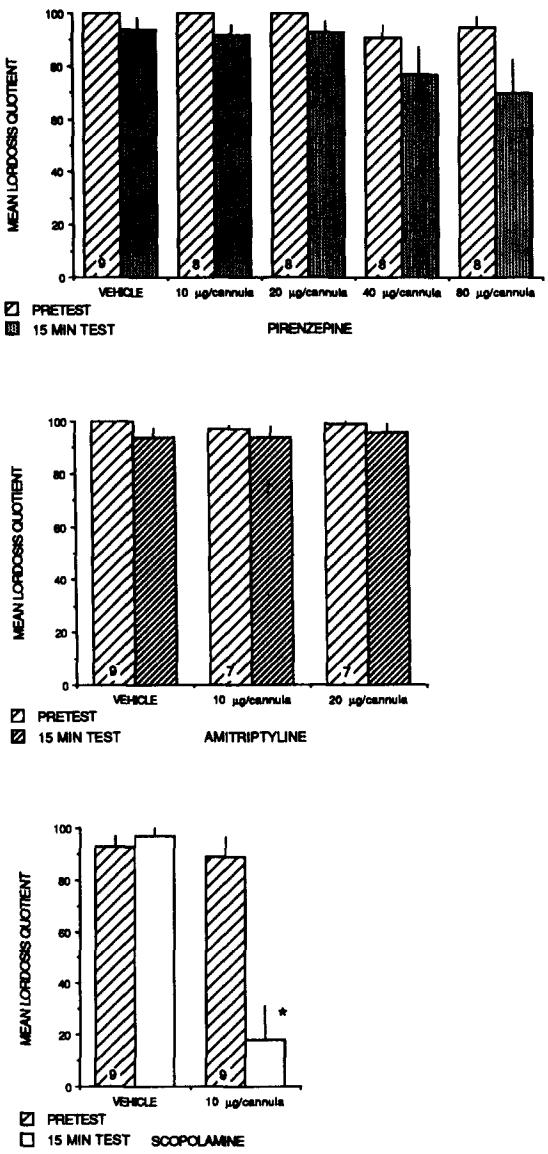

FIG. 3. Effects of antagonists on lordosis behavior. Ovariectomized rats were injected intramuscularly with estradiol benzoate (1 μg/kg) at 72, 48, and 24 h before behavioral testing and progesterone (500 μg) at 4–5 h before testing. Prior to drug treatment all females were pretested for lordosis with stimulus males. During the first 2 weeks of testing, females were infused bilaterally in the lateral ventricles with Krebs-Ringer vehicle (0.5 μl), pirenzepine (10 to 80 μg/cannula), or amitriptyline (10 to 20 μg/cannula). During the third week of testing, random females were infused with vehicle or scopolamine (10 μg/cannula). The effects of these antagonists on lordosis were tested 15 min after infusion. Values represent mean lordosis quotients ± SEM for all animals. *$p<0.01$, scopolamine vs. vehicle at 15 min, t-test.

