Development/Plasticity/Repair

Simultaneous Release of Glutamate and Acetylcholine from Single Magnocellular "Cholinergic" Basal Forebrain Neurons

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Basal forebrain (BF) neurons provide the principal cholinergic drive to the hippocampus and cortex. Their degeneration is associated with the cognitive defects of Alzheimer's disease. Immunohistochemical studies suggest that some of these neurons contain glutamate, so might also release it. To test this, we made microisland cultures of single BF neurons from 12- to 14-d-old rats. Over 1–8 weeks in culture, neuronal processes made autaptic connections onto the neuron. In 34 of 36 cells tested, a somatically generated action potential was followed by a short-latency EPSC that was blocked by 1 mM kynurenic acid, showing that they released glutamate. To test whether the same neuron also released acetylcholine, we placed a voltage-clamped rat myoball expressing nicotinic receptors in contact with a neurite. In six of six neurons tested, the glutamatergic EPSC was accompanied by a nicotinic (hexamethonium-sensitive) myoball current. Stimulation of the M_2-muscarinic presynaptic receptors (characterized using tripitramine and pirenzepine) produced a parallel inhibition of autaptic glutamatergic and myoball nicotinic responses; metabotropic glutamate receptor stimulation produced similar but less consistent and weaker effects. Atropine enhanced the glutamatergic EPSCs during repetitive stimulation by $25 \pm 6\%$; the anti-cholinesterase neostigmine reduced the train EPSCs by $37 \pm 6\%$. Hence, synaptically released acetylcholine exerted a negative-feedback inhibition of coreleased glutamate. We conclude that most cholinergic basal forebrain neurons are capable of releasing glutamate as a cotransmitter and that the release of both transmitters is subject to simultaneous feedback inhibition by synaptically released acetylcholine. This has implications for BF neuron function and for the use of cholinesterase inhibitors in Alzheimer's disease.

Key words: basal forebrain; cholinergic neuron; glutamate; acetylcholine (ACh); neurotransmitter; presynaptic regulation

Introduction

The cholinergic neurons of the basal forebrain (BF) provide a widespread excitatory projection to the cerebral cortex and hippocampus (Mesulam et al., 1983). They are involved in various higher cortical functions such as the maintenance of attention and wakefulness and the processing of short- and long-term memory (for review, see Wenk, 1997; Zaborszky et al., 1999; Lucas-Meunier et al., 2003). Moreover, their degeneration is one of the most highly correlated and consistent features associated with the loss of cognitive function in Alzheimer's disease (Perry et al., 1978; Whitehouse et al., 1982; Coyle et al., 1983).

However, some of the effects of BF degeneration may not solely result from a loss of acetylcholine. Thus, in addition to a substantial GABAergic component to this projection (Zaborszky et al., 1986; Gritti et al., 1993, 1997; Furuta et al., 2004), there is evidence for an important glutamatergic component (Gritti et al., 1993; Manns et al., 2001, 2003). Furthermore, a proportion, at least, of BF neurons appear to contain both ACh and glutamate, as judged from immunocytochemistry and single-cell reverse transcriptase-PCR analysis for specific markers (Manns et al.,

2001; Gritti et al., 2003; Sotty et al., 2003). There is also some experimental evidence to suggest that both of these transmitters might be released from BF axons/terminals. Thus, repetitive stimulation of cholinergic afferents to pyramidal cells in hippocampal slices or slice cultures typically evokes a train of fast EPSPs/EPSCs (most probably glutamatergic) followed by an IPSP/IPSC and a slow cholinergic EPSP/EPSC lasting 20–30 s (Benardo and Prince, 1982; Haas, 1982; Cole and Nicoll, 1984; Gahwiler and Brown, 1985). Corelease of ACh and glutamate has also been reported from a choline acetyltransferase affinity-purified preparation of cortical cholinergic synaptosomes (Docherty et al., 1987).

Nevertheless, these experiments do not unequivocally demonstrate that both neurotransmitters are released simultaneously from the same neuron. This is difficult to test *in situ*, not least because the postsynaptic responses to the two transmitters differ greatly in time course and mechanism: glutamate produces a fast ionotropic response, whereas ACh produces a slow, G-protein-mediated effect via muscarinic receptors.

In previous experiments, we circumvented the problem of recording ACh release by using cultured BF neurons and then creating an "artificial nicotinic synapse" with a skeletal myoball, so that ACh release from BF neuron processes is detected as a fast nicotinic current (Allen and Brown, 1996; Allen, 1999). In the present experiments, we have combined this approach with the "microisland" method to culture single BF neurons (Landis, 1976; Potter et al., 1980; Bekkers and Stevens, 1991; Segal, 1991).

The neuron then forms multiple autaptic contacts onto its own dendrites. As the neurons possess abundant ionotropic (AMPA and NMDA) glutamate receptors (Page and Everitt, 1995; Waters and Allen, 1998), any release of glutamate at the autapses will yield a conventional glutamatergic "synaptic" current. In this way, we show that individual cholinergic BF neurons can simultaneously release both ACh and glutamate and that the release of both transmitters is subject to powerful auto-inhibition by the released ACh, mediated by presynaptic M_2-muscarinic receptors.

Materials and Methods

Microisland cultures. Microisland cultures were prepared in 35 mm plastic Petri dishes. Their surface was first made nonpermissive for cell attachment by coating with a thin layer of agarose. Specifically, a 0.15% solution of agarose type II (Sigma, St. Louis, MO) in distilled water was prepared by heating in a microwave until the agarose was fully melted. A drop of this solution was then spread onto the surface of each Petri dish. As the solution cooled, the height of each drop was reduced as much as possible by removing excess solution with a pipette before the agarose gelled. The dishes were then allowed to thoroughly air dry overnight at room temperature. A mixture of 0.25 mg·ml^{-1} poly-D-lysine (75,000–150,000 molecular weight; Sigma) and 0.5 mg·ml^{-1} collagen (rat tail type I; Sigma) was then spotted onto the dishes using a glass atomizer (Thomas Scientific, Swedesboro, NJ) to create islands of permissive substrate ranging in diameter from 65 to 400 μm. Before use, the dishes were sterilized briefly under strong UV light for 3–5 min.

