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Impaired Modulation of GABAergic Transmission by Muscarinic Receptors in a Mouse Transgenic Model of Alzheimer's Disease*

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It has long been recognized that muscarinic acetylcholine receptors (mAChRs) are crucial for the control of cognitive processes, and drugs that activate mAChRs are helpful in ameliorating cognitive deficits of Alzheimer's disease (AD). On the other hand, GABAergic transmission in prefrontal cortex (PFC) plays a key role in "working memory" via controlling the timing of neuronal activity during cognitive operations. To test whether the muscarinic and γ-aminobutyric acid (GABA) system are interconnected in normal cognition and dementia, we examined the muscarinic regulation of GABAergic transmission in PFC of an animal model of AD. Transgenic mice overexpressing a mutant gene for β-amyloid precursor protein (APP) show behavioral and histopathological abnormalities resembling AD and, therefore, were used as an AD model. Application of the mAChR agonist carbachol significantly increased the spontaneous inhibitory postsynaptic current (sIPSC) frequency and amplitude in PFC pyramidal neurons from wild-type animals. In contrast, carbachol failed to increase the sIPSC amplitude in APP transgenic mice, whereas the carbachol-induced increase of the sIPSC frequency was not significantly changed in these mutants. Similar results were obtained in rat PFC slices pretreated with the β-amyloid peptide (Aβ). Inhibiting protein kinase C (PKC) blocked the carbachol enhancement of sIPSC amplitudes, implicating the PKC dependence of this mAChR effect. In APP transgenic mice, carbachol failed to activate PKC despite the apparently normal expression of mAChRs. These results show that the muscarinic regulation of GABA transmission is impaired in the AD model, probably due to the Aβ-mediated interference of mAChR activation of PKC.

Alzheimer's disease (AD)[1] is a devastating neurodegenerative disorder. Several prominent features consistently found in AD patients include: degeneration of basal forebrain cholinergic neurons and ensuing deficient cholinergic functions in cortex and hippocampus, extracellular protein aggregates containing β-amyloid peptides (Aβ) in these cholinergic target areas, and impairments in mental functions that are characterized by the loss of memory (1–3). So far, the most effective therapeutic strategy in AD treatment is to enhance cholinergic transmission (4, 5). It has long been recognized that muscarinic acetylcholine receptors (mAChRs) are crucial for the control of high level cognitive processes (6, 7). Drugs that antagonize mAChRs worsen the performance of human subjects and animals in learning and memory tasks (8, 9), while drugs that activate mAChRs are helpful in ameliorating cognitive deficits of AD (10, 11). Despite the discovery of correlation between cholinergic hypofunction and AD, the cellular and molecular mechanisms underlying the function and dysfunction of mAChRs in normal cognition and dementia remain elusive.

Prefrontal cortex (PFC), one of the major target areas of basal forebrain cholinergic neurons, has long been associated with high-level, "executive" processes (12), particularly a form of short term information storage described as "working memory" (13). The cholinergic activity in frontal cortex is persistently increased in mice performing a working memory task (14). All the five subtypes of mAChRs, m1–m5 (15), are expressed in cortical pyramidal neurons (16–18). One of the important questions yet to be answered is the targets of muscarinic signaling that are involved in cognition and memory. Recent studies show that GABAergic inhibition in frontal cortex controls the timing of neuronal activities during cognitive processes, therefore, shaping the flow of information in cortical circuits (19). The critical involvement of cortical muscarinic signaling in cognition and AD, combined with the central role of GABAergic inhibition in working memory, prompts us to hypothesize that the GABA system might be a key cellular substrate for muscarinic signaling in cognition and memory, and its dysregulation by mAChRs in AD might contribute to the cognitive impairment.

Emerging evidence suggests that low concentrations of Aβ peptides can potently inhibit various cholinergic neurotransmitter functions independently of concurrent neurotoxicity (20). Aβ peptides are produced by proteolytic cleavage of the β-amyloid precursor protein (APP) (21). Most of the mutations in the APP gene are clustered around the cleavage sites, which increases the rate of cleavage, thereby generating more Aβ (22, 23). Transgenic mice overexpressing mutant APP genes exhibit AD-like symptoms, including increased Aβ deposits and deficits in spatial learning and memory (24–26). In this study, we used this AD model to examine the muscarinic regulation of GABAergic synaptic transmission in PFC pyramidal projection neurons.

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[1] The abbreviations used are: AD, Alzheimer's disease; Aβ, β-amyloid peptide; mAChR, muscarinic acetylcholine receptor; PFC, prefrontal cortex; APP, β-amyloid precursor protein; IPSC, inhibitory postsynaptic current; sIPSC, spontaneous IPSC; GABA, γ-aminobutyric acid; $GABA_A$, γ-aminobutyric acid, type A; PKC, protein kinase C; PKA, cAMP-dependent protein kinase; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; K-S, Kolmogorov-Smirnov; CCh, carbachol; PKI, PKA inhibitor; ANOVA, analysis of variance; RT, reverse transcription.

EXPERIMENTAL PROCEDURES

An AD Model—Transgenic mice carrying the human APP 695 with the double mutation K670N and M671L (Swedish mutation) were created as described previously (25). Eight-week-old transgenic males (on B6SJLF1 hybrid background) were bred with mature B6SJLF1 females. Genetic background of these mice is the same with this breeding scheme. Genotyping were performed by PCR. Male transgenic (1–2 months old) and age-matched wild-type littermates were used in the experiments.

Electrophysiological Recordings in Slices—Young adult rat or mouse slices containing PFC were prepared as described previously (27, 28). All experiments were carried out with the approval of State University of New York at Buffalo Animal Care Committee. In brief, animals were anesthetized by inhaling 2-bromo-2-chloro-1,1,1-trifluoroethane (1 ml/100 g, Sigma) and decapitated, and brains were quickly removed, iced, and then blocked for slicing. The blocked tissue was cut in 300–400-μm slices with a Vibrotome while bathed in a low Ca^{2+} (100 μM), HEPES-buffered salt solution (in mM: 140 sodium isethionate, 2 KCl, 4 $MgCl_2$, 0.1 $CaCl_2$, 23 glucose, 15 HEPES, 1 kynurenic acid, pH = 7.4, 300–305 mosM/liter). Slices were then incubated for 1–6 h at room temperature (20–22 °C) in a $NaHCO_3$-buffered saline bubbled with 95% O_2, 5% CO_2 (in mM): 126 NaCl, 2.5 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 10 glucose, 1 pyruvic acid, 0.05 glutathione, 0.1 N^G-nitro-L-arginine, 1 kynurenic acid, pH = 7.4, 300–305 mosM/liter.