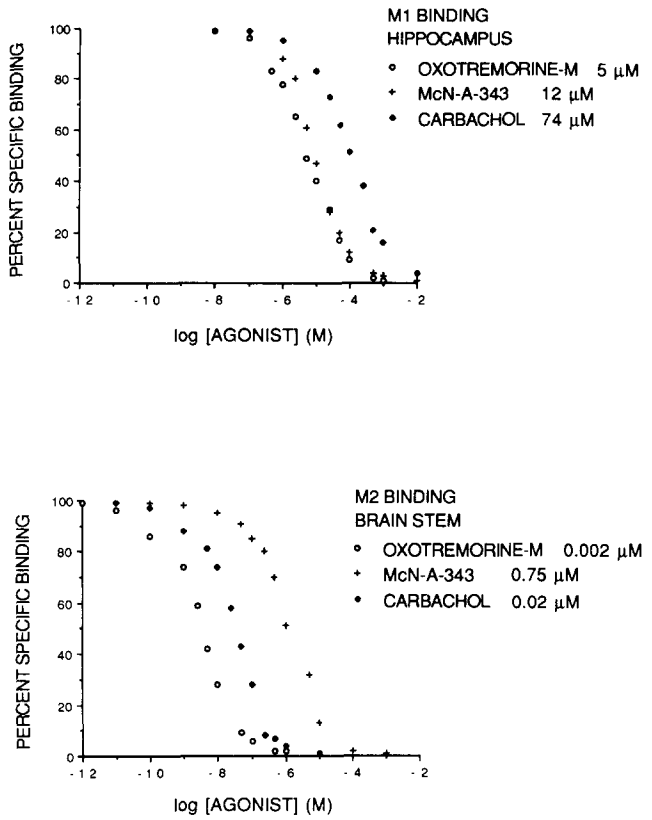

FIG. 4. Competition of agonists for muscarinic subtype binding in rat brain. Various concentrations of oxotremorine-M, McN-A-343, or carbachol were competed against (upper) the M1-selective ligand [^{3}H] pirenzepine (1 nM) in hippocampal homogenates or (lower) the M2-selective ligand [^{3}H] oxotremorine-M (1 nM) in brain stem homogenates. Duplicate samples were incubated for 1 h at room temperature in 500-μl final volumes. Values represent means from 3 separate experiments for each agonist. IC_{50} values were calculated on mean data by linear regression following log/logit transformations.

McN-A-343, displayed a higher affinity for M2 sites than M1 sites.

EXPERIMENT 5

Compounds such as cholinergic receptor agonists, carbachol and oxotremorine, and the acetylcholinesterase inhibitor, physostigmine, activate lordosis in ovariectomized rats primed with low doses of estrogen (5,9). In Experiment 5, the behavioral effects of carbachol, oxotremorine-M, and physostigmine, and McN-A-343 were compared. Carbachol displays a higher affinity for a putative M2 binding site and the facilitative effects of this cholinergic agonist on lordosis have been well characterized under a variety of conditions (7–9). Oxotremorine also activates lordosis in female rats (9), but its methylated form, oxotremorine-M, which displays a higher affinity for a putative M2 binding site, has not been evaluated in sexual behavior experiments. Physostigmine activates lordosis in female rats (5) presumably by reducing degradation of acetylcholine (1), the endogenous neurotransmitter with higher affinity for M2 binding sites (30). According to the results of Experiment 4, McN-A-343 displays a higher affinity for M1 sites than carbachol but a lower affinity for M2 sites than carbachol.

METHOD

Fifty-six ovariectomized Long-Evans rats with indwelling cannulae placed bilaterally in the lateral ventricles were injected intramuscularly with 1 μg/kg estradiol benzoate at 72, 48, and 24 h before behavioral testing. Progesterone was not administered in this paradigm. Following a behavioral pretest, females were infused bilaterally with Krebs-Ringer buffer, physostigmine (10 μg/cannula), carbachol (1 μg/cannula), oxotremorine-M (0.1 μg/cannula), or McN-A-343 (1, 10, or 20 μg/cannula). The effects of intracerebral treatment were determined 15 min after infusion.

RESULTS

As indicated in Fig. 5, bilateral infusion of physostigmine (10 μg/cannula) or carbachol (1 μg/cannula) activated lordosis in Long-Evans females at doses previously found to be effective in Sherman albino females (5,9). Although unmethylated oxotremorine activated lordosis in Sherman females over a dose range of 0.5 to 2 μg/cannula (9), Long-Evans females in preliminary experiments displayed tremor and convulsions following infusion of the methylated form, oxotremorine-M, at doses of 0.5 and 1 μg/cannula. However, bilateral infusion of 0.1 μg/cannula activated levels of lordosis comparable to physostigmine and carbachol without affecting motor performance. In contrast, intraventricular infusion of the putative M1 agonist McN-A-343 failed to significantly increase the incidence of lordosis above vehicle or pretest levels at any dose (1, 10, or 20 μg/cannula). The results of Experiment 5 indicate that lordosis is not activated by stimulation of a putative M1 receptor subtype. Rather, cholinergic compounds that display high affinities for the putative M2 receptor subtype activate lordosis in Long-Evans females primed with estrogen. Furthermore, the potencies of muscarinic agonists in activating lordosis are correlated with the affinities of these agents for M2 binding sites: oxotremorine-M > carbachol > McN-A-343. The ability of these agonists to activate lordosis in female rats is not related to the affinities of these compounds for M1 binding sites: oxotremorine-M > McN-A-343 > carbachol.