Preparation of basal forebrain neurons. A dissociated cell suspension of basal forebrain neurons was prepared as reported previously (Allen and Brown, 2004). Briefly, 12- to 14-d-old Sprague Dawley rat pups were anesthetized by chloroform inhalation before decapitation according to Home Office guidelines. The brain was removed rapidly and placed into ice-cold DMEM supplemented with 8 mM Mg^{2+} and 0.6% glucose. The brain was hemisected and 450-μm-thick coronal sections cut using a McIllwain tissue chopper. Basal forebrain areas, namely, the medial septum (MS), diagonal band of Broca (DBB), and substantia innominata (SI), were isolated and placed into HBSS containing 10 mM HEPES and 1.25 mg·ml^{-1} trypsin and incubated for 60 min at 37°C. The tissue fragments were subsequently washed in HBSS containing 10% fetal bovine serum (FBS) and 8 mM Mg^{2+} before being gently dissociated using a flamed Pasteur pipette. The resulting cell suspension was centrifuged at 600 rpm for 6 min, the supernatant discarded, and the cell pellet resuspended in Neurobasal medium containing B27 supplement (Invitrogen, Paisley, UK), 0.01 mg·ml^{-1} nerve growth factor, L-glutamine (0.5 mM), and 10% FBS. Cells were plated onto the dishes, prepared as detailed above, and left to settle for 2–3 h before the entire dish was gently flooded with medium. The medium was then replaced after 4 h to remove as much cellular debris as possible. Subsequent refeeding took place after 18 h and thereafter weekly with medium of the same composition minus FBS. Cells were maintained at 37°C in a 5% CO_2 incubator for periods of up to 8 weeks before use.

Preparation of skeletal myoball cultures. The method for preparing loosely adherent myoballs suitable for use as ACh detectors was as described previously (Allen, 1999).

Electrophysiology. Petri dishes bearing the microislands were transferred into a recording chamber mounted on stage of an Olympus (Melville, NY) BX50WI microscope equipped with Hoffman modulation optics and a fixed stage/moving microscope assembly. Unless otherwise stated, cells were superfused at a rate of 5–8 ml·min^{-1} at room temperature (22–26°C) with Krebs' solution of the following composition (in mM): 140 NaCl, 3 KCl, 2 $CaCl_2$, 1.2 $MgCl_2$, 12 glucose, and 10 HEPES, adjusted to pH 7.3 with NaOH. Recordings were performed using either the tight-seal whole-cell or amphotericin-B perforated (Rae et al., 1991) patch-clamp technique and either an Axoclamp 2A or Axopatch 200A amplifier coupled to a Digidata 1200 (Molecular Devices, Union City, CA) interface and pClamp 8 (Molecular Devices) acquisition software. Data were acquired at a sampling rate of 10–40 kHz and filtered at 1–5 kHz (voltage clamp) or 20 kHz (bridge voltage recording) before acquisition. When necessary, additional filtering was performed

off-line. Patch pipettes were pulled from 1.5-mm-outer-diameter × 1.17-mm-inner-diameter borosilicate glass (Harvard Apparatus, Holliston, MA) coated to within 100 μm of the tip with Sylgard (Dow Corning, Midland, MI). Electrode resistance ranged between 5 and 9 MΩ for whole-cell and 3 and 5 MΩ for perforated-patch recording. The composition of the pipette solution (unless otherwise stated) was the following (in mM): 108 K acetate, 11 KCl, 40 HEPES, 17 NaOH, 3 EGTA, 0.52 $CaCl_2$, and 1.2 $MgCl_2$, pH 7.3. During perforated-patch recording from neuronal microislands using the Axoclamp 200A amplifier, series resistance values after compensation (50–75%) ranged between 6 and 11 MΩ.

Data acquisition and analysis. Data were analyzed using a combination of the pClamp 8 suite of software (Molecular Devices), Origin 5 (Microcal Software, Northampton, MA), and CorelDraw 10 (Corel, Ottawa, Ontario, Canada). Unless otherwise stated, all values are mean ± SEM.

Drugs. All drugs were applied either via the superfusing solution or by inclusion in the pipette filling solution. Agarose IIA, acetylcholine chloride, D(−)-2-amino-5-phosphonopentanoic acid (D-AP-5), atropine, diazoxide, glycine, hexamethonium, kynurenic acid, N-methyl-lycaconitine (MLA), methoctramine, (±)-muscarine chloride, 1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-benzo[f]quinoxaline-7-sulfonamide disodium salt (NBQX), ±-α-methyl-(4-carboxyphenyl)glycine (MCPG), neostigmine, oxotremorine-M, pirenzepine, trans-(1S,3R)-(±)-1-amino-1,3-cyclopentanedicarboxylic acid (t-ACPD), and tolbutamide were purchased from Sigma. Tripitramine (1, 1, 24-tris[[5, 11-dihydro-6-oxo-6H-pyrido [2,3-b][1,4]-benzodiazepin-11-yl)carbonyl]methyl]-8, 17-dimethyl-1, 8, 17, 24-tetraazatetracosane tetraoxalate) was supplied as a generous gift by Prof. C. Melchiorre (University of Bologna, Bologna, Italy).

Results

Basal forebrain neurons form functional glutamatergic autaptic contacts

After a period of 5–6 d in microisland culture, individual BF neurons began to form functional synapses onto themselves (autapses). Over 2–8 weeks, synaptic responses became larger and more robust as the number of autaptic contacts increased (Fig. 1). Thus, brief (2–3 ms) depolarizing pulses applied to the cell soma through a patch pipette (Fig. 1A) induced an action potential in the soma (Fig. 1B). This was followed by a depolarizing postsynaptic potential (EPSP) as the first action potential propagated along the processes to the autaptic synapses (Fig. 1B,C); if sufficiently large, this EPSP generated a second action potential (Fig. 1B). Under voltage clamp, the postsynaptic response was manifest by a short-latency (inward) EPSC (Fig. 1D). In all but 4 of 36 cells tested, the EPSP/EPSC was reversibly blocked by 1 mM kynurenic acid (Fig. 1B), showing that it was caused by the action of released glutamate. In two of the remaining cells, the current was inhibited by 100 μM picrotoxin, indicating that it was mediated by GABA (or possibly glycine); in two additional cells of the 36, the EPSC required both kynurenic acid and picrotoxin for full inhibition, so resulted from the corelease of both glutamate and GABA/glycine.