To evaluate the regulation of spontaneous IPSCs by muscarinic receptors in PFC slices, the whole-cell patch technique was used for voltage-clamp recordings using patch electrodes (5–9 MΩ) filled with the following internal solution (in mM): 100 CsCl, 30 *N*-methyl-D-glucamine, 10 HEPES, 1 $MgCl_2$, 4 NaCl, 5 EGTA, 12 phosphocreatine, 2 MgATP, 0.2 Na_3GTP, 0.1 leupeptin, pH = 7.2–7.3, 265–270 mosM/liter. The slice (300 μm) was placed in a perfusion chamber attached to the fixed-stage of an upright microscope (Olympus) and submerged in continuously flowing oxygenated artificial cerebrospinal fluid. For blocking glutamate transmission, the α-amino-3-hydroxy-5-methyl-4-isoxazole proprionic acid/kainate (AMPA/KA) receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (10 μM) and *N*-methyl-D-aspartate receptor antagonist D(−)-2-amino-5-phosphonopetanoic acid (25 μM) were added to the recording solution. Cells were visualized with a 40× water-immersion lens and illuminated with near infrared (IR) light, and the image was detected with an IR-sensitive CCD camera. A Multiclamp 700A amplifier (Axon instruments, Union City, CA) was used for these recordings. Tight seals (2–10 GΩ) from visualized pyramidal neurons were obtained by applying negative pressure. The membrane was disrupted with additional suction and the whole-cell configuration was obtained. The access resistances ranged from 13 to 18 MΩ and were compensated 50–70%. Cells were held at −70 mV for the recording of spontaneous IPSCs. To examine the muscarinic regulation of intrinsic firing patterns, current-clamp recordings were performed using patch electrodes filled with the internal solution (in mM): 60 K_2SO_4, 60 *N*-methyl-D-glucamine, 40 HEPES, 4 $MgCl_2$, 5 BAPTA, 12 phosphocreatine, 2 Na_2ATP, 0.2 Na_3GTP, 0.1 leupeptin, pH = 7.2–7.3, 265–270 mosM/liter. The resting membrane potential of the neurons was −61.4 ± 0.96 mV (wild-type, n = 10) and −61.1 ± 1.02 mV (APP transgenic: n = 9) in control Ringer's solution, and −59.3 ± 0.65 mV (wild-type, n = 10) and −59.6 ± 0.73 mV (APP transgenic: n = 9) in the presence of carbachol (10 μM). Glutamatergic and GABAergic transmission was blocked to ensure that the phenomena studied were independent of synaptic transmission. All experiments were performed side by side with cells from wild-type *versus* APP transgenic mice or non-treated *versus* Aβ-treated slices.

Mini Analysis Program (Synaptosoft, Leonia, NJ) was used to analyze synaptic activity. Individual synaptic events with fast onset and exponential decay kinetics were captured with threshold detectors in Mini Analysis software. All quantitative measurements were taken 4–6 min after drug application. IPSCs of 60 s (200–1000 events) under each different treatment were used for obtaining the cumulative distribution plots. The detection parameters for analyzing synaptic events in each cell in the absence or presence of carbachol were the same. Statistical comparisons of the frequency and amplitude of synaptic currents were made using the Kolmogorov-Smirnov (K-S) test. Numerical values were expressed as mean ± S.E.

Muscarinic receptor ligand carbachol (CCh), atropine and pirenzepine (Sigma), as well as second messenger reagents calphostin C, bisindolylmaleimide I (*i.e.* GF109203X; Gö6850), and myristoylated PKI-(14–22) (Calbiochem) were made up as concentrated stocks in water and stored at −20 °C. Stocks were thawed and diluted immediately prior to use. The β-amyloid peptide Aβ_{25-35} and the control peptide containing the reverse sequence Aβ_{35-25} were obtained from Sigma. These peptides were resuspended in sterile distilled water at a concentration of 2 mM and incubated at 37 °C for 1 h to allow fibril formation.

Western Blot Analysis—For detecting activated PKC, a phospho-PKC (pan) antibody that recognizes PKCα, β_I, β_{II}, ϵ, η, and δ isoforms only when phosphorylated at a carboxyl-terminal residue homologous to Ser^{660} of PKCβ_{II} was used in the Western blot analysis. After incubation, slices were transferred to boiling 1% SDS and homogenized immediately. Insoluble material was removed by centrifugation (13,000 × *g* for 10 min), and protein concentration for each sample was measured. Equal amounts of protein from slice homogenates were separated on 7.5% acrylamide gels and transferred to nitrocellulose membranes. The blots were blocked with 5% nonfat dry milk for 1 h at room temperature. Then the blots were incubated with the phospho-PKC (pan) antibody (Cell Signaling, 1:2000) for 1 h at room temperature. After being rinsed, the blots were incubated with horseradish peroxidase-conjugated anti-rabbit antibodies (Amersham Biosciences, 1:2000) for 1 h at room temperature. Following three washes, the blots were exposed to the enhanced chemiluminescence substrate. Then the blots were stripped for 1 h at 50 °C followed by saturation in 5% nonfat dry milk and incubated with a PKC antibody (Santa Cruz, 1:2000) recognizing the α, β, and γ isoforms. Quantitation was obtained from densitometric measurements of immunoreactive bands on autoradiograms. Data correspond to the mean ± S.E. of 5–10 samples per condition and were analyzed by ANOVA tests.

mRNA Detection—PFCs were dissected from wild-type and APP transgenic mouse brain slices (400 μm) and homogenized in 0.5 ml of TRIzol reagent (Invitrogen). Following 5 min of incubation at room temperature, 0.1 ml of chloroform was added and mixed with the homogenized samples. The tubes were incubated at 25 °C for 2–3 min and then centrifuged for 15 min at 4 °C. The upper aqueous phase containing RNA for each sample was transferred to a fresh tube. Then RNA was precipitated from the aqueous phase by mixing with 0.25 ml of isopropyl alcohol, incubating at room temperature for 10 min and then centrifuging for 10 min at 4 °C. The supernatant was removed, and the RNA pellet was washed with 75% ethanol. The RNA pellet was air-dried and then dissolved in diethyl pyrocarbonate-treated water.

Prior to reverse transcription-polymerase chain reaction (RT-PCR), the isolated RNA was treated with DNase I (Invitrogen) to eliminate genomic DNA. The reaction mixture (10 μl) contained 1 μg of RNA, 1 μl of 10× DNase I reaction buffer, 1 μl of DNase I (1 unit/μl), and 8 μl of diethyl pyrocarbonate-treated water. The tube was incubated at room temperature for 15 min. The reaction was terminated by adding 1 μl of 25 mM EDTA and heating for 10 min at 65 °C. RT-PCR analysis of muscarinic receptor cDNAs was performed as described previously (29, 30). PCR products were separated by electrophoresis in a 1.5% agarose gel stained with ethidium bromide. As a control for genomic contamination, samples were prepared as described above except that the RT was omitted in the reverse transcription procedure.