EXPERIMENT 6

Muscarinic receptor binding has been reported to change in some brain regions of female rats following systemic administration of estrogen. Specifically, increases in [^{3}H] quinuclidinyl benzilate binding have been demonstrated in hypothalamic, medial preoptic, and mesencephalic tissues obtained from ovariectomized rats treated with estradiol or estradiol benzoate (12, 28, 29, 31, 32). These alterations in binding appear to be associated with changes in receptor density rather than receptor affinity. However, increases in muscarinic binding induced by estrogen treatment are at best moderate in magnitude (20 to 30%) and at worst unreliable. Since experiments to date have utilized the nonselective muscarinic antagonist [^{3}H] quinuclidinyl benzilate to demonstrate the effects of estrogen, it was hypothesized that estrogen might induce more substantial and consistent changes in binding of a primary receptor subtype. In Experiment 6, the effects of estrogen treatment on M1 and M2 receptor subtype binding were determined in specific brain regions using the putative M1-selective radioligand [^{3}H] pirenzepine and the putative M2-selective radioligand [^{3}H] oxotremorine-M. The brain regions that were evaluated concentrate estrogen and have been implicated by various experiments in female sexual behavior: medial preoptic area, medial basal hypothalamus, central gray, and septum. Estrogen treatment has been found to alter muscarinic binding as measured by [^{3}H] quinuclidinyl benzilate in three of these areas, medial

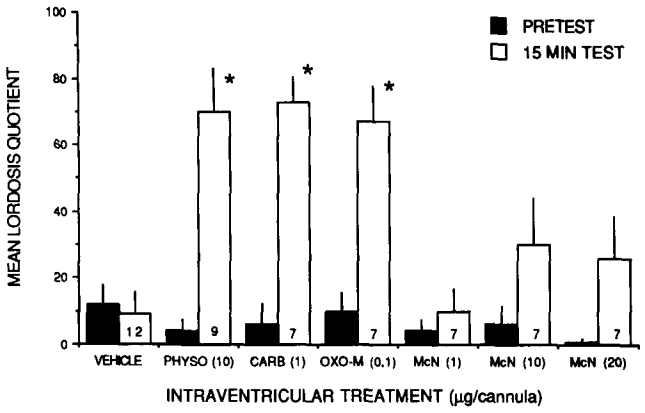

FIG. 5. Effects of agonists on lordosis behavior. Ovariectomized rats were injected intramuscularly with estradiol benzoate (1 μg/kg) at 72, 48, and 24 h before behavioral testing. Prior to drug treatment all females were pretested for lordosis with stimulus males. Females were infused bilaterally in the lateral ventricles with Krebs-Ringer vehicle (0.5 μl), physostigmine (10 μg/cannula), carbachol (1 μg/cannula), oxotremorine-M (0.1 μg/cannula), or McN-A-343 (1, 10, or 20 μg/cannula). The effects of these agonists on lordosis were tested 15 min after infusion. Values represent mean lordosis quotients ± SEM for all animals. *$p<0.01$, agonist vs. vehicle at 15 min, Newman-Keuls test.

TABLE 1

EFFECT OF ESTRADIOL BENZOATE ON MUSCARINIC SUBTYPE BINDING

	Oil	Estradiol Benzoate
[^{3}H] Pirenzepine Binding (fmol/mg)		
Hypothalamus	42 ± 4	46 ± 6
Central Gray	69 ± 7	76 ± 9
Medial Preoptic Area	64 ± 9	85 ± 18
Septal Area	231 ± 64	231 ± 28
[^{3}H] Oxotremorine-M Binding (fmol/mg)		
Hypothalamus	73 ± 5	78 ± 8
Central Gray	195 ± 7	200 ± 11
Medial Preoptic Area	96 ± 5	113 ± 19
Septal Area	326 ± 85	262 ± 44

Ovariectomized rats were injected for 3 days with estradiol benzoate (10 μg per day) or cottonseed oil vehicle (0.1 ml per day). Brain regions were dissected and pooled from 3 animals and subtype binding was determined for M1 binding (10 nM [^{3}H] pirenzepine) and M2 binding (10 nM [^{3}H] oxotremorine-M). Values represent fmol of [^{3}H] ligand specifically bound per mg protein. Each entry is the mean ± SEM from 5 separate pools. Estradiol benzoate did not alter subtype binding significantly in any area.

preoptic area, medial basal hypothalamus, and central gray (12, 28, 29, 31, 32).

