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Effects of the muscarinic antagonists pirenzepine and gallamine on spontaneous and evoked responses of rat cerebral cortical neurones

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1 The muscarinic receptor antagonists gallamine and pirenzepine were iontophoretically applied to rat cerebral cortical cholinoceptive neurones, including corticospinal neurones, to assess their effects on spontaneous firing, and firing induced by: stimulation of the nucleus basalis magnocellularis (NBM); contralateral hindpaw stimulation; application of acetylcholine (ACh); and application of glutamate.

2 Both compounds potently inhibited firing induced by ACh iontophoresis, whilst neither compound consistently altered firing induced by application of glutamate.

3 Gallamine was very effective and pirenzepine less effective, at inhibiting both spontaneous firing and the delayed firing induced by NBM stimulation. The short-latency excitations elicited by NBM stimulation were enhanced by these muscarinic antagonists.

4 Gallamine and pirenzepine enhanced cortical cholinoceptive cell firing induced by contralateral hindpaw stimulation.

5 It is concluded that gallamine depresses spontaneous activity more than pirenzepine, and that both compounds can affect the cortical cell firing evoked by stimulation of the NBM and of thalamo-cortical afferent fibres.

Introduction

The function of acetylcholine (ACh) in the cerebral cortex has been aggressively researched for most of this century. However, the role of this compound in cortical neuronal transmission is still obscure. One focus of current research involves characterization of cortical ACh receptors and delineation of the postsynaptic receptor and effector mechanisms responsible for the physiological actions of this compound on neurones. Determination of these mechanisms is made difficult by the fact that ACh may cause both excitation and inhibition when applied to neurones in the brain (Krnjević, 1974). Further complicating this issue is the fact that muscarinic receptors affecting the responses of a given neurone may be located on the neurone in question, or presynaptically connected to it, and may be found anywhere from the dendrites to the axon terminal.

[3H]-quinuclidinyl benzilate binding studies in the rat cerebral cortex indicate that muscarinic receptors are particularly abundant in this brain region (Watson *et al.*, 1986). In addition, it has been shown that both the M_1 and M_2 subclasses of muscarinic receptors are abundant in rat parietal cortex as determined by binding affinity studies using the muscarinic ligands pirenzepine (Watson *et al.*, 1982; 1986), and oxotremorine (Spencer *et al.*, 1986), respectively. Specific antagonists of brain M_2 receptors are currently being sought, and gallamine, the neuromuscular relaxant, has shown promise in this respect (Eglen & Whiting, 1986; Price *et al.*, 1986). The finding of a neuronal M_2 muscarinic receptor antagonist may be significant since it has been suggested that activation of different subclasses of muscarinic receptors may be resonsible for the excitatory and inhibitory actions of ACh, described above (McCormick & Prince, 1986a). We have, therefore, chosen to assess the physiological actions of pirenzepine and gallamine *in vivo*.

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Parietal cerebral cortical layers 4 and 5 contain the large pyramidal neurones which project to the spinal cord; neurones which have been extensively characterized by both extra- and intracellular recording techniques (Phillis, 1976; McCormick & Prince, 1985). Application of ACh to these neurones in *in vitro* slice preparations produces an initial inhibition followed by a delayed, secondary excitation (McCormick & Prince, 1986b). *In vivo* extracellular recordings from pyramidal neurones have demonstrated an initial decrease followed by an increase in neuronal firing during iontophoretic application of ACh (Phillis, 1976). In addition, peristimulus histograms obtained from pyramidal neurones during stimulation of the globus pallidus have demonstrated a triphasic response: an initial fast excitation, followed by a period of inhibition, followed by a slow secondary period of excitation (Edstrom & Phillis, 1980). These last two phases may correspond to the phases obtained in the *in vitro* ACh response described above. Although the ion conductance changes responsible for these observed complex patterns are poorly defined, evidence from other central neuronal systems suggest that the inhibitory action of ACh may be due to an increase in potassium conductance (McCormick & Prince, 1986c), while the excitatory action may be due to inactivation of a unique voltage-dependent potassium current (the M current) (Brown & Adams, 1980; Halliwell & Adams, 1982; Constanti & Galvan, 1983) or a calcium-dependent potassium current (North & Tokimasa, 1983; Pennefather *et al.*, 1985).