Although EPSCs were primarily mediated by AMPA receptors, their decay phase was biphasic, with time constants of 4.57 ± 0.3 and 58.1 ± 0.09 ms ($n = 35$), the latter contributing 34 ± 9% to the total synaptic current. The slowly declining phase was because of activation of NMDA receptors (Fig. 1C). Thus, after addition of 1–3 μM glycine in the absence of extracellular Mg^{2+} (to enhance NMDA-mediated currents), the fast component could be selectively inhibited by the AMPA antagonist NBQX, whereas the slow component was inhibited by the selective NMDA antagonist D-AP-5 (both at 100 μM; four cells) (Fig. 1C,D).

Individual neurons release both glutamate and acetylcholine

Are these glutamate-releasing neurons truly cholinergic? To test this, we placed a voltage-clamped myoball expressing nicotinic ACh receptors (nAChRs) in contact with the neurites of a cultured BF neuron while at the same time recording the autaptic EPSC from the soma (Fig. 2A). As shown in Figure 2, B and C, on stimulating the neuron, we could then simultaneously record both a glutamatergic EPSC from the soma and a nicotinic current in the myoball. Kynurenic acid almost totally suppressed the somatically recorded EPSC without affecting the myoball current, whereas the nicotinic antagonist hexamethonium (100 μM) selectively suppressed the myoball current. Thus, the same neuron released both glutamate (to generate a glutamatergic autaptic EPSC) and ACh (to activate the nicotinic receptors in the myoball). Such corelease was observed in all of the six neurons we were able to test in this manner.

In some cells (Fig. 2Ci), hexamethonium produced a small (mean, 7.3 ± 3.3%; $n = 5$) reduction in the amplitude of the autaptic EPSC. Although this might be interpreted to suggest a minor cholinergic component to the compound EPSC, this seems unlikely for two reasons. First, only very small nicotinic currents (mean, 54.8 ± 17.4 pA; $n = 19$) could be evoked by somatic application of high concentrations of ACh (1 mM) onto these cells. These were most probably mediated by α7 subunit-containing receptors, because they were replicated by focally applied choline (1–10 mM), desensitized by bath application of choline (30–100 μM), and blocked by MLA (3–10 nM). In only one of a total of 20 cells tested did ACh evoked a much larger (967 pA) MLA-resistant current. Second, hexamethonium did not produce any further reduction of the residual postsynaptic current seen after suppressing the glutamatergic component of the EPSC with kynurenic acid. Hence, the effect of hexamethonium illustrated in Figure 2Ci is most likely the result of a weak inhibitory effect on glutamate receptors.

Presynaptic M$_2$ muscarinic receptors inhibit both ACh and glutamate release

We have shown previously that release of ACh from the processes of BF neurons in culture is strongly suppressed by stimulating the M$_2$ muscarinic ACh receptors (mAChRs) present on these neurons (Allen and Brown, 1996) and that this probably results from inhibition of the presynaptic N- and P/Q-type Ca^{2+} channels (Allen, 1999). We therefore asked whether activating these receptors could also inhibit glutamate release at autapses. Figure 2D indicates that this was indeed the case: application of the muscarinic agonist muscarine (10 μM) produced a parallel suppression of both the glutamatergic EPSC and the simultaneously recorded myoball nicotinic current (mean inhibition, 93.1 ± 2.3 and 96.9 ± 1.2%, respectively; $n = 6$). Muscarinic inhibition of the glutamatergic EPSC was concentration dependent (Fig. 3A). The IC$_{50}$ values for muscarine, oxotremorine-M, and ACh (in the presence of 3 nM N-methyllcaconitine) were 0.49 ± 0.10, 0.38 ± 0.11, and 0.40 ± 0.15 μM, respectively ($n = 5$–7 in each case).
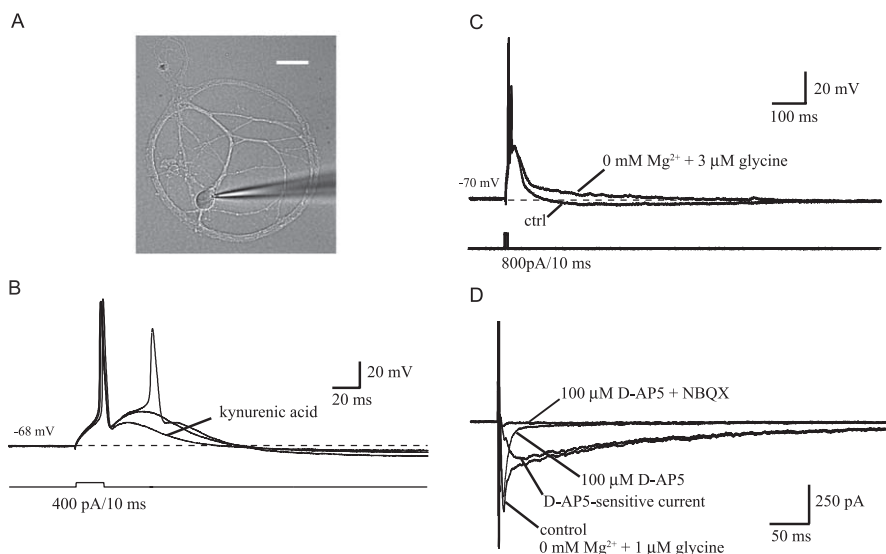

Figure 1. **A**, A typical microisland visualized using Normarski differential interference contrast optics. The neuron shown was obtained from a 12-d-old rat and had been maintained in culture for 22 d. Scale bar, 50 μm. **B**, Somatic action potentials generated by a brief (10 ms, 400 pA) current injection are followed by autaptic EPSPs, one of which generates a second action potential. The autaptic EPSP is attenuated by 1 mM kynurenic acid. [The residual, kynurenic acid-insensitive ADP results from activation of calcium-activated chloride channels (Sim and Allen 1998).] **C**, The EPSPs were prolonged when NMDA-mediated responses were enhanced by removal of extracellular Mg^{2+} and the addition of 3 μM glycine. (Note the slower time base.) ctrl, Control. **D**, Voltage-clamp recording of autaptic EPSCs in 0 [Mg^{2+}], 1 μM glycine solution. The EPSC comprises fast and slow components. The NMDA antagonist D-AP-5 (100 μM) selectively inhibited the slow component; the subtracted D-AP-5-sensitive current shows the characteristic slow rise time and decay of an NMDA current. The residual fast component was inhibited by the AMPA receptor antagonist NBQX (100 μM).