METHOD

Thirty ovariectomized Long-Evans rats were injected intramuscularly 3 weeks after surgery with 10 μg estradiol benzoate (n = 15) or 0.1 ml cottonseed oil vehicle (n = 15) at 72, 48, and 24 h before sacrifice. The medial preoptic area, medial basal hypothalamus, central gray, and septum were dissected as described previously (11,12) and tissues from 3 females with the same treatment were pooled and homogenized in 1 ml of 0.32 M sucrose. Homogenates were stored at −60°C for approximately 1 week prior to assay. For determination of M1 binding, aliquots of tissue were incubated in 10 mM sodium-potassium phosphate buffer (pH 7.4) containing 10 nM [^{3}H] pirenzepine in a final volume of 250 μl for 1 h at room temperature. For determination of M2 binding sites, aliquots of tissue were incubated in 50 mM Tris-HCl buffer + 10 mM $MgCl_2$ (pH 7.4) containing 10 nM [^{3}H] oxotremorine-M in a final volume of 250 μl for 1 h at room temperature.

RESULTS

As indicated in Table 1, estrogen treatment for 3 days did not alter muscarinic subtype binding in the medial preoptic, medial basal hypothalamus, midbrain central gray, or septum of ovariectomized rats. Neither M1 binding as measured by [^{3}H] pirenzepine nor M2 binding as measured by [^{3}H] oxotremorine-M were affected significantly by estradiol benzoate treatment. Similar results were obtained for [^{3}H] pirenzepine binding in ovariectomized Sherman albino rats treated with 10 μg estradiol benzoate for 3 days (unpublished data).

DISCUSSION

The results of this series of experiments confirm previous evidence that cholinergic systems are implicated in the regulation of sexual behavior in female rats as indicated by the occurrence of lordosis behavior (5–9). Additionally, these data indicate that lordosis may be regulated by a muscarinic receptor subtype that corresponds to a putative M2 binding site. The behavioral and biochemical results obtained in these experiments are summarized in Table 2. Intraventricular infusion of pirenzepine, a muscarinic antagonist, failed to reduce the incidence of lordosis in ovariectomized rats treated with estrogen and progesterone. In vitro competition experiments indicated that pirenzepine displayed a much higher affinity for M1 than M2 binding sites, while in vivo experiments demonstrated that a low intraventricular dose of pirenzepine completely inhibited M1 binding in hippocampal tissues without reducing M2 binding in brain stem tissues. In contrast, intraventricular infusion of scopolamine, another muscarinic antagonist, significantly reduced the incidence of lordosis in ovariectomized rats treated with estrogen and progesterone in agreement with previous studies. In vitro competition analyses demonstrated that scopolamine displayed a high affinity for both M1 and M2 binding sites and in vivo administration of scopolamine completely inhibited M1 binding sites in hippocampal tissues and strongly inhibited M2 binding sites in brain stem tissues. Intraventricular infusion of the cholinergic receptor agonist carbachol, the muscarinic receptor agonist oxotremorine-M, and the acetycholinesterase inhibitor physostigmine activated lordosis in ovariectomized rats primed with low doses of estrogen. However, McN-A-343, a putative M1 receptor agonist, did not increase the incidence of lordosis significantly in females following intraventricular infusion. Binding experiments indicated that the ability of receptor agonists to activate lordosis in female rats was related to their affinity for M2 binding sites (oxotremorine-M > carbachol > McN-A-343) not their affinity for M1 binding sites (oxotremorine-M > McN-A-343 > carbachol). Taken together, these results implicate putative M2 binding sites, not M1 binding sites, in the regulation of female sexual behavior in rats.

In agreement with other reports (14,34), the results of Experiment 1 indicate that pirenzepine displays a 45-fold greater affinity for M1 binding sites labelled by [^{3}H] pirenzepine ($IC_{50} = 13.7$ nM) compared to M2 binding sites labelled by [^{3}H] oxotremorine-M ($IC_{50} = 635.8$ nM). Despite this M1 selectivity, pirenzepine

