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Distinct muscarinic receptors inhibit release of γ-aminobutyric acid and excitatory amino acids in mammalian brain

(presynaptic inhibition/M_1 and M_3 receptors/amygdala/striatum/electrophysiology)

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ABSTRACT **Intracellular recordings were made from neurons of rat lateral amygdala, nucleus accumbens, and striatum *in vitro*. Synaptic potentials mediated by γ-aminobutyric acid and by excitatory amino acids were isolated pharmacologically by using receptor antagonists, and their amplitudes were used as a measure of transmitter release. Muscarine and acetylcholine inhibited the release of both γ-aminobutyric acid and excitatory amino acids, but measurements of the dissociation equilibrium constants for the antagonists pirenzepine, 11-{2-[(diethylamino)methyl]-1-piperidinyl}acetyl-5,11-dihydro-6*H*-pyrido[2,3-*b*][1,4]benzodiazepine-6-one, methoctramine, and hexahydrosiladifenidol indicated clearly that different muscarinic receptors were involved (M_1 and probably M_3, respectively). The differential localization of distinct muscarinic receptor subtypes on terminals releasing the major inhibitory and excitatory transmitters of the brain could be exploited therapeutically in some movement disorders and Alzheimer disease.**

Acetylcholine (AcCho) has diverse functional roles in the mammalian brain, including actions at muscarinic receptors that are important for memory (1). Cholinergic neurons are lost in Alzheimer disease, and one therapeutic approach has been to develop drugs that mimic the neurotransmitter action of missing AcCho (1). However, such drugs, like AcCho itself, will have different effects at different muscarinic receptors in the brain, and not all of these effects may be desired. Three muscarinic receptors (M_1–M_3) can be distinguished pharmacologically, but five (m1–m5) have been identified by molecular cloning (m1–m3 correspond to M_1–M_3) (2–8).

Fibers containing γ-aminobutyric acid (GABA) and excitatory amino acids such as glutamate provide synaptic inputs to virtually all central neurons; therefore, in addition to their direct effects on neurons (9, 10), muscarinic agonists will also excite or inhibit cells by acting presynaptically to change release of these neurotransmitters. In the present experiments, the release of GABA and excitatory amino acids (glutamate) was assayed by recording the amplitude of depolarizing synaptic potentials from neurons in three brain regions where AcCho is known to play an important functional role—the amygdala, nucleus accumbens, and striatum.

METHODS

Adult rats were anesthetized with halothane and killed by a heavy blow to the chest. The brain was rapidly removed, and a block of tissue containing nucleus accumbens, dorsal striatum, or amygdaloid complex was sectioned with a Vibratome. Intracellular recordings were made from slices (300 μm thick) by using electrodes containing 2 M KCl (11–13). The superfusing solution was a bicarbonate buffer gassed with 95% O_2/5% CO_2 and contained 2.5 mM KCl, 2.4 mM $CaCl_2$, 1.3 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 26 mM $NaHCO_3$, 126 mM NaCl, and 10 mM glucose. The slice was completely submerged in this flowing solution, which was prewarmed to 37°C. Drugs were applied by changing this solution to one that contained the drug. A bipolar tungsten-in-glass stimulating electrode was used for focal stimulation. The components of the synaptic potentials mediated by glutamate and GABA were both depolarizing because the recording electrodes contained KCl, but the respective contributions could be readily distinguished by selective receptor antagonists (Fig. 1*A*). In 30 μM bicuculline (or 100 μM picrotoxin), the residual synaptic potential was completely blocked by a combination of 10 μM 6-cyano-2,3-dihydroxy-7-nitroquinoxaline (CNQX) and 30 μM 4-aminophosphonovaleric acid (APV), and this is referred to as the glutamate (excitatory amino acid) component; in 30 μM APV and 10 μM CNQX, the residual synaptic potential was completely blocked by 30 μM bicuculline, and this is referred to as the GABA component.

Muscarine superfusion was for 3–10 min, which was sufficient, depending on the flow rate, for the reduction of the synaptic potential to reach a steady state; pirenzepine was superfused for at least 30 min prior to retesting the action of muscarine. Muscarine (3–100 μM) often caused potential changes in the postsynaptic cells, which had resting potentials between −75 and −90 mV, but the membrane potential was restored to the control level for measurements of synaptic potential amplitude. (In amygdala, 20% of cells were hyperpolarized by up to 13 mV, 42% were depolarized by up to 12 mV, and 30% were unaffected: in accumbens and striatum, none were hyperpolarized, and >80% were depolarized by up to 18 mV.) The depolarization was accompanied by an increase in input resistance (11), so this should not contribute to any reduction in the amplitude of the synaptic potential. Dissociation equilibrium constants for antagonists were determined by Gaddum–Schild analysis (14).

