M2 Muscarinic Autoreceptors Modulate Acetylcholine Release in the Medial Pontine Reticular Formation

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ABSTRACT
Muscarinic autoreceptors regulate acetylcholine (ACh) release in several brain regions, including the medial pontine reticular formation (mPRF). This study tested the hypothesis that the muscarinic cholinergic receptor mediating mPRF ACh release is the pharmacologically defined M2 subtype. In vivo microdialysis was used to deliver muscarinic cholinergic receptor (MACHR) antagonists to the feline mPRF while simultaneously measuring endogenously released ACh. The lowest concentration of each antagonist that caused a significant increase in mPRF ACh release was determined and defined as the minimum ACh-releasing concentration. Data obtained from 41 mPRF dialysis sites in 10 animals showed that the order of potency (followed by the minimum ACh-releasing concentration) was scopolamine (1 nM) > AF-DX 116 (3 nM) > pirenzepine (300 nM). Comparison of these minimum ACh-releasing concentrations to the known affinities of the antagonists for the five mACHR subtypes is consistent with the conclusion that the autoreceptor regulating mPRF ACh release is the M2 subtype. Considerable evidence supports a role for cholinergic neurotransmission and postsynaptic M2 receptors in the mPRF in regulating levels of arousal. The present data suggest that presynaptic M2 receptors contribute to the regulation of arousal states by modulating mPRF ACh release.

Autoreceptors are defined as presynaptically localized receptors that respond to a neuron’s own transmitter and function to regulate transmitter release by feedback control (Kalsner, 1990). Functional, anatomic and ligand binding data support the existence of muscarinic, cholinergic autoreceptors in many brain regions. Functionally, in vivo microdialysis studies have shown that mACHRs regulate ACh release in the striatum (Billard et al., 1995), cerebral cortex (Quirion et al., 1994), medial septal area (Moore et al., 1995) and hippocampus (Moore et al., 1995; Nordström and Bartfai, 1980). Anatomically, the presence of mACHRs on presynaptic terminals of basal forebrain cholinergic neurons has been visualized through the use of electron microscopy and antibody staining (Levey et al., 1995). Ligand binding studies have demonstrated that lesions of cholinergic neurons cause changes in terminal field mAChR density, providing evidence for the presence of muscarinic autoreceptors in cerebral cortex (Mash et al., 1985).

Muscarinic autoreceptors regulate ACh release in the føline mPRF (Roth et al., 1996). The brain stem reticular formation modulates level of behavioral arousal, cardiopulmonary control, somatic motor tone and pain sensation (Role and Kelly, 1991). Cholinergic and noncholinergic, cholinceptive pontine neurons play an important role in the control of arousal states, particularly in the generation of rapid eye movement (REM) sleep (Baghdoyan, 1997a; Steriade and McCarley, 1990). Neurons of the mPRF, which are not cholinergic (Jones and Beaudet, 1987), receive their cholinergic input from neurons of the LDT and PPT nuclei (Mitani et al., 1988; Shiromani et al., 1988). ACh is released in the mPRF from LDT/PPT neurons (Lydic and Baghdoyan, 1993), and the muscarinic autoreceptors present in the mPRF are presumed to reside on LDT/PPT terminals (Roth et al., 1996).

Molecular cloning studies have identified five subtypes of mACHRs (m1-m5) linked to different G proteins and intracellular signal transduction pathways (Brown et al., 1997; Felder, 1995; Hosey, 1992). Muscarinic receptors also are classified pharmacologically according to affinity binding profiles of muscarinic antagonists (Caulfield, 1993). The pharmacologically defined M1-M3 mAChR subtypes correspond fairly well to the molecularly identified m1-m3 subtypes, respectively, but it is important to note that there are
Materials and Methods

Animal and drug preparation. All experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (7th ed., National Academy of Sciences Press, Washington, DC, 1996). Adult male cats (N = 10) were anesthetized with halothane or isoflurane (1–2% in O2) and implanted with standard electrodes for recording the cortical electroencephalogram and nuchal electromyogram. As described previously (Leonard and Lydic, 1997), a craniotomy was performed over the cerebellum to allow access of the microdialysis probe to the mPRF. Experiments were started after a 3-wk, postsurgical recovery period.