TABLE 2

SUBTYPE AFFINITIES AND BEHAVIORAL EFFECTS OF MUSCARINIC AGENTS

Antagonists
M1 Affinity: Scopolamine > Pirenzepine = Amitriptyline
M2 Affinity: Scopolamine > Amitriptyline > Pirenzepine
Inhibition of Lordosis: Scopolamine >>> Amitriptyline = Pirenzepine
Agonists
M1 Affinity: Oxotremorine-M > McN-A-343 > Carbachol
M2 Affinity: Oxotremorine-M > Carbachol > McN-A-343
Activation of Lordosis: Oxotremorine-M > Carbachol > McN-A-343

has much lower affinities for M1 as well as M2 binding sites than scopolamine which displays high but unequal affinities for M1 sites labelled by [^{3}H] pirenzepine ($IC_{50}=0.3$ nM) and M2 sites labelled by [^{3}H] oxotremorine-M ($IC_{50}=2.9$ nM). Consequently, scopolamine competes for both muscarinic receptor subtypes at affinities many times greater than pirenzepine. Although only scopolamine inhibited lordosis in female rats in Experiment 3, it would be difficult to conclude that M1 binding sites are not implicated in cholinergic regulation of lordosis based on the subtype binding affinities of scopolamine and pirenzepine.

However, the results of Experiment 2 indicate that at an intraventricular dose of 10 μg bilaterally, pirenzepine and scopolamine completely inhibited M1 binding of [^{3}H] pirenzepine in hippocampal tissues enriched in M1 sites. Thus, pirenzepine infusion of 10 μg bilaterally completely blocked M1 binding but this intraventricular dose as well as doses up to 80 μg bilaterally failed to inhibit lordosis in female rats in Experiment 3. These data indicate that lordosis behavior can occur normally even when M1 binding sites in the brain are pharmacologically blocked, results which strongly suggest that M1 receptor subtypes are not involved in the control of this behavior.

In contrast, following intraventricular infusion of 10 μg bilaterally, only scopolamine and not pirenzepine inhibited M2 binding of [^{3}H] oxotremorine-M in brain stem tissues enriched in M2 sites. From these data, it could be concluded that lordosis will not occur when M2 binding sites in the brain are pharmacologically blocked, results that implicate M2 receptor subtypes in the regulation of lordosis behavior. However, this interpretation must be tempered since methodological factors prevented an accurate determination of the effects of intraventricular infusion of pirenzepine on M2 binding. Although infusion of 10 μg bilaterally did not alter M2 binding of [^{3}H] oxotremorine-M in brain stem homogenates, the in vivo concentration of pirenzepine in these brain areas following infusion was probably much higher than in the incubation medium since tissues were diluted significantly by homogenation in 200 μl volumes and addition of 10 μl of buffer to 40 μl of homogenate for incubation. In addition, the binding effects of higher intraventricular doses of pirenzepine used in behavioral experiments (20, 40, and 80 μg bilaterally) were not determined. Consequently, the present procedures do not rule out the possibility that intraventricular infusion of pirenzepine, particularly at high doses, may have inhibited both M1 and M2 binding in brain but failed to affect lordosis behavior. Despite these considerations, the extremely low in vitro affinity of pirenzepine for M2 binding sites demonstrated in Experiment 1 implies that even at high in vivo doses pirenzepine would not inhibit M2 binding in rat brain.

The results of Experiment 4 indicate that the three agonists, carbachol, oxotremorine-M, and McN-A-343 display different affinities for muscarinic receptor subtypes in agreement with previous reports (34,38). While each agonist displayed a higher affinity for M2 rather than M1 binding sites, the order of potency in competing for M1 and M2 binding sites differed for these three agents. For M1 sites labelled by [^{3}H] pirenzepine in hippocampal homogenates, oxotremorine-M displayed a slightly higher affinity ($IC_{50}=5$ μM) than McN-A-343 ($IC_{50}=12$ μM), while the affinity of carbachol for M1 binding sites was considerably lower than either of these two compounds ($IC_{50}=75$ μM). Although oxotremorine-M also exhibited the highest affinity for M2 sites labelled by [^{3}H] oxotremorine-M in brain stem homogenates ($IC_{50}=0.002$ μM), carbachol displayed a higher affinity for M2 binding sites ($IC_{50}=0.02$ μM) than McN-A-343 ($IC_{50}=0.75$ μM). In Experiment 5, intraventricular infusion of oxotremorine-M and carbachol activated lordosis in ovariectomized rats primed with estrogen while McN-A-343 was behaviorally ineffective. Consequently, the ability to activate lordosis was paralleled by the affinities of these agonists for M2 not M1 binding sites: oxotremorine-M > carbachol > McN-A-343. In addition, the acetylcholinesterase inhibitor physostigmine also activated lordosis presumably by elevating synaptic levels of acetylcholine, the endogenous neurotransmitter reported to have a higher affinity for M2 rather than M1 sites (30). These data provide additional evidence that muscarinic regulation of lordosis in female rats is mediated by a putative M2 binding site and not the M1 binding site. However, conclusions about subtype specificity based on agonist data must be qualified since most putative M1 agonists display low efficacy and partial activation of muscarinic functions (20). Consequently, it is possible that the behavioral ineffectiveness of McN-A-343 is accounted for by a weak biological efficacy rather than an M2 affinity lower than oxotemorine-M and carbachol.