These IC$_{50}$ values for ACh and oxotremorine-M are similar to those determined previously for the inhibition of the somatic Ca^{2+} current in BF neurons (0.2 and 0.36 μM, respectively) (Allen and Brown, 1993).

We also determined the subtype of muscarinic receptor responsible for inhibition of glutamate release, using ACh as agonist and the antagonists pirenzepine and tripitramine (which are relatively selective for M$_1$/M$_4$ and M$_2$ receptors, respectively) (Caulfield and Birdsall, 1998). Mean IC$_{50}$ values for ACh-induced EPSC inhibition in the presence of 100 nM pirenzepine or 3 nM tripitramine were 0.64 ± 0.017 μM ($n = 7$) and 8.47 ± 2.4 μM ($n = 6$), respectively (Fig. 3B). Antagonist dissociation equilibrium constants (K_B, nanomolar) were calculated from the standard competitive antagonism equation as follows: $r = 1 + ([B]/K_B)$, where r is the ratio of the ACh IC$_{50}$ in the presence of antagonist to that in its absence, and [B] is the concentration of antagonist (nanomolar). Deduced values for K_B were as follows: pirenzepine, 166 nM; tripitramine, 0.148 nM. The ratio of these values (1121:1) is in good agreement with those reported previously for their relative affinities for M$_2$ receptors (800:1 to 1250:1) (Caulfield and Birdsall, 1998). Hence, muscarinic inhibition of glutamate release is mediated by the same (M$_2$) subclass of receptor as that responsible for inhibiting the Ca^{2+} current (Allen and Brown, 1993) and ACh release from BF neurons (Allen and Brown, 1996).

Synaptically released ACh inhibits glutamate release from the same BF neuron

Because the evoked release of glutamate from BF neurons is inhibited by exogenously applied ACh (see above), we asked whether glutamate release might also be inhibited when ACh is coreleased from the same neuron. Our first test for this was simply to record the glutamatergic EPSC during low-frequency (2
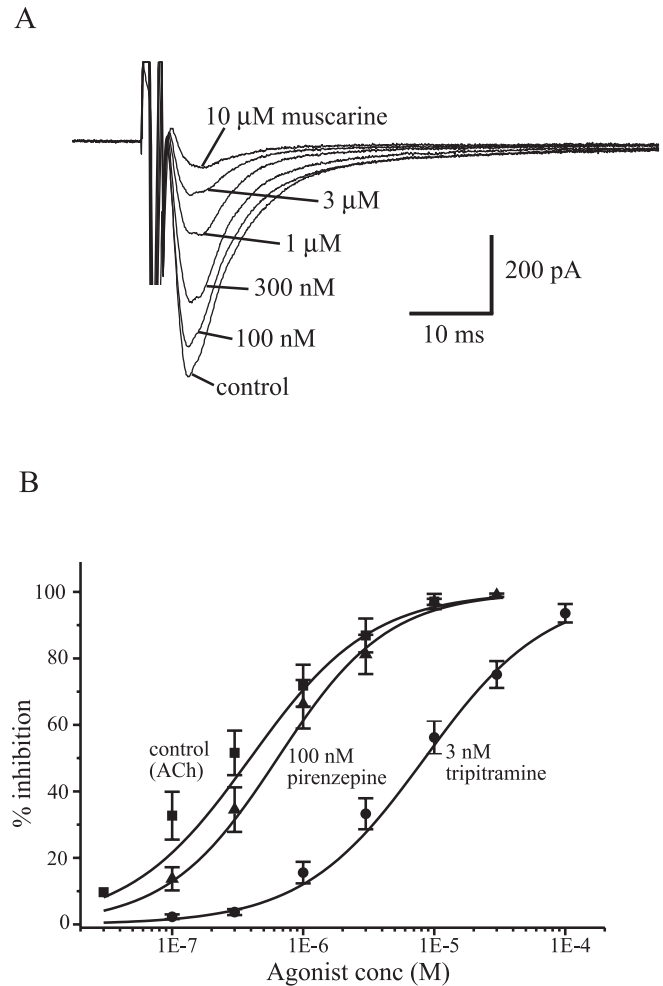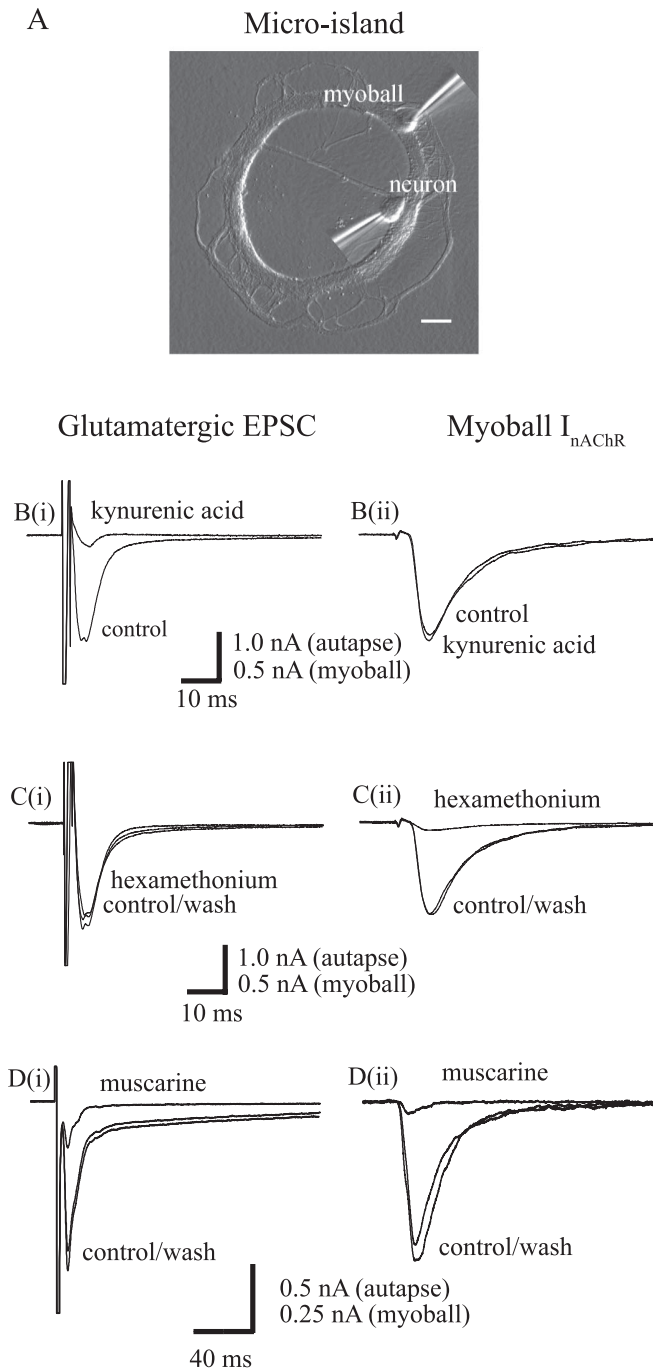
Figure 2. Corelease of glutamate and acetylcholine from single magnocellular basal forebrain neurons grown in microisland culture and suppression of their release by stimulating mAChRs. **A**, Photographic montage of a single-cell microisland from which the simultaneous release of glutamate (detected in the form of an autaptic EPSC by the neuronal recording electrode) and ACh (detected in the form of a nicotinic current in a voltage-clamped skeletal myoball placed in contact with the neurites of the cell) were detected from the same cell. Scale bar, 30 μm. **Bi, Ci, Di**, Glutamatergic autaptic EPSCs evoked by single action potentials elicited in response to a brief (2 ms) depolarizing step from a holding potential of −70 mV. **Bii, Cii, Dii**, Simultaneous release of ACh from the same cells detected using single perforated-patched skeletal myoballs voltage clamped at −70 mV and placed in contact with the neurites of the cell (Allen, 1999). Note that the records shown in **B** and **C** were obtained from the same neuron/myoball pair; records in **D** are from a different neuron/myoball pair. In **B**, the glutamate antagonist kynurenic acid (1 mM) abolished the autaptic EPSC without affecting the myoball response. Conversely, in **C**, the nicotinic ACh-receptor antagonist hexamethonium (100 μM) abolished the response of the myoball but had almost no effect on the autaptic EPSC. In **D**, the mAChR agonist muscarine (10 μM) reversibly inhibits both glutamate and ACh release from a single neuron.