RESULTS AND DISCUSSION

Muscarine reversibly depressed the synaptic potentials mediated by both glutamate and GABA (Fig. 1 *B* and *C*): it was equally effective in inhibiting both glutamate and GABA release. The concentrations that gave 50% reduction (EC_{50})

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Abbreviations: AcCho, acetylcholine; AFDX 116, 11-{2-[(diethylamino)methyl]-1-piperidinyl}acetyl-5,11-dihydro-6*H*-pyrido[2,3-*b*][1,4]benzodiazepine-6-one; APV, 4-aminophosphonovaleric acid; CNQX, 6-cyano-2,3-dihydroxy-7-nitroquinoxaline; EC_{50}, concentration giving half-maximal effect; GABA, γ-aminobutyric acid; HHSD, hexahydrosiladifenidol; McN-A-343, 4-(3-chlorophenylcarbamoyl)-2-butynyltrimethylammonium chloride.

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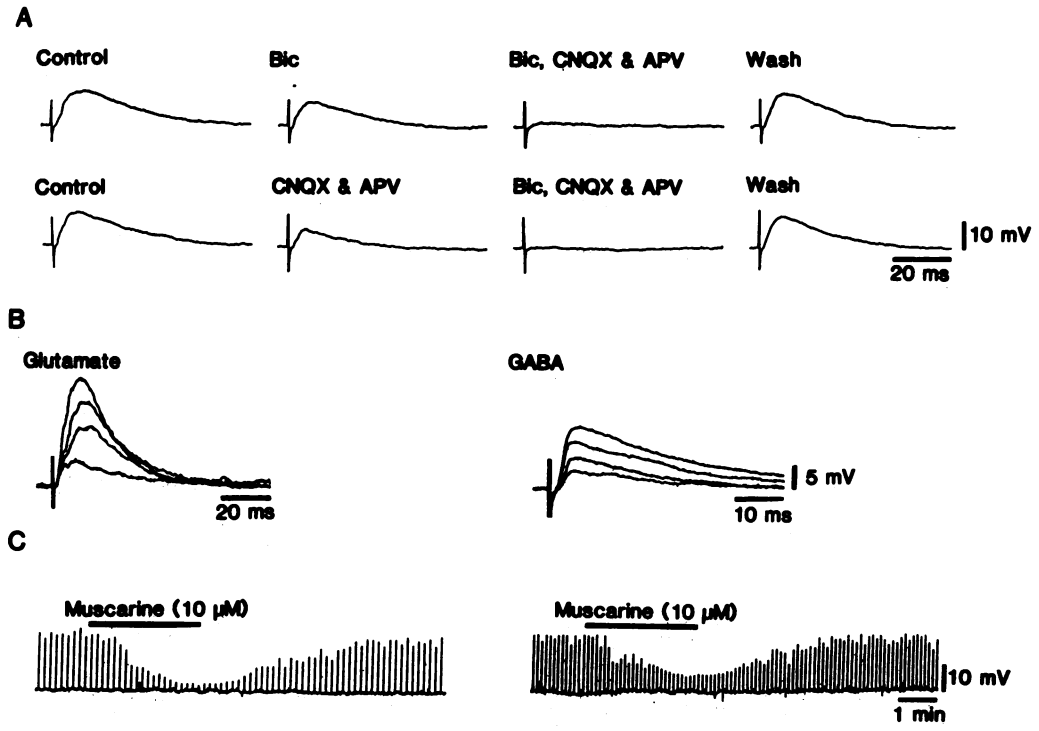

FIG. 1. Muscarine depresses glutamate and GABA components of depolarizing synaptic potentials. (*A*) Pharmacological separation of glutamate and GABA components. The synaptic potential was reduced in part by 30 μM bicuculline (Bic) and in part by a combination of 10 μM CNQX and 30 μM 4-APV. When excitatory amino acid antagonists and bicuculline were added together, the synaptic potential was completely blocked. (*B*) Superimposed synaptic potentials to show control responses, and responses in the presence of muscarine (0, 3, 10, and 30 μM). Here and in other figures, the glutamate component (*B Left*) was recorded in 30 μM bicuculline (or 100 μM picrotoxin), and the GABA component (*B Right*) was in 30 μM APV and 10 μM CNQX. (*C*) Time course of action of muscarine to inhibit synaptic potential. (*C Left*) Glutamate component. (*C Right*) GABA component.

of the glutamate component of the synaptic potential were 13 ± 0.9 μM (n = 31), 9.7 ± 1.3 μM (n = 30), and 13 ± 2.2 μM (n = 18) and of the GABA component were 12 ± 1.5 μM (n = 10), 9.8 ± 2.0 μM (n = 16), and 10 ± 2.7 μM (n = 10) (amygdala, accumbens, and striatum, respectively).