RESULTS

Muscarinic Modulation of the Spontaneous IPSC Amplitude Is Abolished in APP Transgenic Mice—To test the impact of muscarinic receptors (mAChRs) on GABAergic inhibitory transmission in PFC, we first examined the effect of mAChR agonist carbachol on spontaneous inhibitory postsynaptic currents (sIPSCs) in mouse pyramidal neurons located in deep layers of PFC. Application of the $GABA_A$ receptor antagonist bicuculline (10 μM) completely blocked the sIPSCs (n = 5, data not shown), indicating that these synaptic currents are mediated by $GABA_A$ receptors. Bath application of carbachol (20 μM), a broad-spectrum cholinergic agonist, caused a reversible increase in the amplitude and frequency of sIPSCs (Fig. 1, *A–C*). The increase developed gradually and reached a plateau 4–6 min after the application of carbachol. A tonic increase of sIPSCs, rather than periodical bursts of sIPSCs (31), was observed in response to carbachol in most of our cells. In a sample of PFC pyramidal neurons examined, carbachol increased the mean amplitude of sIPSCs by 62.4 ± 9.7% (n = 18, $p < 0.001$, K-S test) and the mean frequency of sIPSCs by 220 ± 35.1% (n = 18, $p < 0.001$, K-S test). In the presence of pirenzepine (1 μM), an antagonist for m1/m4 receptors, carbachol failed to cause a significant change in sIPSCs (mean amplitude: 5.5 ± 3.0%; mean frequency: 5.3 ± 7.0%; n = 5, $p > 0.05$, K-S test),

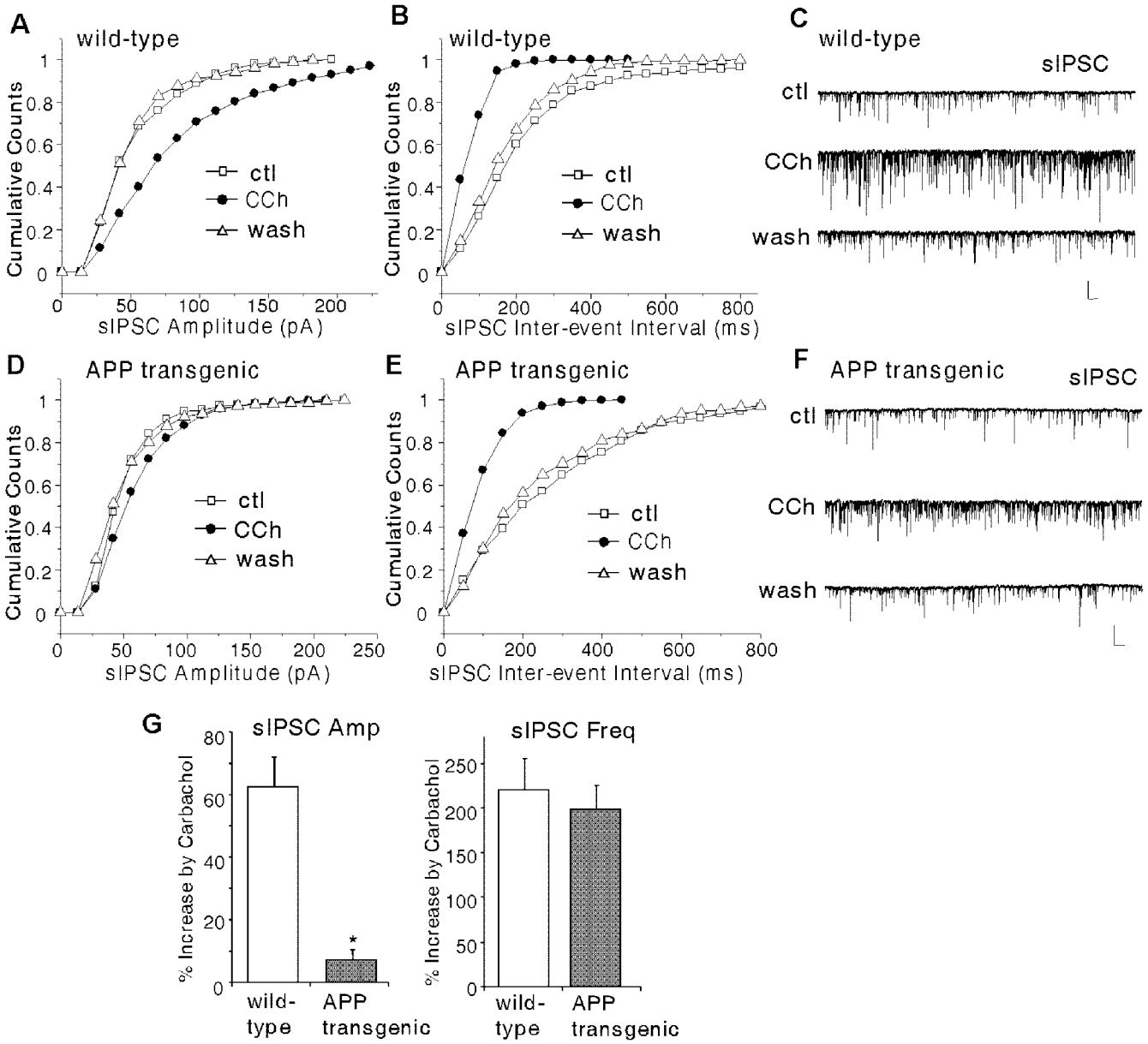

FIG. 1. **Muscarinic receptors enhanced the amplitude of sIPSCs in PFC pyramidal neurons from wild-type mice, but not from APP transgenic mice.** *A* and *B*, cumulative plots indicating that the distribution of sIPSC amplitude (*A*) and frequency (*B*) was reversibly increased by CCh (20 μM) in a neuron from a wild-type mouse. *C*, representative sIPSC traces recorded from the neuron used to construct *A* and *B* under control condition, during bath application of carbachol and after washing off the agonist. *Scale bars*: 100 pA, 1 s. *D* and *E*, cumulative plots indicating that carbachol (20 μM) did not increase the sIPSC amplitude (*D*), but increased the sIPSC frequency (*E*), in a neuron from an APP transgenic mouse. *F*, representative sIPSC traces recorded from the neuron used to construct *D* and *E*. *G*, histograms (mean ± S.E.) showing the percent modulation of sIPSC amplitudes and frequencies by carbachol in PFC pyramidal neurons from wild-type ($n = 18$) *versus* APP transgenic mice ($n = 29$). *, $p < 0.001$, ANOVA.

suggesting the mediation by m1 or m4 mAChRs. Since m1 is the most prominent subtype abundantly expressed in the majority of cortical neurons (16, 18), the potent effect of carbachol on GABA transmission found in about 90% of the PFC pyramidal neurons we examined is likely to be mediated by the m1 receptor.