Figure 3. Muscarinic receptor-mediated inhibition of the glutamatergic autaptic EPSC. **A**, EPSCs in a single cultured neuron in response to single action potentials elicited by imposing brief (2 ms) depolarization steps from V_h −80 mV under control conditions and in the presence of increasing concentrations of muscarine (0.1–10 μM). Each response is an average of four to six EPSCs after equilibration at each of the different agonist concentrations. **B**, Dose–response relationships for inhibition of EPSC by ACh recorded under control conditions and the presence of the M_1/M_4 mAChR antagonist pirenzepine or the M_2 antagonist tripitramine. Inhibition was measured as percentage reduction of peak EPSC amplitude. Curves are least-squares fits to the following Hill equation: $y = y_{max} \times x^{n_H}/(x^{n_H} + K^{n_H})$, where y = percentage inhibition, $y_{max} = 100$, x = agonist concentration (conc; micromolar), K is a constant (equivalent to the IC_{50}; micromolar), and n_H is the Hill slope. Fitted curves were constructed using the mean IC_{50} and Hill slope values obtained from the individual cells. All data points are the mean $\pm$ SEM values obtained after normalization of data obtained from the individual cells ($n = 5$–7 cells for each point). Mean values for K (micromolar; n_H in parentheses) were as follows: control, 0.395 ± 0.152 (slope, 0.95); pirenzepine, 0.639 ± 0.017 (slope, 1.23); and tripitramine, 8.47 ± 2.4 (slope, 0.93).

Hz) trains of stimuli delivered before and after adding the muscarinic antagonist atropine (30–100 nM). The EPSC declined substantially after the first stimulus but was then maintained at a steady, lower level throughout the rest of the train (Fig. 4A–C). Atropine did not affect the amplitude of the first EPSC (showing that it did not affect the postsynaptic action of glutamate) but consistently increased the amplitude of subsequent EPSCs, by on average $25 \pm 5.5\%$ ($n = 4$) (Fig. 4C).

In the experiment illustrated in Figure 4, A and B, atropine produced full enhancement of the second EPSC, which was then sustained throughout the train of impulses. Thus, muscarinic receptor activation must have attained its full effect within the

500 ms between the first and second stimuli and then reached a steady state thereafter. To examine how rapidly the activation occurs, we repeated these experiments using 10 Hz stimuli delivered in short trains (to minimize transmitter depletion; see legend to Fig. 4). In this case, atropine had no effect on the first and second EPSCs but showed progressively increasing effects on the third and fourth EPSCs (mean enhancement of third and fourth EPSC amplitudes being 4.9 ± 1.2 and $10.9 \pm 2.8\%$, respectively; $n = 10$) (Fig. 4D,E). The fifth EPSC evoked 1 s later without intervening stimuli was still enhanced (mean increase, $23.7 \pm 5.9\%$; $n = 10$), showing that inhibition was sustained (Fig. 4F). Thus, this form of presynaptic inhibition had a delay of at least 100 ms, a rise time of ≥ 200 ms, and a duration of >1 s. It seems most likely that inhibition results from activation of M_2 muscarinic receptor coupled to presynaptic Ca^{2+} channels by $\beta\gamma$ subunits of the G-protein G_o (Allen and Brown, 1996). The equivalent form of mAChR-induced Ca^{2+} current inhibition in sympathetic neurons has an onset time constant of ~ 1 s and a recover time constant of $6–10$ s after rapid agonist application (Zhou et al., 1997). However, auto-inhibition in the CNS by a synaptically released transmitter can be somewhat faster than this (e.g., ~ 0.2 and 2 s for onset and offset of $GABA_B$-mediated inhibition in the hippocampus) (Pfrieger et al., 1994). Our observations match the latter quite well; at the very least, they imply that the site of ACh release must have been very close to the Ca^{2+} channels that triggered the release of glutamate.