Scopolamine methyl bromide, pirenzepine dihydrochloride and carbachol (Sigma Chemical Co., St. Louis, MO) were dissolved in Ringer’s solution (147 mM NaCl, 2.4 mM CaCl2, 4.0 mM KCl, 10 μM neostigmine bromide). Cholinesterase inhibitors are required to prevent the enzymatic degradation of ACh, and are used routinely for microdialysis (Billard et al., 1995; Leonard and Lydic, 1997; Moor et al., 1997; Quirion et al., 1994). AF-DX 116 (Boehringer Ingeheim, Ridgefield, CT) was dissolved in 0.05 N HCl and p-FHHSID (Research Biochemicals International, Natick, MA) was dissolved in 100% EtOH. It was possible to use these solvents because they were shown to have no effect on ACh release. All drugs were diluted serially to their final concentrations using Ringer’s solution.

In vivo microdialysis and HPLC/EC. Microdialysis probes (CMA/10; CMA Microdialysis, Acton, MA) had a 2-mm long polycarbonate membrane, a 0.5-mm diameter and a 20-kDa cut-off. Quantification of pontine ACh release using HPLC/EC (Bioanalytical Systems Inc., West Lafayette, IN) has been described in detail (Keifer et al., 1996; Leonard and Lydic, 1997; Lydic and Baghdoyan, 1993). Briefly, a CMA/100 pump ensured continuous flow of Ringer’s solution through the dialysis probe at a rate of 3 μl/min. Dialysis samples were collected every 10 min, and the dialysate was injected immediately into the HPLC/EC system to determine the amount of ACh. Upon injection, a 50 mM Na2HPO4 mobile phase (pH = 8.5) carried the dialysate through a column that separated ACh and choline. An immobilized enzyme reactor column produced hydrogen peroxide in concentrations proportional to the amount of ACh present in the sample. Hydrogen peroxide then was quantified using a platinum electrode with an applied potential of 500 mV in reference to a silver/silver chloride electrode. The resulting signal was recorded on a flatbed recorder and simultaneously digitized for off-line analysis. The areas under the chromatogram peaks were integrated using the Inject program (Bioanalytical Systems Inc.).

Experimental design. Every experiment began by creating a standard curve based on seven known concentrations of ACh. In addition, before positioning the microdialysis probe in the brain, the probe was placed in an ACh solution of known concentration and dialyzed to determine the percent ACh recovery. This procedure was repeated after collection of the last brain dialysis sample. Pre- and postexperimental probe recoveries were compared by t test to ensure that any changes in ACh observed during the experiment were due to pharmacological manipulations rather than to alteration of the dialysis probe membrane. Data reported below were obtained from experiments showing no significant difference between pre- and postexperimental probe recovery.

Anesthesia began by mask induction with halothane (3–3.5% in O2). After the loss of wakefulness, laryngoscopy was performed, lidocaine (1%) was sprayed on the vocal cords and auffed endotracheal tube was inserted in the trachea. The animal was placed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA) equipped with a Kopf 880 semi-chronic head holder. Electroencephalogram and electromyogram were recorded continuously on a Grass model 7 Polygraph (Astro-Med Inc., West Warwick, RI). End-tidal carbon dioxide and halothane concentrations were measured using a Raman spectrometer (Ohmeda Rascal II, Louisville, CO) and maintained at 28 to 30 mm Hg and 1.4%, respectively. Core body temperature was held at 37°C using a T/Pump Heat Therapy System (Gaymar, TP 400 Series, Orchard Park, NY) and a rectal thermometer. Oxygen saturation and heart rate were monitored continuously using an Ohmeda Biox 3700 Pulse Oximeter (Boulder, CO). Blood pressure readings were taken noninvasively every 10 min using a Dinamap (Critikon, Tampa, FL), and heart and breath sounds were monitored regularly using an esophageal stethoscope. The microdialysis probe was placed in the mPRF through the craniotomy according to the stereotoxic coordinates of Berman (1968) and perfused continuously with Ring- er’s solution.

Once mPRF ACh release was determined to be stable, five dialysis samples (50 min) were collected to determine baseline (control) levels of ACh release. Using a CMA/110 liquid switch, the mPRF then was dialyzed with Ringer’s solution containing either scopolamine, AF-DX 116, pirenzepine, p-FHHSID or carbachol. Nine sequential dialysis samples were obtained during drug administration, and only one dose of one drug was dialyzed per experiment. On completion of each experiment, the dialysis probe was removed from the brain and the craniotomy was closed. After extubation, animals were kept under continuous observation until recovery from anesthesia. A minimum of 7 days separated experiments in the same animal. Each animal was used for six to eight experiments. The dialysis probe was placed in a different site within the mPRF for each experiment.