In Experiment 6, estrogen treatment of ovariectomized rats failed to alter the binding of subtype selective ligands. Three days of treatment with estradiol benzoate (10 μg per day) did not affect the binding of the M1-selective ligand [^{3}H] pirenzepine or the M2-selective ligand [^{3}H] oxotremorine-M in septum, medial preoptic area, medial basal hypothalamus, or central gray. Previous experiments demonstrated increases in the binding and number of muscarinic receptors labelled by [^{3}H] quinuclidinyl benzilate in these areas following administration of estrogen to ovariectomized rats (12, 28, 29, 31, 32). These changes in muscarinic binding, however, proved to be small in magnitude and not always reproducible. The objective of Experiment 6 was to determine if the effects of estrogen on muscarinic subtype binding would be greater in magnitude than found with the nonselective muscarinic ligand [^{3}H] quinuclidinyl benzilate. There was no evidence that estrogen treatment affected muscarinic subtype binding with the binding methodology employed in Experiment 6. Despite the putative role of M2 binding sites in the regulation of lordosis indicated by Experiments 1 through 5, the hormone primarily responsible for lordosis, estrogen, did not alter M2 binding sites in brain areas traditionally associated with female sexual behavior in rats. These results suggest that either muscarinic receptor regulation by estrogen in these brain areas is not involved in hormonal control of lordosis or our procedures failed to detect relevant changes in muscarinic binding induced by estrogen. The anatomical sites at which cholinergic systems act to regulate lordosis are unknown. Consequently, estrogen could alter muscarinic binding in critical brain areas or subareas that were not analyzed in Experiment 6.

Although recent evidence confirms the existence of at least 5 protein forms that bind muscarinic ligands (3), the pharmacological tools to selectively manipulate these distinct binding sites are only emerging. The results of the present experiments indicate that sexual behavior, specifically lordosis, may be regulated preferentially by a muscarinic subtype that corresponds to a binding site identified as M2 according to earlier classification schemes. The functional relationship of the putative M2 site to other binding sites in the muscarinic series remains unresolved. However, a role for M2 binding sites in the regulation of sexual behavior is supported by the effectiveness of M2 selective agents in altering lordosis in female rats and the predominance of M2 binding sites in diencephalic and mesencephalic regions traditionally implicated in female sexual behavior. A further understanding of the muscarinic mechanisms that regulate female sexual behavior may evolve as information accumulates on muscarinic receptor subtypes, their subcellular location, their transmembrane signaling, and their modulation by hormones.

ACKNOWLEDGEMENTS

Supported by USPHS grant HD-22235 and Louisiana Educational Quality Support Fund 86-TUU (16)-133-11. The technical assistance of Jeffrey Finkelstein and Debra Pugh and the statistical expertise of Dr. Mary Brazier, Loyola University of New Orleans, are gratefully acknowledged.