The extent to which glutamate release would be inhibited by synaptically released ACh will not just depend on the local concentration but also the number of receptors it has access to. This in turn will depend on its lifetime before it is broken down by endogenous cholinesterases. To determine what influence cholinesterases exert on the strength of presynaptic inhibition, we examined the effect of the anti-cholinesterase drug neostigmine (3 μM) on the amplitude of the glutamatergic EPSC, using the same short-train protocol illustrated in Figure 4D. Again, like atropine, it had no effect on the amplitude of the first two EPSCs but reduced the amplitude of all subsequent EPSCs. The mean inhibition of third, fourth, and fifth EPSC amplitudes were 10.8 ± 1.3, 15.8 ± 1.9, and $36.7 \pm 5.7\%$, respectively ($n = 12$) (Fig. 5). These effects were fully reversed by $30–100$ nM atropine. Thus, inhibiting cholinesterase clearly intensified the cross-inhibitory effect of ACh on glutamate release.

Do BF neurons possess presynaptic glutamate receptors?
Because these neurons are both cholinergic and glutamatergic, the question arises whether, like some other glutamatergic neurons, they possess inhibitory presynaptic "metabotropic" glutamate receptors (mGluRs), in addition to presynaptic muscarinic
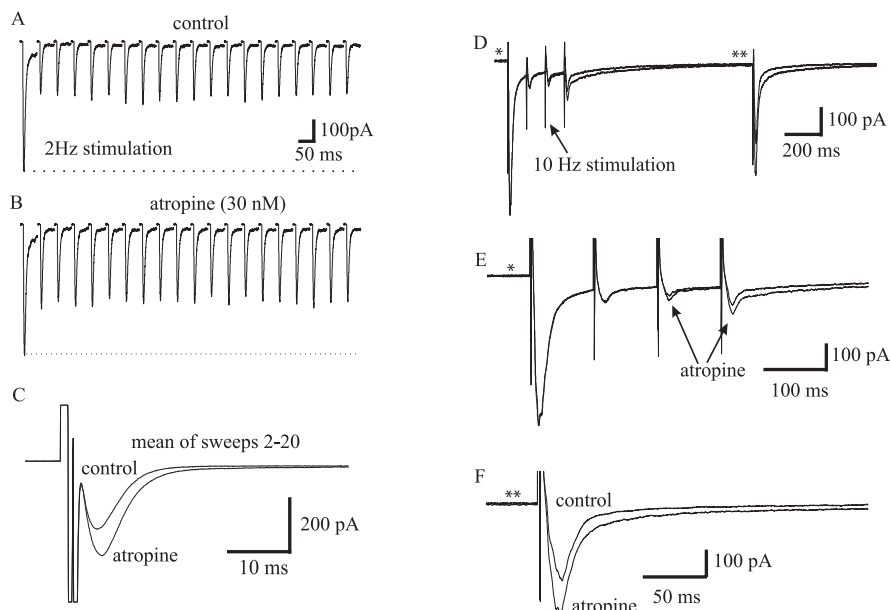

Figure 4. Glutamate-mediated EPSCs are enhanced when muscarinic autoreceptors are blocked by atropine, showing that glutamate release is depressed by coreleased ACh. *A*, *B*, Autaptic glutamatergic EPSCs evoked at 2 Hz under control conditions and in the presence of the mAChR antagonist atropine (30 nM). *C*, Mean amplitudes of the second to 20th EPSCs in *A* and *B* recorded before and after adding atropine. *D*, A brief burst of four glutamatergic EPSCs evoked at a frequency of 10 Hz followed by a delay of 1 s before a final (5th) EPSC, recorded before and after adding 100 nM atropine. (This protocol was repeated every 60 s.) *E*, *F*, The initial four (*) and final (5th; **) EPSCs on an expanded scale. In the presence of atropine, the amplitudes of the first and second EPSCs remained unchanged, whereas the amplitude of the subsequent EPSCs became larger. This shows that the presynaptic muscarinic autoreceptors activate within $150–200$ ms of the start of the first action potential/release event. Once activated in this way, functional inhibition of glutamate release persists for a period in excess of 1 s (*F*). EPSCs were evoked by briefly stepping to a depolarized potential for 2 ms from a holding potential of -70 mV; all traces are the mean of three or four repetitions. Note that the dramatic decline in the glutamatergic EPSC after the first stimulus mostly persisted in the presence of atropine and therefore could not be attributed to cholinergic presynaptic inhibition. One possibility is that it results from AMPA receptor desensitization. We tested this using 200 μM diazoxide (to reduce desensitization) (Yamada and Tang, 1993) in the presence of 100 μM tolbutamide (the latter to block K_{ATP} channel activation) using the protocol in *D*. Diazoxide increased both amplitude and half-decay times of the first EPSC [by $51 \pm 6.4\%$ ($n = 9$) and $212 \pm 34.7\%$ ($n = 7$), respectively] but did not significantly affect the depression of the second EPSC (second/first EPSC amplitudes 35 ± 5.5 and $43.4 \pm 5.7\%$ in the absence and presence of drug, respectively; $n = 8$). Because the release of ACh from these neuron processes shows a comparable fall-off (Allen and Brown 1996), it most likely results from a temporary depletion of transmitter or exhaustion of the release machinery. The 1 s rest period in *D* allows some recovery from this depletion.

receptors; and, if so, is there reciprocal mGluR-mediated inhibition of ACh release? We tested this using the mGluR agonist t-ACPD. t-ACPD (30 μM) inhibited the glutamatergic EPSC in 12 of 21 cells tested (mean inhibition, $23.7 \pm 5.1\%$; $n = 12$) and the myoball nAChR current in four of seven cells (mean inhibition, $50.1 \pm 13.8\%$; $n = 4$). Of six neurons in which ACh and glutamate release from the same cell were recorded simultaneously, t-ACPD inhibited the release of both transmitters in three cells (Fig. 6), ACh release alone in one cell, and had no effect in the remaining two cells. Thus, the effects of mGluRs differ from those of muscarinic AChRs in two important ways. First, they only inhibit release in a subpopulation of the cells. Second, the effects of t-ACPD were much weaker than those of muscarinic agonists, which produced virtually complete inhibition of release of both transmitters in all of the neurons tested (see above). We also tested the effect of the mGluR antagonist MCPG (30–300 μM) on evoked glutamatergic EPSCs but could detect no significant increase in EPSC amplitude in any of five cells tested.