Since application of exogenous ACh to pyramidal neurones may or may not mimic a physiological occurrence, the response of these neurones to endogenously released ACh is of interest. Kainic acid lesioning studies, in conjunction with direct immunofluorescent antibody labelling of cortical cholinergic markers, have demonstrated that cholinergic innervation in rat cerebral cortex derives principally from the nucleus basalis magnocellularis (NBM), although as much as 30% of the ACh present in cortex may derive from intrinsic neurones (Lehman *et al.*, 1980; Johnston *et al.*, 1981). Thus, recording cortical responses to NBM stimulation may offer a novel, *in vivo*, method of determining the physiological effects of drugs which modify the actions of ACh on a potentially cholinergic input. Since neuronal destruction in the nucleus basalis of Meynert, the equivalent nucleus in man, consistently occurs in patients with the dementia of Alzeheimer's and Parkinson's disease (Whitehouse *et al.*, 1981; 1982; Gasper & Gray, 1984; Nakano & Hirano, 1984), the pharmacological and physiological characterization of this brain pathway is also of significant clinical interest.

In the present study, we have examined the effects of pirenzepine and gallamine on the spontaneous firing of cholinoceptive pyramidal neurones, and on firing induced by iontophoretic application of ACh. In addition, we have examined the effects of these two compounds on cortical evoked activity brought about by stimulating the NBM and, for control comparison, the contralateral hindlimb.

Methods

Twenty adult male Sprague-Dawley rats weighing 350–400 g were used. Detailed descriptions of the surgical and recording procedures have been published previously (Phillis *et al.*, 1979). Briefly, the animals were anaesthetized with halothane and maintained on a mixture of methoxyflurane and 60% nitrous oxide/40% oxygen via a tracheal cannula. Temperature was maintained at 37°C via an electric heating pad controlled by a rectal thermal probe. Following reflection of the scalp, a small hole was drilled through the parietal bone, 2 mm lateral to the sagittal suture and 1.5 mm posterior to the coronal suture, permitting insertion of both the iontophoretic electrode and the bipolar, coaxial (SNEX, 100 × 70 mm) electrode used to stimulate the NBM (stimulating surface area 0.45 mm × 1.0 mm; stimulation parameters 220 Hz, 15 ms duration, 1–5 μA). In some animals, a coaxial stimulating electrode was placed in the pyramidal tract in order to identify pyramidal neurones via antidromic stimulation. All electrodes were stereotaxically placed according to the atlas of Paxinos & Watson (1982). Hindlimb stimulation was produced by a small bipolar pin electrode in the contralateral hindpaw. The exposed cortex was continuously covered by a solution of 4% agar in Ringer solution to prevent desiccation. The stimulating electrodes were attached to a Grass stimulator via an RF stimulus isolation unit. The stimulator also triggered an Ortec time histogram analyzer and memory control unit used to compile peristimulus histograms (PSH). Electrode position was verified on formalin-fixed, 100 μm thick stained sections cut on a freezing microtome.

Seven barrelled iontophoretic electrodes were broken to a tip diameter of approximately 10 μm, with a recording barrel resistance under 3 MΩ. The recording electrode barrel and one side barrel used for automatic current balancing by the Dagan polarizer were filled with 2 M NaCl, and the remaining barrels filled with one of the following solutions: acetylcholine chloride (0.1 M; pH 5.0), gallamine (0.1 M; pH 4.5), pirenzepine (0.1 M; pH 5.0) or glutamate (0.1 M; pH 7.5). Retaining currents of 10–12 nA were applied to all drug barrels when drugs were not being applied. Application currents ranged from 40–50 nA.

All of the studies reported in the Results section involved ACh-excitable cerebral cortical neurones. To evaluate the effects of ACh antagonists on the response of neurones to NBM or hindlimb stimulation, the following testing procedure was followed. After collecting a control PSH, each drug was applied for 3 min before and during the recording of a second PSH. Iontophoretic pulses of ACh were then applied until recovery from the drug had occurred, at which time a third histogram was recorded. Typically, 150–250 stimulus cycles were required to accumulate each histogram, at a repetition frequency of $1\,s^{-1}$. Bin widths for histogram compilations were 5 ms. Drugs were scored as either increasing or decreasing the control. Since control baseline variability was between 3–5%, as judged by the height of histogram recordings, the criterion for scoring an increase or decrease in the response was a 15% or greater change from control.