We next examined whether the muscarinic modulation of GABAergic inhibitory transmission is altered in the AD model. Compared with wild-type mice, APP transgenic mice exhibited significantly higher (~20–30-fold) levels of Aβ peptides at 2 months of age, even though no amyloid plaques, neuronal death, or cognitive deficit were observed at the early stage (25). Amyloid deposits were found in frontal cortex, along with other brain regions, in aged APP transgenic mice (25), suggesting that the elevated Aβ expression is present in frontal cortical neurons at the presymptomatic period. We first compared the basal properties of sIPSCs in wild-type *versus* APP transgenic mice. No significant difference was found between the two groups (mean amplitude: WT, 36.2 ± 3.7 pA, $n = 18$; APP transgenic, 34.4 ± 2.9 pA, $n = 29$, $p > 0.05$, ANOVA; mean frequency: WT, 4.2 ± 0.6 Hz, $n = 18$; APP transgenic, 3.8 ± 0.4 Hz, $n = 29$, $p > 0.05$, ANOVA). The lack of changes on the basal GABAergic transmission in APP transgenic mice suggests that PFC GABAergic interneurons are not lost or significantly impaired. We then examined the effect of carbachol on sIPSCs in APP transgenic mice. As shown in Fig. 1, *D–F*, bath application of carbachol (20 μM) failed to increase the sIPSC amplitude in the mutant cell, but the carbachol-induced enhancement of sIPSC frequency was intact. In a sample of PFC pyramidal neurons from APP transgenic mice, carbachol caused little

change in the mean amplitude of sIPSCs (7.16 ± 3.2%, $n = 29$, $p > 0.05$, K-S test), but still significantly increased the mean frequency of sIPSCs (198.6 ± 27.0%, $n = 29$, $p < 0.001$, K-S test). The effects of carbachol on the sIPSC amplitude and frequency in PFC neurons from wild-type *versus* APP transgenic mice are summarized in Fig. 1*G*. It is evident that muscarinic modulation of the sIPSC amplitude, but not the sIPSC frequency, was significantly ($p < 0.001$, ANOVA) impaired in APP transgenic mice.

Muscarinic Modulation of the sIPSC Amplitude Is Eliminated in Rat PFC Slices Pretreated with the β-Amyloid Peptide—We then examined whether the altered muscarinic modulation of GABA transmission in APP transgenic mice is attributable to the elevated β-amyloid protein levels at an early age (25). To do so, we treated rat PFC slices with β-amyloid peptides (Aβ) before examining carbachol effects on sIPSCs. $A\beta_{25–35}$, which represents the biologically active region of Aβ (32, 33), was aged to produce aggregated $A\beta_{25–35}$. In non-treated rat slices, bath application of carbachol caused a reversible increase in the amplitude and frequency of sIPSCs (Fig. 2, *A–C*), similar to what was found in wild-type mice. However, in $A\beta_{25–35}$-treated slices, carbachol failed to increase the sIPSC amplitude, but still induced a potent enhancement of the sIPSC frequency. A representative example from an $A\beta_{25–35}$-treated neuron is shown in Fig. 2, *D–F*.

To confirm the specificity of the action of $A\beta_{25–35}$, its control peptide containing the reverse sequence $A\beta_{35–25}$ was used to pretreat PFC slices. Similar to non-treated slices, in $A\beta_{35–25}$-treated slices, bath application of carbachol induced a reversible increase in the sIPSC amplitude (Fig. 2*G*). As summarized in Fig. 2*H*, in $A\beta_{25–35}$-treated pyramidal neurons, carbachol caused little change in the mean amplitude of sIPSCs (4.27 ± 2.6%, $n = 17$, $p > 0.05$, K-S test), which was significantly ($p < 0.001$, ANOVA) different from the carbachol effect on the sIPSC amplitude in non-treated neurons (78.6 ± 10.5%, $n = 35$, $p < 0.001$, K-S test) or neurons pretreated with the control peptide $A\beta_{35–25}$ (77.4 ± 12.5%, $n = 5$, $p < 0.001$, K-S test). However, the carbachol-induced increase in the mean frequency of sIPSCs in $A\beta_{25–35}$-treated neurons (221.5 ± 31.4%, $n = 17$, $p < 0.001$, K-S test) was similar to the carbachol effect in non-treated neurons (245.4 ± 32.4%, $n = 35$, $p < 0.001$, K-S test) or $A\beta_{35–25}$-treated neurons (231.7 ± 38.3%, $n = 5$, $p < 0.001$, K-S test). Despite the ability of $A\beta_{25–35}$ to alter the muscarinic regulation of sIPSCs, $A\beta_{25–35}$ itself had little direct effect on the sIPSC amplitude (5.3 ± 2.1%, $n = 10$, $p > 0.05$, K-S test) and frequency (8.9 ± 3.1%, $n = 10$, $p > 0.05$, K-S test).

In prefrontal cortex, serotonin, by activating 5-HT_2 receptors, can also potentiate GABA transmission (34). To test whether β-amyloid impairs the actions of 5-HT_2 receptors, we examined the serotonergic regulation of sIPSCs in $A\beta_{25–35}$-treated PFC slices. Application of serotonin (20 μM) caused a potent increase in the mean amplitude and frequency of sIPSCs in $A\beta_{25–35}$-treated PFC pyramidal neurons (amplitude: 80.7 ± 14.4% (Fig. 2*I*); frequency: 387.4 ± 75.0%, $n = 6$, $p < 0.001$, K-S test), which was not significantly different from the serotonin effect on sIPSCs in non-treated neurons (amplitude: 85.8 ± 16.8% (Fig. 2*I*); frequency: 392.3 ± 65.6%, $n = 10$, $p < 0.001$, K-S test), suggesting the lack of Aβ effect on serotonin functions. Taken together, these results indicate that β-amyloid selectively alters the muscarinic regulation of GABA transmission.

Muscarinic Modulation of GABA Transmission Is through a PKC-dependent Mechanism—To find out the potential reason for the impairment of muscarinic modulation of GABA transmission in PFC from APP transgenic mice, we first examined the cellular mechanisms underlying the modulation of GABA transmission by mAChRs. It is known that activation of m1 receptors stimulates the hydrolysis of membrane phosphoinositol lipids, leading to PKC activation, while activation of m4 receptors inhibits adenylyl cyclase. To test whether the muscarinic modulation of GABA transmission is through the m1-activated PKC, we tested the effect of carbachol on IPSCs when PKC activation was blocked.