REFERENCES

1. Bartolini, A.; Bartolini, R.; Domino, E. F. Effects of physostigmine on brain acetylcholine content and release. Neuropharmacology 12: 15–25; 1973.
2. Birdsall, N. J. M.; Burgen, A. S. V.; Hulme, E. C. The binding of agonists to brain muscarinic receptors. Mol. Pharmacol. 14:723–736; 1978.
3. Bonner, T. I. New subtypes of muscarinic acetylcholine receptors. Trends Pharmacol. Sci. (Suppl.) December:11–15; 1989.
4. Bradford, M. M. A rapid and sensitive method for the quantitative determination of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248–254; 1976.
5. Clemens, L. G.; Barr, P. J.; Dohanich, G. P. Cholinergic regulation of female sexual behavior in rats demonstrated by manipulation of endogenous acetylcholine. Physiol. Behav. 45:437–442; 1989.
6. Clemens, L. G.; Dohanich, G. P. Inhibition of lordotic behavior in female rats following intracerebral infusion of anticholinergic agents. Pharmacol. Biochem. Behav. 13:89–95; 1980.
7. Clemens, L. G.; Dohanich, G. P.; Witcher, J. A. Cholinergic influences on estrogen-independent sexual behavior in female rats. J. Comp. Physiol. Psychol. 95:763–770; 1981.
8. Clemens, L. G.; Humphrys, R. R.; Dohanich, G. P. Cholinergic brain mechanisms and the hormonal regulation of female sexual behavior in the rat. Pharmacol. Biochem. Behav. 13:81–88; 1980.
9. Dohanich, G. P.; Barr, P. J.; Witcher, J. A.; Clemens, L. G. Pharmacological and anatomical aspects of cholinergic activation of female sexual behavior. Physiol. Behav. 32:1021–1026; 1984.
10. Dohanich, G. P.; McMullan, D. M.; Brazier, M. M. Cholinergic regulation of sexual behavior in female hamsters. Physiol. Behav. 47:127–131; 1990.
11. Dohanich, G. P.; Witcher, J. A.; Clemens, L. G. Prenatal antiandrogen feminizes behavioral but not neurochemical response to estrogen. Pharmacol. Biochem. Behav. 23:397–400; 1985.
12. Dohanich, G. P.; Witcher, J. A.; Weaver, D. R.; Clemens, L. G. Alteration of muscarinic binding in specific brain areas following estrogen treatment. Brain Res. 241:347–350; 1980.
13. Dutar, P.; Nicoll, R. A. Classification of muscarinic responses in hippocampus in terms of receptor subtypes and second-messenger systems: Electrophysiological studies in vitro. J. Neurosci. 8:4214–4224; 1988.
14. Freedman, S. B.; Beer, M. S.; Harley, E. A. Muscarinic M1, M2 receptor binding: Relationship with functional efficacy. Eur. J. Pharmacol. 156:133–142; 1988.
15. Gil, D. W.; Wolfe, B. B. Pirenzepine distinguishes between muscarinic receptor-mediated phosphoinositide breakdown and inhibition of adenylate cyclase. J. Pharmacol. Exp. Ther. 232:608–616; 1985.
16. Gillard, M.; Waelbroeck, M.; Christophe, J. Muscarinic receptor heterogeneity in rat central nervous system. II. Brain receptors labelled by [^{3}H] oxotremorine-M correspond to heterogeneous M2 receptors with very high affinity for agonists. Mol. Pharmacol. 32: 100–108; 1987.
17. Goldman, P.; Scranton, T.; Messer, W. S. Interaction of amitriptyline with muscarinic receptor subtypes in the rat brain. Neurochem. Int. 14:447–454; 1989.
18. Goyal, R. K.; Rattan, S. Neurohumoral, hormonal, and drug receptors for the lower oesophageal sphincter. Gastroenterology 74:598–619; 1978.
19. Hagan, J. J.; Jansen, J. H. M.; Broekkamp, C. L. E. Blockade of spatial learning by the M1 muscarinic antagonist pirenzepine. Psychopharmacology (Berlin) 93:470–476; 1987.
20. Hagan, J. J.; Tonnaer, J. A. D. M.; Broekkamp, C. L. E. Cholinergic stimulation of drinking from the lateral hypothalamus: Indications for M2 muscarinic receptor mediation. Pharmacol. Biochem. Behav. 26:771–779; 1987.
21. Hammer, R.; Berrie, C. P.; Birdsall, N. J. M.; Burgen, A. S. V.; Hulme, E. C. Pirenzepine distinguishes between different classes of muscarinic receptors. Nature 283:90–92; 1980.
22. Hammer, R.; Giachetti, A. Muscarinic receptor subtypes: M1 and M2 biochemical and functional characterization. Life Sci. 31:2991–2998; 1982.
23. Kaufman, L. S.; McEwen, B. S.; Pfaff, D. W. Cholinergic mechanisms of lordotic behavior in rats. Physiol. Behav. 43:507–514; 1988.
24. Mash, D. C.; Flynn, D. D.; Potter, L. T. Loss of M2 muscarine receptors in the cerebral cortex in Alzheimer's disease and experimental cholinergic denervation. Science 228:115–117; 1985.
25. Mash, D. C.; Potter, L. T. Autoradiographic localization of M1 and M2 muscarine receptors in the rat brain. Neuroscience 19:551–564; 1986.
26. Menard, C. S.; Dohanich, G. P. Scopolamine inhibition of lordosis in naturally cycling female rats. Physiol. Behav. 45:819–823; 1989.
27. Messer, W. S., Jr.; Hoss, W. Selectivity of pirenzepine in the central nervous system: I. Direct autoradiographic comparison of the regional distribution of pirenzepine and carbamylcholine binding sites. Brain Res. 407:27–36; 1987.
28. Meyers, T. C.; Clemens, L. G. Dissociation of cholinergic facilitation of feminine sexual behavior and estrogen induction of muscarinic cholinergic receptors. Conf. Reprod. Behav. Prog. Abstr. 17:68; 1985.
29. Olsen, K. L.; Edwards, E.; Schechter, N.; Whalen, R. E. Muscarinic receptors in preoptic area and hypothalamus: effects of cyclicity, sex and estrogen treatment. Brain Res. 448:223–229; 1988.
30. Quirion, R.; Araujo, D.; Regenold, W.; Boksa, P. Characterization and quantitative autoradiographic distribution of [^{3}H]acetylcholine muscarinic receptors in mammalian brain. Apparent labelling of an M2-like receptor sub-type. Neuroscience 29:271–289; 1989.
31. Rainbow, T. C.; DeGroff, V.; Luine, V. N.; McEwen, B. S. Estradiol 17-β increases the number of muscarinic receptors in hypothalamic nuclei. Brain Res. 198:239–243; 1980.
32. Rainbow, T. C.; Snyder, L.; Berck, D. J.; McEwen, B. S. Correlation of muscarinic receptor induction in the ventromedial hypothalamic nucleus with activation of feminine sexual behavior by estradiol. Neuroendocrinology 39:476–480; 1984.
33. Spencer, D. G.; Horvath, E.; Traber, J. Direct autoradiographic determination of M1 and M2 muscarinic acetylcholine receptor distribution in the rat brain: Relation to cholinergic nuclei and projections. Brain Res. 380:59–68; 1986.
34. Spencer, D. G.; van Rooijen, L. A. A.; Horvath, E.; Dompert, W.; Traber, J. Neurochemical and behavioural effects of muscarinic acetylcholine receptor agonists. Trends Pharmacol. Sci. (Suppl) February:61–68; 1988.
35. Stone, W. S.; Cottrill, K. L.; Walker, D. L.; Gold, P. E. Blood