Results

The PSH generated during NBM stimulation was characterized by an immediate, fast excitation (phase 1) of 0–10 ms latency and 5–10 ms duration, followed by a prolonged, 80–100 ms period of inhibition (phase 2), followed by a late, slower period of excitation lasting 300–500 ms (phase 3), (see Figures 3 and 4). Phase 3 was variable, in some cells occurring in a broad, somewhat flattened peak, and in other cells consisting of several discrete peaks. The PSH obtained during hindlimb stimulation was characterized primarily by an early phase of excitation, occurring within 8 ms after the stimulus pulse, and of 10–15 ms duration.

Gallamine and pirenzepine were applied with 50 nA currents to deep cortical cells. On 24 out of 28 cells tested, gallamine decreased spontaneous firing. Pirenzepine, on the other hand, when applied by 50 nA currents to 26 cells, decreased the firing of 7, increased firing of 7, and had no effect on 12 cells. When these drugs were applied during ACh-induced firing, gallamine decreased firing in 30 out of 36 cells tested, and pirenzepine inhibited firing of 37 out of 37 cells tested (see Figure 1). Both drugs were applied onto cortical cells which were being driven by repetitive glutamate applications, to determine if a non-specific action on neuronal firing was responsible for the effects observed. Results of these experiments showed: on 35 cells tested, gallamine had no effect on 16, decreased slightly the glutamate-induced firing rate of 14, and increased the glutamate-induced firing rate of 5; on 32 cells tested, pirenzepine had no effect on the induced firing rate of 18, decreased slightly the induced firing rate of 12, and increased the induced firing rate of 2. The above

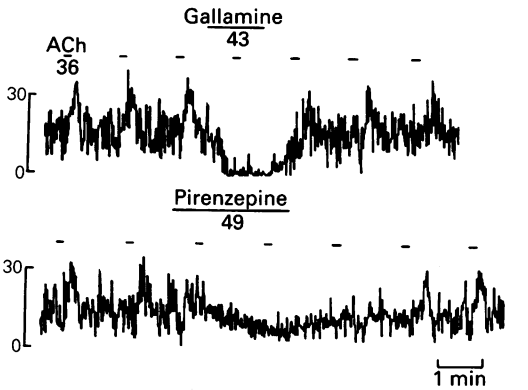

Figure 1 Ratemeter recording from a pyramidal tract neurone. Vertical bars represent firing frequency in spikes min^{-1}. Horizontal bars depict duration of iontophoresis of designated compounds at current in nA given below each compound. Time scale is as shown. Acetylcholine (ACh) was pulsed at approximately 1.5 min intervals, after which time gallamine and pirenzepine were iontophoresed as shown.

data are summarized in Table 1. Figure 2 depicts a representative ratemeter recording of the effects of gallamine and pirenzepine on spontaneous firing and on ACh and glutamate-induced activity. Gallamine increased glutamate induced firing of this neurone.

The effect of gallamine on the PSHs generated by NBM stimulation recorded from 18 cortical neu-

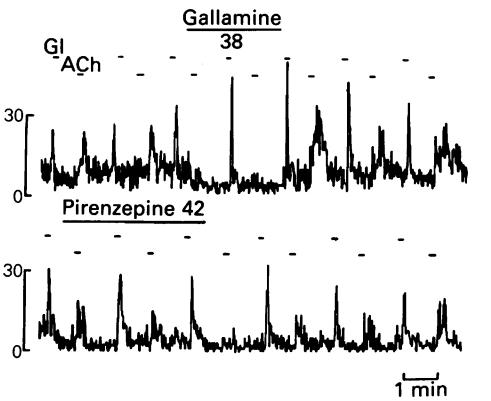

Figure 2 Ratemeter recordings from the same pyramidal tract neurone. Vertical bars represent firing frequency in spikes min^{-1}. Horizontal bars represent duration of iontophoresis of designated compounds, at currents in nA designated by numbers. In this neurone, glutamate (Gl, 32 nA) was pulsed for 10 s alternating with acetylcholine (ACh, 40 nA) for 10 s, during which time gallamine and pirenzepine were iontophoresed as shown.