We first preincubated rat PFC slices with the cell-permeable and specific PKC inhibitor calphostin C (1 μM) for 1 h, followed by the examination of carbachol effects on sIPSCs. As shown in Fig. 3, *A* and *B*, carbachol failed to enhance sIPSC amplitudes in the calphostin C-treated slice. The carbachol enhancement of sIPSC frequencies was not significantly affected by calphostin C (Fig. 3*C*). Another potent and selective PKC inhibitor, bisindolylmaleimide (1 μM), gave similar results as calphostin C, eliminating the carbachol enhancement of sIPSC amplitudes (data not shown). To confirm the specific involvement of m1/PKC in the muscarinic regulation, PFC slices were preincubated with the cell-permeable myristoylated PKA inhibitor PKI-(14–22) (1 μM) to test the potential role of m4/PKA in this process. As shown in Fig. 3, *D–F*, carbachol still induced a potent increase in sIPSC amplitudes and frequencies in the PKI-(14–22)-treated slice, similar to what was obtained in the non-treated slice (Fig. 2, *A–C*), indicating that PKA inhibition did not affect the muscarinic regulation of GABA transmission. As summarized in Fig. 3*G*, the carbachol effect on sIPSC amplitudes in the presence of PKC inhibitor calphostin C (−21.6 ± 5.8%, $n = 6$, $p > 0.05$, K-S test) or bisindolylmaleimide (−19.6 ± 4.3%, $n = 7$, $p > 0.05$, K-S test) was significantly ($p < 0.001$, ANOVA) different from that in the presence of PKA inhibitor PKI-(14–22) (76.7 ± 11.3%, $n = 5$, $p < 0.01$, K-S test). The carbachol effect on sIPSC frequencies was similar with these treatments (Fig. 3*H*, calphostin C: 180.4 ± 32.7%, $n = 6$; bisindolylmaleimide: 200.8 ± 41.7%, $n = 7$; PKI-(14–22): 210.8 ± 38.8%, $n = 5$). These data suggest that the muscarinic modulation of sIPSC amplitudes (but not frequencies) depends on the activation of PKC.

Muscarinic Activation of PKC Is Lost in APP Transgenic Mice—Given the PKC dependence, we speculated that the underlying mechanism for the loss of muscarinic modulation of GABA transmission in APP transgenic mice is the impaired muscarinic activation of PKC in these mutants. To test this, we compared the muscarinic activation of PKC in PFC slices from wild-type and APP transgenic mice. Because the catalytic competence of many PKC isozymes depends on autophosphorylation at the carboxyl terminus on a conserved residue (35), a phosphospecific pan PKC antibody that detects PKC isoforms only when phosphorylated at this residue was used for measuring activated PKC. As shown in Fig. 4, *A* and *B*, carbachol potently increased the activated PKC in wild-type slices (2.51 ± 0.17-fold, $n = 12$, $p < 0.001$, ANOVA), but this effect was almost completely abolished in slices from APP transgenic mice (1.10 ± 0.06-fold, $n = 12$, $p > 0.05$, ANOVA). These results suggest that the mAChR-mediated second messenger cascade is altered by Aβ, which apparently leads to the loss of muscarinic modulation of GABAergic signaling.

To examine whether the impaired PKC activation by carbachol in APP transgenic mice is due to the loss of mAChR expression, we compared the m1–m5 mRNA levels in PFC from wild-type *versus* APP transgenic mice. As shown in Fig. 4*C*, the expression levels of m1–m5 mRNAs detected with RT-PCR were almost identical in PFC tissues from wild-type *versus* APP transgenic mice. To provide a semiquantative analysis of m1 mRNA, serial dilution experiments were performed (30). As shown in Fig. 4*D*, using increasing dilutions (1:10 to $1:10^5$) of the cDNAs from wild-type *versus* APP transgenic mice pro-