One goal of the present study was to determine the lowest concentration of each antagonist that caused a significant increase in mPRF ACh release (defined as the minimum ACh-releasing concentration) (Billard et al., 1995). This required a series of experiments conducted according to the following procedure (Billard et al., 1995). An initial concentration of each antagonist was dialedyzed in one experiment, and the effects on ACh release were quantified. If that initial concentration caused no change in ACh release, then the antagonist concentration was increased by one-half log unit and the experiment was repeated until a concentration was found that caused a statistically significant increase in ACh release. Similarly, if the initial concentration did cause increased ACh release, then the antagonist concentration was reduced sequentially by one-half log unit until a concentration was found that caused no change in ACh release. As described above, only one dose of one drug was delivered during each experiment. The criteria for establishing the minimum ACh-releasing concentration of an antagonist required three experiments showing a significant increase in ACh release at a given concentration, and each antagonist was tested in at least three different animals. Similar requirements were applied for establishing the highest antagonist concentration that had no effect on ACh release (defined as the highest no-effect concentration) (Billard et al., 1995). Descriptive statistics and one-way ANOVA with Dunnett’s multiple comparison test were used to determine the effect of dia- lysised drugs on ACh release. The significance level was P < .05.

Histology. After the last dialysis experiment, halothane administra- tion was discontinued and pentobarbital was injected i.v. (35–40

no mACHr antagonists with exclusive selectivity for any one of the molecularly identified subtypes (Buckley, 1990; Dörje et al., 1991). Although the functional roles of mACHr sub- types are beginning to be elucidated, lack of subtype-specific ligands makes such studies difficult (Felder, 1995). The pharmacologically defined M2 subtype has been demonstrated to regulate ACh release in several brain areas (Billard et al., 1995; Moor et al., 1995), satisfying the operational definition of an autoreceptor. Given the importance of the pontine brain stem for regulating behavioral arousal (Steriade and McCar- ley, 1990), and given that the predominant mACHr subtype in the mPRF is M2 (Baghdoyan, 1997b; Baghdoyan et al., 1994), the present study used in vivo microdialysis to test the hypothesis that the mACHr mediating ACh release in the mPRF is the pharmacologically defined M2 subtype.
mg/kg). The brain was perfused in situ with 10% formalin, removed and fixed in formalin for at least 2 wk. The brain stem then was placed in 30% sucrose + 10% formalin for 7 days and serial sections were cut on a freezing microtome at 40 μ in thickness. All sections were slide-mounted and stained with Cresyl violet. The laterality of every section that included the mPRF was identified by comparing each section with the sagittal plates of Berman’s atlas (Berman, 1968). The stereotaxic coordinates of all dialysis sites were determined from the probe-induced lesions appearing in these sections, as described previously (Lydic and Baghdoyan, 1993).

Results

Figure 1 shows a digitized image of a Cresyl violet-stained, sagittal section through the cat brain stem. This section contains a typical lesion made in the mPRF by a dialysis probe (arrow). The lesion was used to confirm probe placement in the mPRF. The ACh measurements reported below were obtained from 41 mPRF dialysis sites in 10 animals. The mPRF dialysis sites ranged from P 1.0 to P 4.0, L 0.3 to L 2.3 and H −4.0 to H −6.5 (stereotaxic coordinates according to the sagittal plates in Berman, 1968). Mean ± S.E.M. stereotaxic coordinates for all dialysis sites were P 2.6 ± 0.1, L 1.2 ± 0.1 and H −5.3 ± 0.1. The H coordinate indicates the midpoint of the 2-mm long dialysis membrane.

Results illustrating how each dialysis experiment was performed are shown in figure 2. The Ringer’s (control) and scopolamine ACh chromatograms (fig. 2A) correspond to 0.7 and 2.2 pmol/10 min, respectively. During the first 10 min of dialysis with scopolamine, mPRF ACh was increased 90% over the mean control level (fig. 2B, first scopolamine sample vs. the mean of the five Ringer’s samples). By 20 min of mPRF dialysis with scopolamine, ACh was increased 221% over the average control ACh level (fig. 2B, second scopolamine sample). This scopolamine-induced increase in ACh release (fig. 2C) is typical of recent findings demonstrating the presence of muscarinic autoreceptors in the mPRF (Roth et al., 1996).