Glutamic acid (n = 6) and GABA (n = 3) were also applied directly to the neurons by changing to a superfusing solution that contained 10 mM agonist for 4 s so that reproducible depolarizations could be obtained with repeated applications: these depolarizations by exogenous glutamate and GABA were unaffected by muscarine, indicating that the depression of the synaptic potential was most likely presynaptic.

Bethanechol (n = 3) and AcCho (n = 6) had effects similar to that of muscarine. The release of GABA was more sensitive than the release of glutamate to McN-A-343 [4-(3-chlorophenylcarbamoyl)-2-butynyltrimethylammonium chloride] and its 4-chloro analog (Fig. 2), suggesting that the presynaptic muscarinic receptors on the two sets of nerve terminals might differ; these compounds are somewhat selective for M_1 receptors in other tissues (15).

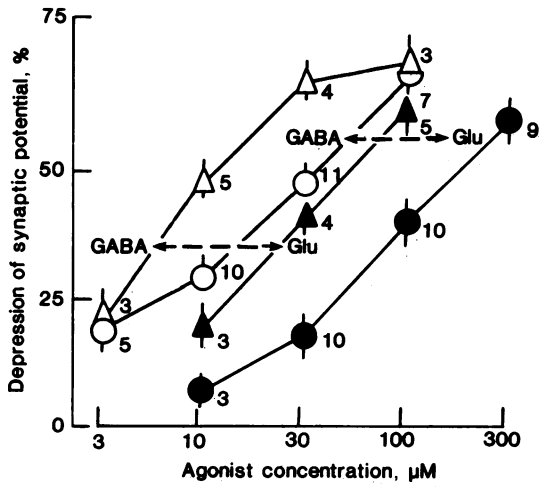

FIG. 2. McN-A-343 (triangles) and its 4-chloro analog (circles) inhibit synaptic potentials mediated by glutamate (filled symbols) and GABA (open symbols). Graphs show concentration–response curves. The number beside each point indicates the number of cells, and the vertical bars show the SEM. Data with the McN-A-343 4-chloro analog are from nucleus accumbens. Data with McN-A-343 are pooled from amygdala, accumbens, and striatum: the respective EC_{50} values to depress the glutamate component were 199 ± 28 μM (n = 4), 130 ± 29 μM (n = 5), and 194 ± 53 μM (n = 4) and to inhibit the GABA component were 33 ± 9.0 μM (n = 3), 28 ± 4.2 μM (n = 6), and 35 ± 9.0 μM (n = 3).

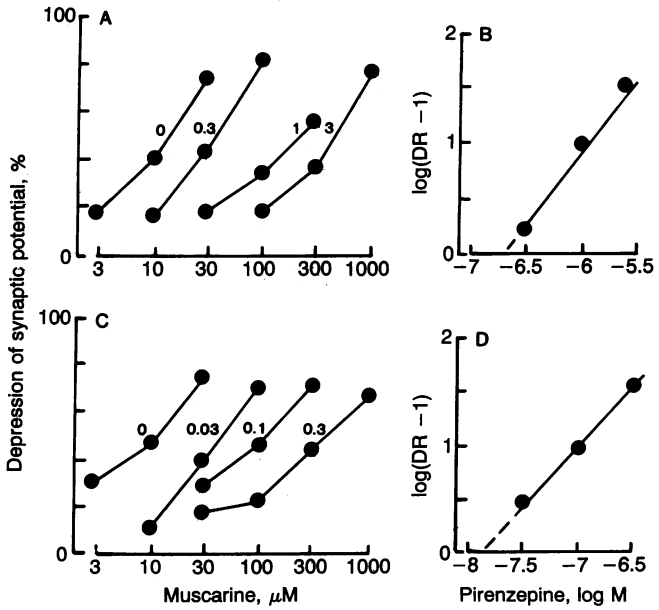

FIG. 3. Pirenzepine differentiates between muscarinic receptors on glutamate-releasing and on GABA-releasing terminals. (*A*) Concentration–response curves for muscarine to inhibit glutamate release in the absence and presence of pirenzepine at concentrations (μM) indicated by each trace. Results are from one amygdala neuron. (*B*) Gaddum–Schild plot from the results in *A*: pirenzepine acts as a competitive antagonist. (*C*) Similar experiment for GABA release in another amygdala neuron. (*D*) Gaddum–Schild plot shows that pirenzepine acts as a competitive antagonist but has a higher affinity for the receptors on GABA terminals than on the glutamate terminals.