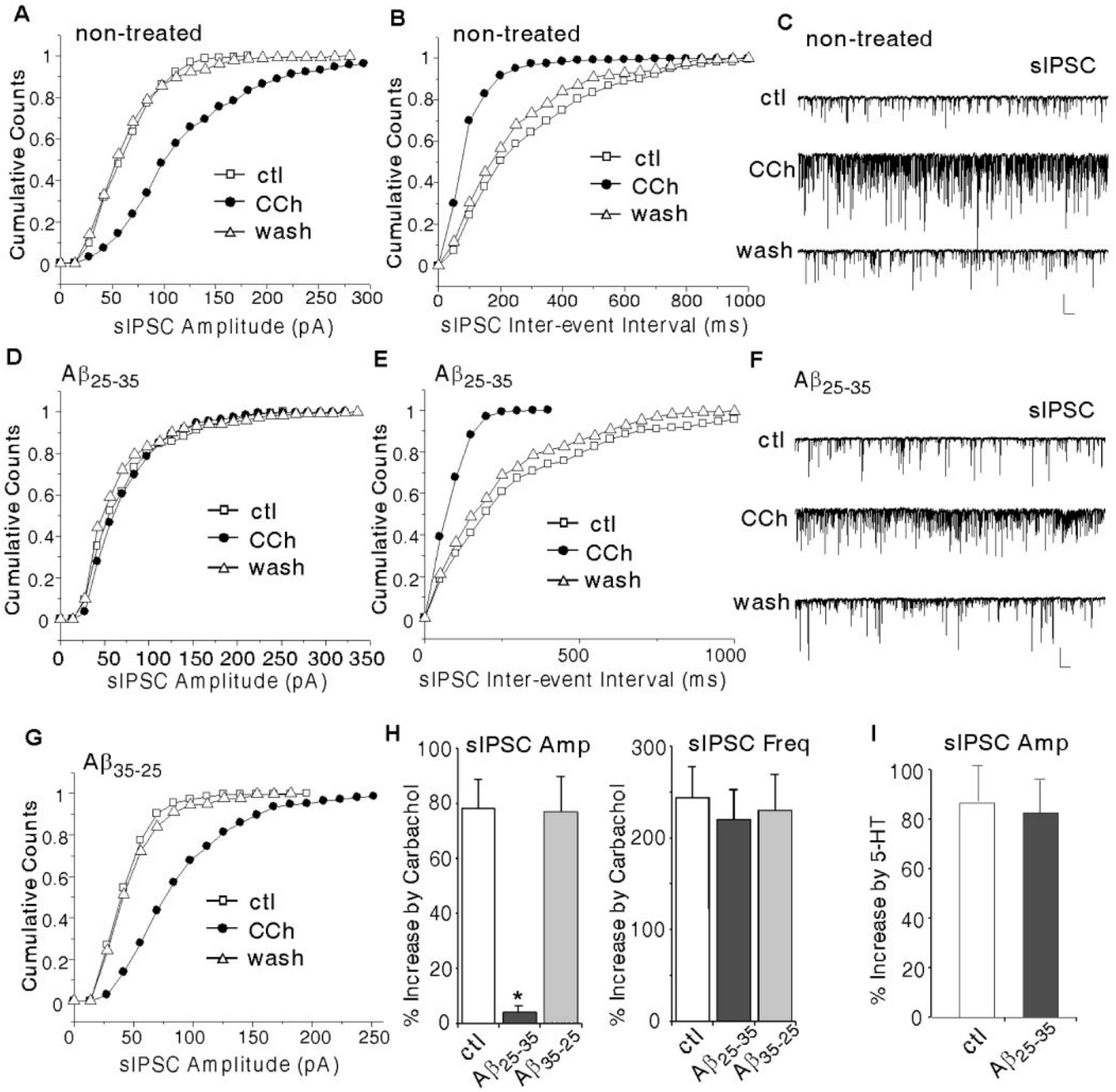

FIG. 2. **Muscarinic receptors failed to enhance the amplitude of sIPSCs in Aβ_{25-35}-treated PFC pyramidal neurons.** *A* and *B*, cumulative plots indicating that carbachol (20 μM) increased both the amplitude (*A*) and the frequency (*B*) of sIPSCs in a rat PFC pyramidal neuron. *C*, representative sIPSC traces recorded from the neuron used to construct *A* and *B* under control condition, during bath application of carbachol and after washing off the agonist. *Scale bars*: 100 pA, 1 s. *D* and *E*, cumulative plots indicating that carbachol (20 μM) failed to increase the sIPSC amplitude (*D*), but increased the sIPSC frequency (*E*), in a neuron pretreated with Aβ_{25-35} (10 μM) for 4 h. *F*, representative sIPSC traces recorded from the neuron used to construct *D* and *E*. *G*, cumulative plots indicating that carbachol (20 μM) increased the amplitude of sIPSCs in a neuron pretreated with the control peptide Aβ_{35-25} (10 μM) for 4 h. *H*, histograms (mean ± S.E.) showing the percent modulation of sIPSC amplitudes and frequencies by carbachol in Aβ_{25-35}-treated neurons ($n = 17$) *versus* non-treated ($n = 35$) or Aβ_{35-25}-treated neurons ($n = 5$). *, $p < 0.001$, ANOVA. *I*, histograms (mean ± S.E.) showing the percent modulation of sIPSC amplitudes by serotonin (20 μM) in Aβ_{25-35}-treated ($n = 6$) *versus* non-treated neurons ($n = 10$).

duced almost the same amount of PCR products of m1, implicating similar abundance of the m1 mRNA in wild-type and APP transgenic mice. These results suggest that the expression of mAChRs is not significantly altered in APP transgenic mice.

Muscarinic Modulation of Intrinsic Firing Is Not Altered in APP Transgenic Mice—Since muscarinic modulation of GABAergic transmission is impaired in PFC neurons from APP transgenic mice or Aβ_{25-35}-treated rat slices, we would like to know whether the impairment is due to a selective change in the signaling mechanisms underlying muscarinic regulation of the GABA system or a general dysfunction of muscarinic receptors caused by elevated β-amyloid peptides. To test this, we examined the effects of mAChRs on the intrinsic firing pattern of pyramidal neurons in PFC slices when glutamatergic and GABAergic synaptic transmission was blocked.

As found in several neuronal populations (36, 37), bath application of carbachol (5 or 10 μM) to PFC slices blocked the slow afterhyperpolarization that followed a train of action potentials elicited by current steps, induced a slow after depolarization that gives rise to a plateau potential accompanied by