glucose and brain function: Interactions with CNS cholinergic systems. Behav. Neural Biol. 50:325–334; 1988.
36. Tonnaer, J. A. D. M.; van Vugt, M. A.; de Boer, Th.; de Graaf, J. S. Differential interactions of muscarinic drugs with binding sites of [^{3}H] pirenzepine and [^{3}H] quinuclidinyl benzilate in rat brain tissue. Life Sci. 40:1981–1987; 1987.
37. Watson, M.; Roeske, W. R.; Yamamura, H. I. [^{3}H]Pirenzepine and (−)-[^{3}H]quinuclidinyl benzilate binding to rat cerebral cortical and cardiac muscarinic cholinergic sites. II. Characterization and regulation of antagonist binding to putative muscarinic subtypes. J. Pharmacol. Exp. Ther. 237:419–427; 1986.
38. Watson, M.; Yamamura, H. I.; Roeske, W. R. A unique regulatory profile and regional distribution of ^{3}H-pirenzepine binding in the rat provide evidence for distinct M1 and M2 muscarinic receptor subtypes. Life Sci. 32:3001–3011; 1983.
39. Watson, M.; Yamamura, H. I.; Roeske, W. R. [^{3}H]Pirenzepine and (−)-[^{3}H]quinuclidinyl benzilate binding to rat cerebral cortical and cardiac muscarinic cholinergic sites. I. Characterization and regulation of agonist binding to putative muscarinic subtypes. J. Pharmacol. Exp. Ther. 237:411–418; 1986.