Table 1. Logarithm of K_d values for antagonism of inhibition of synaptic potential by muscarine

Neurons	Pirenzepine		AFDX-116	Methoctramine	HHSD	
	Glu-RN	GABA-RN	Glu-RN	Glu-RN	Glu-RN	GABA-RN
AMYG	−7.0 ± 0.20 (9)	−7.9 ± 0.07 (6)	−5.8 ± 0.06 (4)	—	−7.8 ± 0.01 (4)	−7.5 ± 0.07 (4)
ACCU	−6.8 ± 0.07 (7)	−7.7 ± 0.08 (4)	—	−6.14 ± 0.19 (5)	−7.8 ± 0.12 (7)	—
STRI	−6.9 ± 0.32 (4)	−7.5 ± 0.15 (4)	—	—	−7.85 ± 0.16 (3)	—

In experiments in which three or four antagonists were applied while recording from a single neuron (e.g., Figs. 3 and 4) the slope of the Gaddum–Schild plot did not differ from 1. Therefore, K_d values were computed from K_d = [B]/(DR − 1), where [B] is the antagonist concentration and DR is the ratio of equi-effective agonist concentrations in the absence and presence of pirenzepine. If two or more antagonist concentrations were applied to one neuron, a single estimate of the K_d for that cell was obtained from the mean. Each neuron was used to provide an independent estimate of K_d, whether one or more antagonist concentrations were applied to it; the number of neurons is in parentheses beside each estimate. K_d values are expressed as their logarithms ± SEM. AMYG, ACCU, and STRI, experiments in lateral amygdala, nucleus accumbens, and striatum; Glu-RN, glutamate-releasing neurons; GABA-RN, GABA-releasing neurons.

The muscarinic receptors on the presynaptic terminals that released GABA were distinguished by antagonists from those of the glutamate-releasing terminals. Fig. 3 illustrates experiments that show that more pirenzepine was required to antagonize muscarinic inhibition of the release of glutamate than of the release of GABA. The high affinity of pirenzepine for the receptors on the GABA-releasing nerves in all three tissues (Table 1) clearly implicates the M_1 receptor, and the K_d for hexahydrosiladifenidol (HHSD) determined in the amygdala is consistent with this interpretation.

Presynaptic inhibition by activating M_1 receptors has been shown in brain and other tissues (16, 17), including the release of GABA (18). The presence of M_1 receptors on GABA-containing neurons in these three brain regions is quite consistent with the finding that they all express moderately high levels of m1 receptor RNA (8) because most of the GABA terminals arise from intrinsic GABA neurons.

The dissociation constant (K_d) for pirenzepine as an antagonist of muscarine inhibition of glutamate release (Table 1) does not agree well with the affinity of pirenzepine for M_1 or M_2 receptors but is closer to the values reported for the m3 and m5 receptors, cloned and expressed in oocytes (19) or Chinese hamster ovary cells (20). Experiments with more selective antagonists were therefore carried out. In amygdala, 11-{2-[(diethylamino)methyl]-1-piperidinyl}-acetyl-5,11-dihydro-6*H*-pyrido[2,3-*b*][1,4]benzodiazepine-6-one (AFDX-116; ref. 5) and HHSD (7) both competitively antagonized the action of muscarine, and the K_d values (Table 1) agreed well with those for the M_3 (and expressed m3) receptor (5, 7, 19–21). In accumbens, methoctramine (6) and HHSD were used as antagonists: the K_d values determined were also very close to those reported for M_3 receptors (6, 19–21). The K_d of methoctramine suggests M_3 rather than m4 (19, 20), and BM-5, an agonist selective for m2 and m4 receptors (22), was ineffective at 30–300 μM (n = 6). However, the results with these antagonists do not distinguish unequivocally between M_3 (m3), m4, and m5 receptors or a combination of these. Glutamate-containing fibers to the accumbens, dorsal striatum, and amygdala arise in part from cell bodies in cortical cell layer V (23, 24), and m3 receptor mRNA is strongly expressed in this layer (8): m5 mRNA is expressed in the CA1 region of the hippocampus (25), which