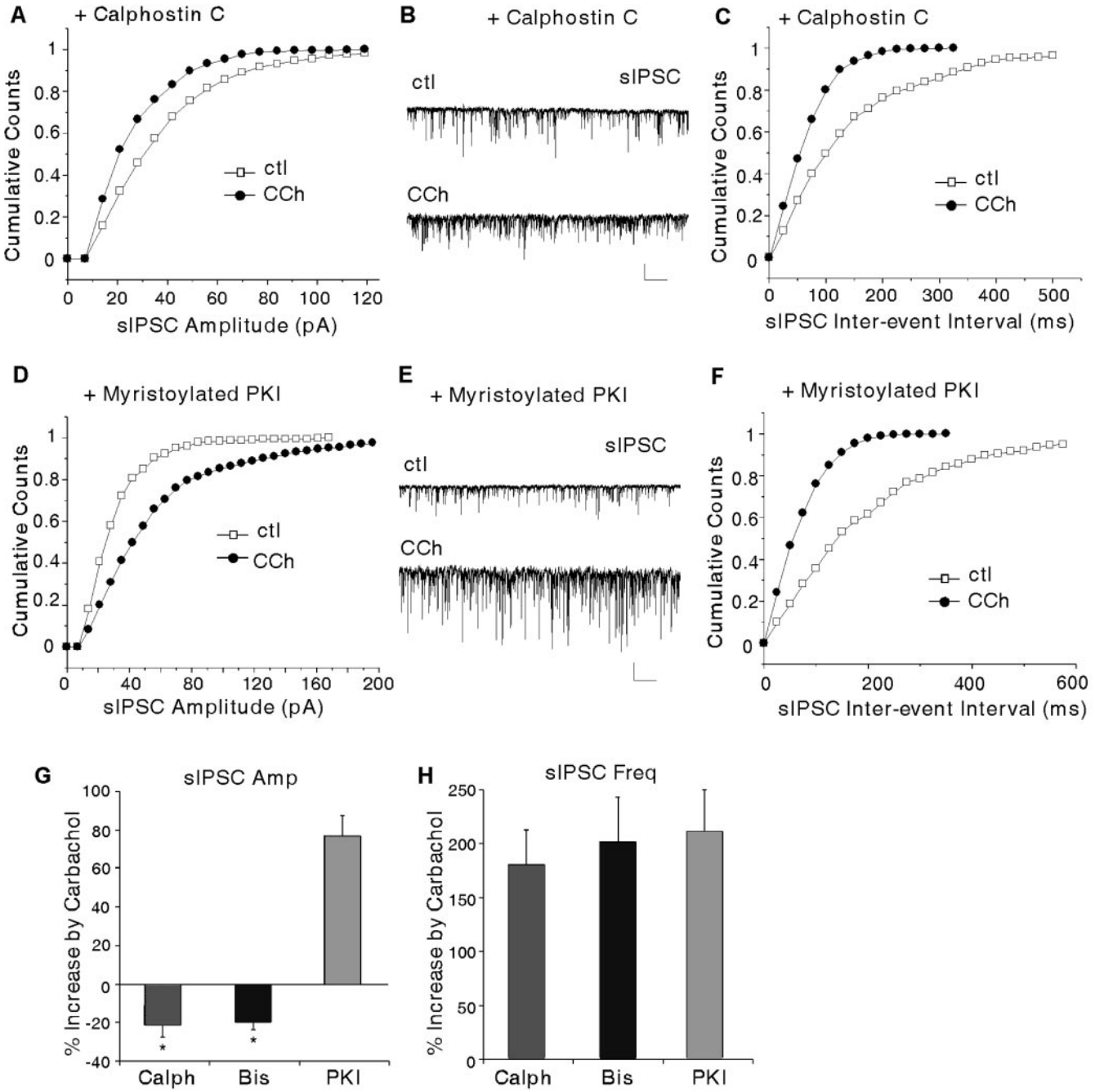

FIG. 3. **Muscarinic receptors failed to enhance sIPSC amplitudes in PFC slices pretreated with PKC inhibitors.** *A* and *D*, cumulative plots indicating that carbachol (20 μM) did not increase the sIPSC amplitude recorded in a PFC slice pretreated with the PKC inhibitor calphostin C (1 μM) (*A*), but increased the sIPSC amplitude recorded in a PFC slice pretreated with the myristoylated PKA inhibitor PKI-(14–22) (1 μM) (*D*). *B* and *E*, representative sIPSC traces recorded from the neuron used to construct *A* (*B*) or *D* (*E*) under control condition and during bath application of carbachol. *Scale bars*: 50 pA, 2.5 s. *C* and *F*, cumulative plots indicating that the distribution of sIPSC frequency was increased by carbachol (20 μM) recorded in a PFC slice pretreated with calphostin C (*C*) or PKI-(14–22) (*F*). *G* and *H*, histograms (mean ± S.E.) showing the percent modulation of sIPSC amplitudes (*G*) or frequencies (*H*) by carbachol in PFC slices pretreated with calphostin C (*Calph*, $n = 6$), bisindolylmaleimide I (*Bis*, $n = 7$), or myristoylated PKI-(14–22) (*PKI*, $n = 5$). *, $p < 0.001$, ANOVA.

spiking ($n = 10$). A representative example from a wild-type mouse is shown in Fig. 5*A*. This carbachol-induced generation of persistent activity has been proposed to provide a cellular mechanism for the delayed activity observed during working memory tasks (38). It is mediated by m1 activation of a Ca^{2+}-sensitive, voltage-dependent nonspecific cationic current (39, 40, 37). In PFC pyramidal neurons from APP transgenic mice, bath application of carbachol also elicited a persistent firing ($n = 9$; a representative example shown in Fig. 5*B*), which was similar to what was found in wild-type mice. Moreover, the ability of carbachol to trigger sustained spiking activity was intact in PFC neurons from Aβ_{25-35}-treated slices ($n = 4$, data not shown).

We next examined the potential reason for the intact muscarinic regulation of intrinsic firing in APP transgenic mice. Since the Aβ-caused loss of muscarinic activation of PKC apparently leads to the impaired muscarinic regulation of GABA transmission, we tested whether PKC activation is required for the muscarinic regulation of firing. To do so, we preincubated rat PFC slices with the cell-permeable and specific PKC inhibitor bisindolylmaleimide (1 μM), followed by the examination of carbachol effect on the intrinsic firing pattern. Application of

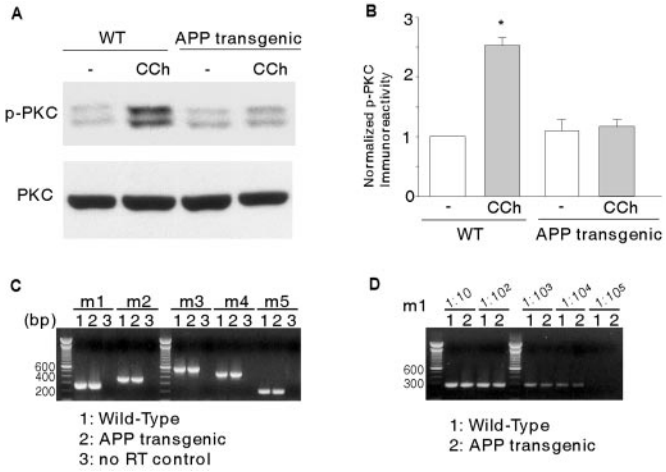

FIG. 4. **Muscarinic activation of PKC was lost in PFC slices from APP transgenic mice, but mAChR expression was unchanged in the mutant mice.** *A*, immunoblots of activated PKC autophosphorylated at carboxyl terminus (*top*) and total PKC (*bottom*) in PFC slices from a wild-type and an APP transgenic mouse treated with or without carbachol (20 μM) for 5 min. *B*, quantitation of the activated PKC in PFC slices from wild-type *versus* APP transgenic mice in response to carbachol ($n = 12$; *, $p < 0.001$, ANOVA). *C*, photograph of an ethidium bromide-stained agarose gel in which PCR amplicons have been separated by electrophoresis. Note that m1–m5 mRNAs were similarly expressed in PFCs from wild-type *versus* APP transgenic mice. No RT control consistently yielded negative results, indicating that only mRNAs were amplified and detected. *D*, gel showing the serial dilution analysis of m1 mRNA from wild-type *versus* APP transgenic mice. Note that PCR amplicons were similarly detectable with dilutions of 1:10 to $1:10^4$ of the cDNA from wild-type *versus* APP transgenic mice, but not at the higher dilution ($1:10^5$). Similar results were obtained from three other pairs of mice.