Table 1 Effects of gallamine and pirenzepine on spontaneous activity, glutamate-induced firing, and acetylcholine-induced firing of deep cortical pyramidal cells

	Spontaneous activity			*Glutamate firing*			*Acetylcholine firing*		
Compound	inc	nc	dec	inc	nc	dec	inc	nc	dec
Gallamine	3 (1)	11 (3)	86 (24)	14 (5)	46 (16)	40 (14)	0	17 (6)	83 (30)
Pirenzepine	27 (7)	46 (12)	27 (7)	6 (2)	57 (18)	37 (12)	0	0	100 (37)

inc = increased firing rate, nc = no change in firing rate, dec = decrease in firing rate. Numbers represent percentage of all cells tested. Numbers in parentheses represent actual number of cells tested (*n*).

rones was as follows: gallamine increased the initial fast excitation in 12 cells, while in 2 cells it was decreased and in 4 cells there was no change. Phase three excitation was inhibited in 15 cells, increased in 1 cell, while no change was noted in 2 cells. The effects of gallamine on the inhibitory phase were less clear with the majority of responses being unaltered (see Table 2). Pirenzepine was tested on 17 cells during NBM stimulation, with the following results: the initial fast excitation was increased in 9 cells, decreased in 2 cells, and no change was noted in 6 cells. An example of the enhancement of this initial fast excitation is depicted in Figure 3. The most notable effect of pirenzepine was on the late excitation, where 9 of 17 cells were inhibited. The NBM PSH results are summarized in Table 2. Representative NBM PSHs are depicted in Figure 4.

Hindpaw stimulation resulted in a single or multiple short latency peaks of excitation with no associated inhibition. The initial short latency peak, probably relayed through the ventral posterior lateral nucleus of the thalamus, is thought to represent glutamatergic input onto pyramidal cells (Jones, 1986). In 7 of 15 cells tested (47%), gallamine increased the response, while no change was noted in the other 8 cells (53%). Pirenzepine increased the response of 5 of 11 cells (45%), decreased the response in 2 of 11 cells (18%), and had no effect on the remaining 4 cells (36%). Figure 5 represents PSH recordings from one cell. Table 3 summarizes the above results.

Discussion

The application of muscarine to ACh receptors results in a variety of conductance changes, not all of which have been assigned to a particular receptor subtype. Both decreases and increases in potassium conductance have been described, thought to be the result of M_1- and M_2-receptor activation respectively (North, 1986; Egan & North, 1986). Inhibition of a voltage-dependent calcium conductance by muscarinic agonists has also been described (North, 1986), however the receptor subtype effecting this action has not been identified. Lastly, an increase in sodium conductance has been linked to an M_2 receptor (Egan & North, 1986). The criteria for assigning a receptor to the M_1 or M_2 subclasses rests principally with its affinity for binding pirenzepine, those with high affinity (circa 60 nanomolar) being assigned to the M_1 receptor subclass, and all others designated as belonging to the M_2 subclass. However, the discovery of more specific receptor antagonists, especially of the M_2 subclass, may further distinguish subpopulations of the M_2 subclass of muscarinic receptors, as suggested by Egan & North (1986). Currently, two compounds, gallamine and pirenzepine, are being used in *in vitro* receptor experiments to classify muscarinic receptor subtypes. It is thus of interest to define the physiological actions of these compounds on neurones responding to ACh to extend the receptor binding data.

Table 2 Effects of gallamine and pirenzepine on cortical pyramidal somatosensory unit peristimulus histograms generated by stimulation of the nucleus basalis magnocellularis

	Phase 1			*Phase 2*			*Phase 3*		
Compound	inc	nc	dec	inc	nc	dec	inc	nc	dec
Gallamine	67 (12)	22 (4)	11 (2)	11 (2)	78 (14)	11 (2)	5 (1)	11 (2)	83 (15)
Pirenzepine	53 (9)	35 (6)	12 (2)	12 (2)	76 (13)	12 (2)	18 (3)	29 (5)	53 (9)

Phase 1 = initial fast excitation, phase 2 = duration of inhibition following phase 1, phase 3 = secondary excitation following phase 2. Numbers represent percentage of all cells tested, with actual numbers (*n*) of cells tested in parentheses. inc = increased activity or duration, nc = no change in activity or duration, dec = decreased activity or duration.