We wondered why t-ACPD had such a weak effect. We therefore tested whether t-ACPD affected the somatic Ca^{2+} current in the same manner as mAChR agonists (Allen and Brown, 1993).
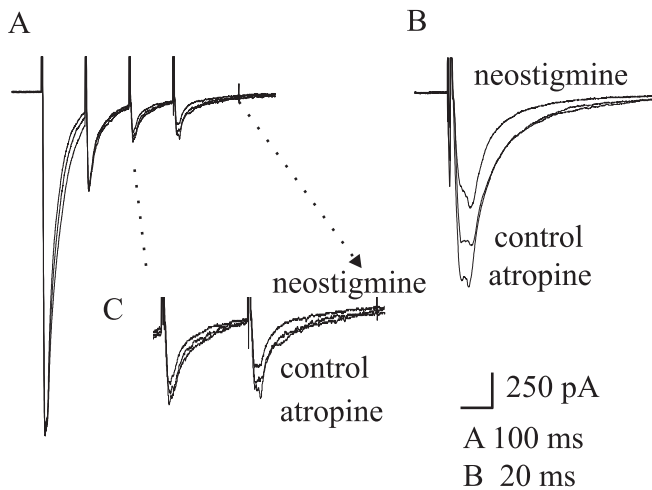
Figure 5. Effects of the anti-cholinesterase neostigmine (3 μM) on presynaptic muscarinic receptor-mediated inhibition of glutamatergic EPSCs. ***A***, Four EPSCs were evoked at a frequency of 10 Hz, with a delay of 1 s before a fifth EPSC was elicited (***B***). This protocol repeated every 60 s. Addition of neostigmine had no effect on the initial two EPSCs but depressed all subsequent ones, including that recorded 1 s later (***B***, ***C***). Addition of atropine (100 nM) in the presence of neostigmine fully reversed this effect and even enhanced the later EPSCs.
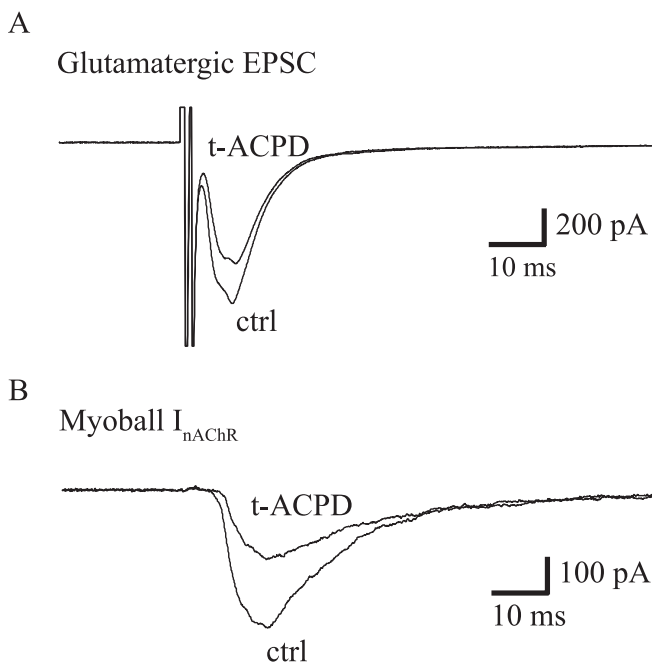

Figure 6. Stimulation of presynaptic metabotropic glutamate receptors (mGluRs reduces the release of both glutamate and ACh). Records show the depressant effect of the mGluR agonist t-ACPD (30 μM) on glutamatergic autaptic EPSCs (***A***) and myoball nicotinic ACh currents (***B***), respectively, recorded simultaneously on stimulating a single BF neuron in a microisland culture (see Fig. 2). Note that all records are the mean of four repetitions in the presence and absence of agonist. For clarity, washout responses have been omitted, but autaptic and myoball responses recovered to 97 and 103%, respectively, of the control (ctrl) values.

Using perforated-patch recording and constructing current–voltage curves by the protocol described previously, we found that t-ACPD, in concentrations up to 300 μM, had no effect on the Ca^{2+} current in 10 of 12 neurons tested, although mAChR agonists were fully effective under the same conditions and produced <10% inhibition in the remaining two cells. We also

looked for other potential release-inhibiting effects that might be detectable by somatic recording, such as activation of inwardly rectifying K^+ currents, by constructing full current–voltage curves in the presence of tetrodotoxin using square voltage steps between -140 and 0 mV. We could not discern any effect of t-ACPD on the amplitudes or kinetics of the inward-rectifier, A-type, or delayed-rectifier K^+ currents on K_{ATP} currents (recorded in the presence and absence of the activator diazoxide) or on the "leak" K^+ current. We conclude either that the sample of neurons we used for these experiments did not possess somatic mGluRs or that the (irregular) effect of mGluR stimulation on evoked transmitter release stems from some other, perhaps intracellular, effect that differs from the effect of mAChR stimulation.

Discussion

The principle of cotransmission, the process whereby a neuron can store and release two or more different neurotransmitters, is now widely accepted (Burnstock, 2004). Corelease of ACh and glutamate has been described previously in *Torpedo* electric organ synaptosomes (Israel et al., 1993) and from amphibian neurons (Li et al., 2004), and very recently, a component of the synaptic current at the classical nicotinic motoneuron–Renshaw cell synapse in the spinal cord has been attributed to the release of glutamate (Mentis et al., 2005; Nishimura et al., 2005).