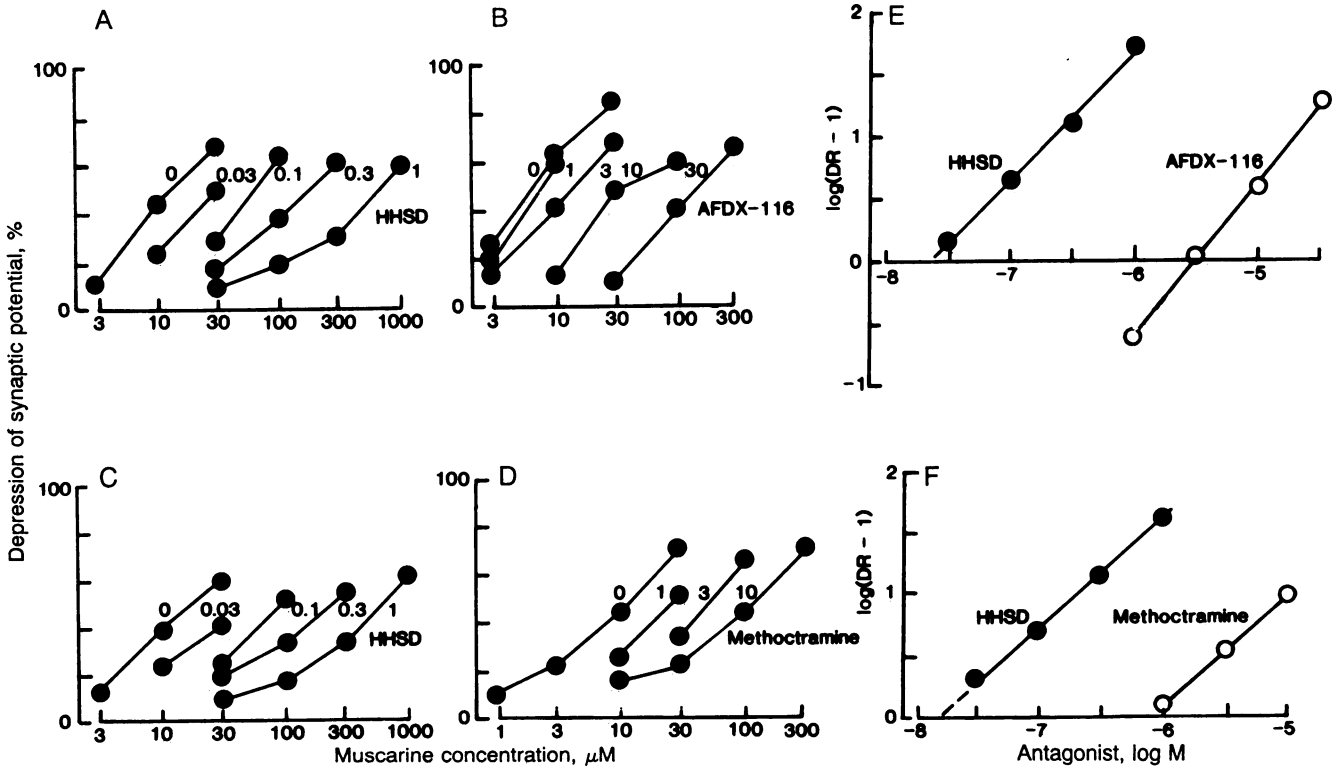

FIG. 4. Glutamate release is inhibited through M_3-like receptors. The inhibition of glutamate release by muscarine was measured, and this was antagonized by HHSD (*A* and *C*) AFDX-116 (*B*), and methoctramine (*D*). Concentrations (μM) of antagonist present in each case are indicated. Four different neurons were used: amygdala in *A* and *B* and accumbens in *C* and *D*. (*E* and *F*) Gaddum–Schild plots from experiments in *A–D*.

also provides some excitatory input to accumbens and amygdala.

The powerful presynaptic inhibitory actions of muscarine, at concentrations similar to or lower than those needed to produce direct postsynaptic effects (11), suggest that the presynaptic receptors will be an important substrate both for physiologically released AcCho and for exogenously administered drugs. A selective M_1 antagonist would inhibit the cells by increasing GABA release whereas a selective M_3 agonist would inhibit the cells by decreasing glutamate release. In the case of the accumbens and striatum, this might benefit certain dyskinesias (26). In the case of the amygdala, selective inhibition of these neurons could be helpful in certain epilepsies; the amygdala is among the most readily kindled of brain regions, and this is blocked by muscarinic antagonists (27, 28). Conversely, selective agonists at M_1 receptors would be expected to excite the principal cells of these regions by direct excitation (11, 29) and by reduction of GABA-mediated feedback inhibition, while having no effect on the excitatory drive to the cells mediated by glutamate. The cholinergic innervation of the amygdala from the nucleus basalis is one site of the typical neurodegenerative changes associated with Alzheimer disease (1, 30), and the present findings therefore point to the possible value of M_1 agonists and M_3 antagonists in this disease.

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