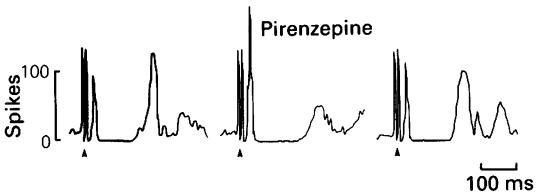

Figure 3 Peristimulus histogram (PSH) recording from a pyramidal tract neurone during stimulation of the nucleus basalis magnocellularis. The height of a histogram window containing 100 events is represented by the vertical bar. The first 50 ms represents spontaneous activity, which is followed by the stimulation artifact, and 8 ms later by phase 1 of the PSH. Phase 2 is designated as the period of inhibition following phase 1, and phase 3 is the period of excitation following phase 2. The figure depicts a predrug control PSH, followed by the PSH generated during application of 50 nA of pirenzepine, concluded by the post drug control PSH, obtained 5 min after cessation of drug iontophoresis.

In the present studies, gallamine inhibited the spontaneous firing of 86% of cells tested, while pirenzepine inhibited firing of 27%. Thus, gallamine is much more potent than pirenzepine at inhibiting spontaneous activity of these neurones.

When applied concomitantly with ACh, pirenzepine inhibited ACh-induced firing in 100% of cells

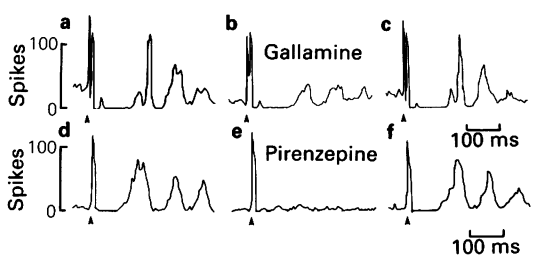

Figure 4 (a–c) Peristimulus histogram (PSH) recording from a pyramidal tract neurone during stimulation of the nucleus basalis magnocellularis. The height of the histogram window containing 100 events is represented by the vertical bar. The first 50 ms represents spontaneous activity, followed by the stimulation artifact, which in the top figure is followed by phase 1, the fast excitation described in the text. (d–f) Depicts a PSH recording from another neurone where phase 1 merges with the tail of the stimulation artifact. The inhibitory phase (phase 2) follows phase 1 and precedes phase 3, the latent excitatory phase. The figure shows the effects of 3 min, 50 nA applications of gallamine and pirenzepine on the responses to nucleus basalis magnocellularis stimulation with the corresponding pre- and post-drug controls. Arrows indicate stimulation of the nucleus basalis magnocellularis.

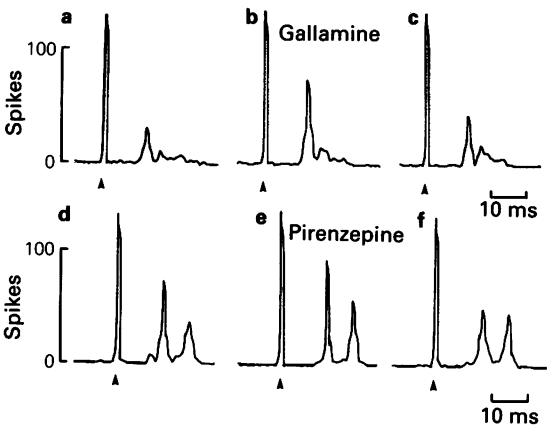

Figure 5 Peristimulus histogram (PSH) recordings from pyramidal tract neurones during stimulation of the contralateral hindlimb. The height of the histogram window containing 100 events is represented by the vertical bar. The first 10 ms shows a lack of spontaneous activity. This figure contains PSH recordings obtained during 3 min, 50 nA applications of gallamine and pirenzepine, with corresponding pre- and post-drug control PSHs.

tested, while gallamine inhibited ACh-induced firing in 83%. This indicates that both drugs are potent antagonists of ACh-induced firing in these cells.