Further evidence supporting the regulation of mPRF ACh release by muscarinic autoreceptors is provided by the finding that mPRF dialysis with the agonist carbachol (10 mM) significantly decreased ACh release below control levels (t = 1.8, d.f. = 28). Thus, although the Ringer’s solution contained 10 μM neostigmine, the autoreceptors were not maximally inhibited during control dialysis and could respond to an agonist by decreasing ACh release.

This study next sought to identify the subtype of mACHR regulating ACh release by comparing the relative potencies of different mACHR antagonists in their ability to increase mPRF ACh release. The data are reported in figures 3, 4 and 5. Figure 3 illustrates the time course of one experiment using AF-DX 116 (squares), another experiment with pirenzepine (circles) and a third experiment with p-FHHSiD (triangles). Mean ± S.E.M. basal levels of mPRF ACh were
0.59 ± 0.03, 0.52 ± 0.01 and 0.32 ± 0.14 pmol/10 min preceding dialysis with AF-DX 116, pirenzepine and p-FHHSiD, respectively. Dialysis of the mPRF with AF-DX 116 and with pirenzepine significantly increased mPRF ACh release to 1.48 ± 0.05 and 0.70 ± 0.23 pmol/10 min, respectively. In contrast, administration of p-FHHSiD did not alter mPRF ACh release (0.36 ± 0.01 pmol/10 min). Figure 4 summarizes results based on 27 experiments in 10 cats. Cross-hatched histograms indicate average ACh level (pmol/10 min) in the mPRF during control (Ringer’s) dialysis and solid histograms indicate mPRF ACh levels during dialysis with the minimum concentration of antagonist needed to produce a significant increase in ACh release over control levels. This concentration of antagonist is referred to as the minimum ACh-releasing concentration (Billard et al., 1995), and was determined to be 1 nM for scopolamine (fig. 4A), 3 nM for AF-DX 116 (fig. 4B) and 300 nM for pirenzepine (fig. 4C). p-FHHSiD was tested at concentrations ranging from 10 to 1000 nM, but did not cause a significant increase in mPRF ACh release. Figure 4 also shows the highest concentria...
centration of each antagonist that had no effect on ACh release (diagonally hatched histograms). This highest no-effect concentration (Billard et al., 1995) was one half log unit below the minimum ACh-releasing concentration (fig. 4, A–C).

The relative potencies of the mAChR antagonists for increasing ACh release are compared in figure 5. The solid histograms plot the mean percent increase in ACh release induced by the minimum ACh-releasing concentration for each antagonist. The order of potency was scopolamine > AF-DX 116 > pirenzepine, indicating that the mAChR regulating ACh release in the mPRF is the pharmacologically defined M2 subtype.

**Discussion**

The present study establishes the minimum ACh-releasing concentration for scopolamine, AF-DX 116 and pirenzepine in the mPRF; demonstrates that mPRF carbachol administration decreases mPRF ACh release; and supports the view that the M2 subtype of mAChR functions as an autoreceptor in the mPRF. The ensuing discussion considers the use of muscarinic antagonists for identifying mAChR subtypes, and the potential role of muscarinic autoreceptors in modulating the REM phase of sleep.

**Muscarinic receptor pharmacology and the use of in vivo microdialysis to identify autoreceptor subtypes.** Evidence for the existence of muscarinic autoreceptors that modulate ACh release in the mPRF; that modulate ACh release with known affinities of these antagonists for m1-m5 mAChRs, inferences can be made about the mAChR subtype that functions as an autoreceptor in the mPRF. This approach recently was used to identify the muscarinic autoreceptor subtype as M2 in rat striatum (Billard et al., 1995).