In the latter experiments, detection of cotransmission at a single synapse was facilitated by the fact that both transmitters activated fast ionotropic receptors. Comparable experiments on BF–cortical or hippocampal target cell synapses are not possible because, at these synapses, ACh stimulates slowly operating muscarinic ACh receptors. In the present experiments, we therefore converted the normal target synapse to a glutamatergic autapse and converted the muscarinic response to a nicotinic response with a myoball. In this way, we have unequivocally demonstrated the corelease of glutamate and ACh from single basal forebrain neurons. Virtually all of the BF neurons displayed short-latency fast autaptic EPSCs mediated by glutamate on stimulating the soma of the same neuron, and, in all six neurons so tested, an ACh-mediated nicotinic current could be simultaneously recorded in a detector myoball apposed to one of its processes. Moreover, even in the absence of direct recording of ACh release with the myoball, in an additional 14 cells, addition of atropine and/or neostigmine modified glutamatergic EPSCs in the manner expected for simultaneous corelease of ACh (see below).

Although autaptic connections are favored in microisland cultures (Mennerick et al., 1995), the high frequency of glutamate-mediated EPSCs seen on stimulating presumed cholinergic BF neurons is unlikely to be an artifact of culture, because glutamatergic EPSPs and EPSCs have also been noted to precede the slow cholinergic postsynaptic response of pyramidal cells in slices of adult hippocampus on stimulating afferents from the medial septum of the BF (Cole and Nicoll, 1984) or in long-term organotypic cultures (Gahwiler and Brown, 1985). What was not clear in those experiments was whether the glutamate and the ACh were released from axons of the same or different neurons. Our observations indicate that, in most cases at least, the two transmitters were likely to have been released from the same neuron. We have not been able to directly determine whether ACh and glutamate are coreleased from the same vesicles or release sites, because the BF neurons possess too few nicotinic receptors to permit simultaneous detection of a nicotinic component to the autaptic EPSC. However, the rapid time course of the cholinergic feedback inhibition of glutamate release would suggest a very close apposition of the release foci for the two transmitters.

This raises the interesting question whether these neurons should be regarded as phenotypically cholinergic or glutamatergic neurons. This we cannot directly answer: we would only note that they correspond in all respects to those categorized previously as magnocellular cholinergic neurons. Thus, in previous experiments using these neurons in long-term culture, they showed the same electrophysiological properties as those reported for identified cholinergic BF neurons *in situ* (Allen and Brown, 1993; Sim and Allen, 1998) and released ACh from their processes (Allen and Brown, 1996). Furthermore, neurons isolated in the same manner, grown on the same substrate, and maintained in identical growth media as those used in the present experiments (albeit as mass cell cultures) expressed the cholinergic marker choline acetyltransferase as determined by single-cell PCR (Allen and Brown, 2004). In this respect, therefore, they belong to that class of BF neurons conventionally regarded as cholinergic.

Functionally, the corelease of glutamate with ACh from these neurons is likely to have several important consequences. First, it suggests that some re-evaluation of the role of BF neurons in regulating cortical and hippocampal excitability may be necessary. Thus, BF neurons have previously been considered to have a rather slow, tonic, and "diffusive" effect on cortical and hippocampal neuron activity (for review, see Harkany et al., 1999). This would accord with the slow and sustained effect of released ACh on the target pyramidal neurons (Cole and Nicoll, 1984; Gahwiler and Brown, 1985). However, the ability to generate fast EPSCs through corelease of glutamate would allow for additional, rapid, and more punctate effects, more closely geared to the discharge patterns of the BF neurons. Furthermore, because much of the cholinergic innervation is onto dendritic shafts as opposed to pyramidal cell bodies (Beaulieu and Somogyi, 1991; Umbriaco et al., 1994; Mrzljak et al., 1995), it is likely that ACh also modulates the active conductance properties of these dendrites to facilitate or attenuate throughput and summation of synaptic inputs from other sources. If some of these afferents also release glutamate, then this could provide an additional excitatory input with much faster onset and offset rates than that mediated by mAChRs.

Second, the release of glutamate raises the possibility of cross-excitation between cholinergic BF neurons. No such excitation would be likely to occur as a result of ACh release, because magnocellular BF neurons express predominantly M_2 mAChRs, which are (if anything) inhibitory, and, as noted above, the density of nAChRs is too low to generate of fast EPSPs. However, within the MS–DBB region, many of the projecting cholinergic BF neurons give off axon collaterals that innervate other septohippocampal cholinergic and GABAergic neurons (Leranth and Frotscher, 1989). Thus, 93% of the ChAT-positive neurons within the MS–DBB receive contacts from at least one vesicular glutamate transporter 2-containing bouton (Hajszan et al., 2004). This glutamatergic innervation has both extrinsic and intrinsic origins (Csaki et al., 2000, 2002; Fremeau et al., 2001; Manns et al., 2001; Kiss et al., 2002; Lin et al., 2003; Hajszan et al., 2004). Because the projections of individual septohippocampal neurons are topographically organized with relatively small terminal fields (<1–1.5 mm in diameter) (Price and Stern, 1983), lateral interconnectivity within the MS–DBB mediated by glutamate could provide a mechanism for rapid radiation of excitation within the hippocampus. Furthermore, glutamatergic cross-transmission within the BF may be subject to negative-feedback regulation by coreleased ACh (see below), because glutamergic EPSCs recorded from BF neurons are subject to the same (M_2-

mediated) muscarinic inhibition as that observed at our autapses (Sim and Griffith, 1996). In addition, our finding that in a proportion of cells, activation of mGluRs attenuated the glutamatergic EPSC suggests that glutamate might be released and act presynaptically to modulate its own release (and possibly the release of ACh as well). However, we could not substantiate this because the mGluR blocking agent MCPG did not enhance glutamatergic EPSCs; this probably reflects the much weaker and less consistent effects of exogenously applied mGluR agonists compared with those produced by mAChR agonists.

Finally, it has long been established that the release of ACh from cholinergic projection neurons in the cortex is subject to very strong negative-feedback inhibition through the presynaptic M_2 mAChRs (Dudar and Szerb, 1969). The present experiments show that this is accompanied by an equally strong feedback inhibition of glutamate release, mediated through the same (M_2) mAChRs as those responsible for the inhibition of ACh release and furthermore, that this is intensified by anti-cholinesterase drugs. This means that glutamate release may also be inhibited when attempts are made to use such drugs in the treatment of Alzheimer's disease. The consequent reduction of glutamatergic excitation may be one reason why these drugs appear to have rather limited effects.

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