The effects of gallamine and pirenzepine on glutamate-induced firing were assessed, revealing either no change in firing rate (46% and 58% of cells tested respectively), or a mild inhibition of firing (40% and 37% of cells tested respectively), which was usually associated with a decrease in spontaneous firing rates. Thus, the effect of gallamine and pirenzepine on glutamate-induced firing was variable in comparison to the clear and reproducible effects of both antagonists on ACh-induced and spontaneous firing rates. The actions of pirenzepine and gallamine observed in these experiments argue for a specific interaction with cholinoceptors. In 14% of the cells tested, gallamine enhanced glutamate-

Table 3 Effects of gallamine and pirenzepine on cortical pyramidal somatosensory units peristimulus histograms generated by stimulation of the contralateral hindpaw

	inc	nc	dec
Gallamine	47 (7)	53 (8)	0
Pirenzepine	45 (5)	36 (4)	18 (2)

Numbers represent percentage of all cells tested, with actual numbers (*n*) of cells tested in parentheses. inc = increased activity, nc = no change in activity, dec = decreased activity.

induced firing, an example of which is depicted in Figure 2.

Interpretation of the PSH data is made difficult by the complexity of the responses obtained following stimulation of NBM. The three phases observed in the PSHs following NBM stimulation may result from direct actions of ACh on pyramidal cells, or from effects on interneurones which then synapse on pyramidal cells. Recent intracellular recording data from *in vitro* slices of guinea-pig cerebral cortical pyramidal cells suggests that the actions of applied ACh on these cells represents both direct effects on the pyramidal soma (the delayed excitation), and activation of GABAergic interneurones which impinge on pyramidal cells (the inhibitory phase; McCormick & Prince, 1986b). The inhibitory effects of these interneurones would not be expected to be susceptible to antagonism by locally (iontophoretically) applied cholinoceptor agents, a prediction supported by both the present data, and the data of McCormick & Prince (1986b).

Results of the present study reveal that gallamine is a potent inhibitor of phase 3 (late excitation) of the PSHs obtained through NBM stimulation, inhibiting this response in 83% of cells tested. Pirenzepine was not as effective, inhibiting phase 3 in only 53% of neurones tested. Phase 2 (inhibition) was not affected by gallamine in 78% of cells tested, or by pirenzepine in 76% of cells tested, suggesting that neither M_1 nor M_2 muscarinic receptor activation is important in the generation of this response, which is consistent with the suggestion made above, that this response could represent non-cholinergic inhibitory interneurone activation. In this light, it is of interest that bicuculline, an antagonist of GABA, reduced the duration of phase 2 when applied iontophoretically onto 8 cortical cells (W. Marszalec, personal communication). The effect of gallamine on stage 1 was to enhance the response in 67% of cells tested, while pirenzepine enhanced the response in only 53% of cells tested. This response occurred with a short latency (less than 10 ms, see Figure 4), and was at times obscured by the stimulus artifact (see Figure 5). However, expanded PSH collections run with 1 ms bin widths revealed this phase to be a consistent component of the NBM response. The fact that the response was enhanced by both gallamine and pirenzepine suggests that this early response may not be cholinergic at all, but may represent some other input on the pyramidal cell from the NBM, whose response can be enhanced during muscarinic receptor blockade.

The effects of gallamine and pirenzepine on modulation of the response obtained from pyramidal neurones following hindlimb stimulation represents the interaction of these muscarinic antagonists with non-cholinergic, possibly glutamatergic, inputs from the ventral posterior lateral nucleus of the thalamus (Jones, 1986). Gallamine and pirenzepine enhanced the response in 47% and 45% of cells tested respectively. This lends further support to the idea that ACh may exert a modulatory role over incoming neuronal activity, and that blockade of cholinergic activity can lead to enhanced outputs from certain neuronal systems. However, which neurones specifically affect this control cannot be determined from these experiments.

The findings in this study are significant in that, until now, there has not been a demonstration that locally applied cholinolytic agents are capable of inhibiting the spontaneous firing of pyramidal cells. The above data clearly show that gallamine is very potent at inhibiting the spontaneous firing of pyramidal neurones, and that gallamine and pirenzepine both affect pyramidal cell activity. This information should prove valuable in interpreting receptor localization data such as that published by Spencer *et al.* (1986), who found autoradiographic evidence for M_2 receptors in the pyramidal cell layer. The data also add further support to the idea that the nucleus basalis magnocellularis is a major cholinergic input to the cortex, and that its responses may be influenced by muscarinic drugs. Clinically the decrease in cortical cholinoceptor sites found in Alzeheimer's disease (Mash *et al.*, 1985) may correlate with the increased destruction of the nucleus basalis of Meynert, also a consistent finding in this disease (Whitehouse *et al.*, 1981).

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