The results showed that the minimum ACh-releasing concentration for scopolamine was 1 nM (fig. 4A). Scopolamine has high affinity for all mAChR subtypes (Billard et al., 1995; Bolden et al., 1992), and thus was predicted to be the most potent antagonist (fig. 5). The minimum ACh-releasing concentrations for AF-DX 116 and pirenzepine were 3 nM (fig. 4B) and 300 nM (fig. 4C), respectively. p-FHHSiD was ineffective in increasing ACh release at concentrations up to 1000 nM (see below). Based on the binding affinities of AF-DX 116 and pirenzepine for mAChR subtypes (Billard et al., 1995; Bolden et al., 1992; Dörje et al., 1991; Jones et al., 1992), only the m2 receptor would be expected to produce a functional response at concentrations of less than 100 nM AF-DX 116 and more than 100 nM pirenzepine. The conclusion that the m2 subtype modulates mPRF ACh release is consistent with data from in vitro autoradiographic studies demonstrating that the mPRF contains predominantly M2 binding sites in cat (Baghdoyan et al., 1994) and rat (Baghdoyan, 1997b), and with a new, preliminary report demonstrating the presence of m2 receptors on the terminals of cholinergic neurons in cat pontine reticular formation (Ray et al., 1997).

One limitation of the present study is the impossibility, for all practical purposes, of determining the antagonist concentration in the mPRF region sampled by the dialysis probe. This issue has been discussed in detail (Billard et al., 1995), and emphasizes the importance of testing a range of dosages for several different antagonists in their ability to increase mPRF ACh release. Only by comparing relative (vs. absolute) potencies of these antagonists is it possible to make conclusions about which mAChR subtype modulates mPRF ACh release.

Another potential methodological limitation is the concentration of neostigmine in the Ringer’s solution with which the dialysis probe was perfused. The rapid and powerful degrading actions of acetylcholinesterase require that neostigmine be used to prevent the breakdown of ACh. Previous studies have shown that neostigmine in the microdialysis buffer can stimulate the autoreceptor such that no further reduction in ACh release can be evoked (Moor et al., 1995). The finding that dialysis with carbachol significantly decreased ACh release below control levels demonstrates that the autoreceptors were not maximally inhibited during the present experiments.

It is unclear why p-FHHSiD had no effect on ACh release at concentrations (100–1000 nM) which would be expected to block m2 receptors (Dörje et al., 1991). Previous work also found that 1000 nM p-FHHSiD failed to increase ACh release when delivered directly to cortical and hippocampal slices, where muscarinic autoreceptors of the M1, M2 and M4 subtype were identified (Vannucchi and Pepeu, 1995). Further studies are necessary to reconcile the apparent lack of potency of p-FHHSiD in functional studies with its high affinity in binding assays.

As has been emphasized elsewhere (Billard et al., 1995), more than one subtype of mAChR may function as an autoreceptor, and the generalization that muscarinic autoreceptors are always of the M2 subtype is premature. For example, a preliminary report shows that m3 receptors are present on some presynaptic, cholinergic terminals (Rye et al., 1995). An
M3-like autoreceptor has been suggested to regulate ACh release in rat striatum (DeBoer et al., 1990), and both M1 and M4 receptors have been suggested to regulate ACh release in the hippocampus (Vannucchi and Pepeu, 1995). The agreement between the present minimum ACh-releasing concentrations of mAChR antagonists (fig. 4) and previously determined affinities of these antagonists at cloned mAChR subtypes (Billard et al., 1995; Bolden et al., 1992; Dörje et al., 1991; Jones et al., 1992) supports the conclusion that the M2 receptor functions as an autoreceptor in the feline mPRF. Converging lines of evidence obtained using a variety of methodologies will be helpful in establishing the subtype identity of muscarinic autoreceptors in additional brain regions.

**mPRF ACh release and REM sleep generation.** ACh in the mPRF contributes to the generation of REM sleep (reviewed in Baghdoyan, 1997a; McCarley et al., 1995; Sterr and McCarley, 1990). During REM sleep, pontine ACh release is increased over both waking and non-REM sleep levels (Kodama et al., 1990; Leonard and Lydic, 1997). LTD/PPT neurons, which regulate ACh release in the mPRF (Lydic and Baghdoyan, 1993), increase their discharge rates before and during REM sleep (El Mansari et al., 1989; Kayama et al., 1992), and neurotoxic lesions of the dorsolateral pontomesencephalic tegmentum cause a long-term suppression of REM sleep that is correlated with the degree of damage to cholinergic LDT/PPT neurons (Webster and Jones, 1988). Most recently, electrical stimulation of LTD/PPT has been shown to enhance REM sleep (Thakkar et al., 1996).

Microinjection of cholinergic agonists or neostigmine into the mPRF causes a REM sleep-like state, and considerable evidence supports the conclusion that in the mPRF both endogenous ACh and exogenous cholinomimetics cause REM sleep by activating postsynaptic M2 receptors (reviewed in Baghdoyan, 1997a; McCarley et al., 1995). For example, postsynaptic M2 receptors have been visualized in the mPRF (Ray et al., 1997; Rye et al., 1995; Vilaró et al., 1992), and natural REM sleep can be blocked by pontine microinjections of methoctramine but not pirenzepine or p-FHHSiD (Imeri et al., 1994). In addition, the cholinergically evoked REM sleep-like state can be inhibited by blocking an m2/m4-linked signal transduction pathway involving a pertussis toxin-sensitive G protein, adenylyl cyclase, cAMP and protein kinase A (Capece and Lydic, 1997; Shuman et al., 1995), and carbachol is now known to cause a dose-dependent, atropine-sensitive activation of G proteins in the pontine reticular formation (Capece et al., 1998). Intracellular recordings made in vitro have shown that carbachol depolarizes mPRF neurons by activating a postsynaptic, pirenzepine insensitive (i.e., non-M1) mAChR (Greene et al., 1989). Taken together, these multiple lines of evidence are consistent with the hypothesis that ACh released from LTD/PPT terminals into the mPRF causes REM sleep, in part, by activating postsynaptic M2 receptors localized to the same mPRF region explored in the present study. The data reported here suggest, for the first time, that presynaptic M2 receptors in the mPRF also may contribute to REM sleep generation by modulating mPRF ACh levels.

Electrophysiological studies have shown that activation of M2 receptors hyperpolarizes neurons via an inwardly rectifying K+ current (reviewed in Jones et al., 1992). The membrane potential effects of muscarinic agonists on cholinergic terminals with the mPRF are unknown, but the present finding that mPRF carbachol decreased mPRF ACh release is consistent with the possibility of an M2-mediated hyperpolarization of the presynaptic terminal. Activation of M2 receptors also has been shown to inhibit a high-voltage-activated component of the Ca++ current in rat basal forebrain cholinergic neurons, suggesting another possible mechanism by which M2 autoreceptors may modulate ACh release (Allen and Brown, 1993).

This study was conducted using halothane anesthesia, which eliminates REM sleep. Thus, the putative role of M2 autoreceptors in REM sleep regulation was not addressed. Because ACh release in many brain areas changes with arousal state (Jasper and Tessier, 1971; Kodama et al., 1990; Leonard and Lydic, 1997; Lydic et al., 1991; Williams et al., 1994), the present rationale for using anesthetized animals was to hold arousal state constant. Administration of muscarinic agonists and antagonists into the pontine reticular formation of conscious cat by microdialysis recently has been shown to cause significant changes in behavioral state (Sakai and Onoe, 1997), and pontine ACh release is known to change with changes in behavioral state (Kodama et al., 1990; Leonard and Lydic, 1997; Lydic and Baghdoyan, 1993; Lydic et al., 1991). By holding arousal state constant with inhalation anesthesia, it was possible in the present study to make inferences regarding presynaptic receptor modulation of ACh release.

The present data do not prove, unequivocally, that muscarinic autoreceptors exist in the mPRF. In fact, the existence of any autoreceptors regulating transmitter release has been questioned (Kalsner, 1990). It is possible, for example, that a neuronal feedback loop between the mPRF and LDT/PPT terminals, and that postsynaptic M2 receptors on neurons in that feedback loop modulate ACh release. The most direct explanation of the present finding that mPRF ACh release was decreased in response to an agonist and increased in response to antagonists is that mPRF ACh release is modulated by M2 autoreceptors, putatively localized to LDT/PPT neuron terminals. This interpretation is supported by a recent study using immunohistochemistry in conjunction with electron microscopy to demonstrate that, in cat pontine reticular formation, m2 receptors are localized to terminals of cholinergic neurons (Ray et al., 1997).

In conclusion, this study showed for the first time that in the mPRF, AF-DX 116 was more potent than pirenzepine or p-FHHSiD in causing increased ACh release. These findings are most consistent with the interpretation that mAChRs of the M2 subtype function as autoreceptors to modulate pontine ACh release.

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**References**


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