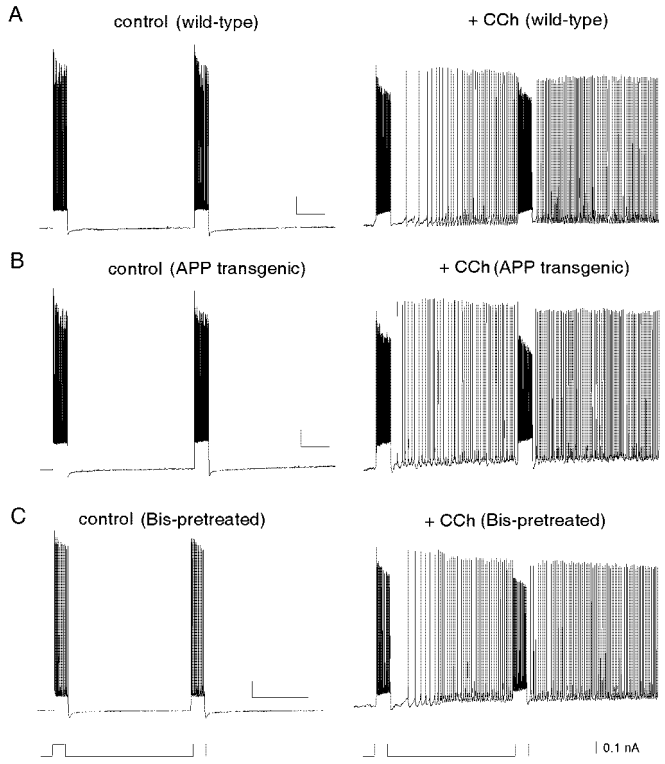

FIG. 5. **Muscarinic receptors induced persistent spiking activity in PFC pyramidal neurons from both wild-type and APP-transgenic mice, and this effect was PKC-independent.** *A* and *B*, responses to current steps before (*control*) and after CCh (10 μM) application in neurons from a wild-type mouse (*A*) and an APP transgenic mouse (*B*). *C*, responses to current steps before (*control*) and after CCh (10 μM) application in a neuron pretreated with the PKC inhibitor bisindolylmaleimide (*Bis*, 1 μM). *Scale bars*: 10 mV, 5 s.

carbachol (10 μM) triggered sustained spiking activity in bisindolylmaleimide-treated neurons ($n = 7$, a representative example shown in Fig. 5*C*), which was almost identical to the carbachol effect in non-treated cells ($n = 3$, data not shown). These results show that the muscarinic induction of persistent firing is through a PKC-independent mechanism, which is not disrupted in APP transgenic mice. Taken together, these functional studies suggest that elevated levels of Aβ are unable to affect muscarinic receptors *per se*, at least at the early stage, but are more likely to impair selective signaling events mediated by muscarinic receptors.

DISCUSSION

Over the past decade, considerable efforts have been focused on the development of cholinergic agonists for the treatment of AD, based on the key role of cortical cholinergic systems in cognitive processes (41) and the consistent finding of severe degeneration and dysfunction of basal forebrain cholinergic systems in AD (1, 8). Corresponding to the degeneration, there is a significant loss of nicotinic ACh receptors and certain types of muscarinic ACh receptors in the cortical and hippocampal regions of AD brains (42, 43). There is a general consensus that m1 muscarinic receptors are preserved in most AD patients (44). However, several studies indicate that m1/G-protein-mediated signal transduction may be disrupted in AD (45, 46). These results suggest that m1 signaling could be a point of pharmacological intervention in the treatment of AD (47). As m1 receptors are largely intact, if dysfunctional downstream signaling cascade can be restored, there is a good chance to improve cholinergic functions. Thus, it is crucial to have a better understanding on muscarinic signaling in the normal situation and how it might be altered in AD. In this study, we found that activation of m1 receptors exerts a potent enhancement on GABAergic synaptic transmission in PFC pyramidal neurons. Since antagonizing GABA transmission in dorsolateral PFC disrupts the performance of a delayed response task, a measurement of spatial working memory (48, 49), the muscarinic enhancement of GABA signaling in PFC projection neurons may underlie the facilitating role of mAChRs in cognition.

Muscarinic receptors could enhance GABAergic synaptic transmission through a presynaptic and a postsynaptic mechanism. In this study, activation of mAChRs with carbachol induced an increase in the frequency of sIPSCs and shifted their amplitude distribution toward larger sizes. Several mechanisms could account for the carbachol effects on GABA transmission. First, mAChRs increase the excitability of GABAergic interneurons, therefore leading to the elevated probability of GABA release. Direct excitation of GABAergic interneurons by activation of cholinergic receptors has been reported in the cortex *in vitro* (50–52, 34). The ionic basis for the excitatory actions of muscarinic receptors on these interneurons is not clear yet. Muscarinic suppression of potassium conductances and potentiation of cation currents (53, 39) could be potential mechanisms. Second, mAChRs enhance the probability of action potential-dependent GABA release from axon terminals, therefore leading to the increase of the contribution of large size (multiquantal) sIPSCs to the overall population of synaptic events. The underlying mechanisms for this muscarinic action await to be elucidated. One possibility is that the m1/phospholipid/PKC signaling regulates the presynaptic protein synaptotagmine that functions as a calcium sensor to trigger synchronous vesicle fusion events (54–56), thus facilitating the Ca^{2+} cooperativity of transmitter release. Third, mAChRs potentiate the postsynaptic $GABA_A$ receptor functions, therefore resulting in the bigger response to GABA. The muscarinic modulation of

postsynaptic $GABA_A$ receptor properties could be attributed to changes in the phosphorylation state of $GABA_A$ receptor subunits by PKC-activated Src kinase in response to m1 receptor stimulation (57, 58, 18).

In addition to cholinergic hypofunction, genetic studies have implicated the importance of Aβ deposition in the pathogenesis of AD (3). Aβ is synthesized and secreted by brain cells (59, 60) and deposited proximal to nerve terminals by axonally transported APP (61). It is generally accepted that accumulation of diffuse deposits of Aβ in the brain is an early event in the development of AD, which emphasizes the importance of elucidating the neuronal response to Aβ fibrils before clinical symptoms arise. We propose that the early pathophysiological changes could contribute significantly to later cognitive impairments, which are not accompanied by massive neurodegeneration (26). Several lines of evidence have suggested that Aβ has pleiotropic actions on the cholinergic system (20), including the suppression of acetylcholine synthesis in primary cultures of basal forebrain neurons (62), the inhibition of acetylcholine release from hippocampal slices (63), and the disruption of muscarinic receptor-G-proteins coupling in cortical cultures (64). In the present study, we found that in neurons from APP transgenic mice or neurons exposed to fibrillar $A\beta_{25-35}$, the carbachol enhancement of sIPSC amplitudes was impaired, whereas the carbachol-induced increase in sIPSC frequencies was intact. It suggests that Aβ partially affects the muscarinic modulation of GABAergic interneuron excitability, leading to the impairment in muscarinic regulation of multiquantal release of GABA. Since young APP transgenic mice demonstrate little or no neuronal loss (25, 65) and apparently normal expression of mAChRs (Fig. 4, *C* and *D*), the altered regulation of GABA transmission by muscarinic receptors in these mutants are most likely due to the specific impairment of muscarinic signaling. Our result is consistent with the notion that Aβ can directly induce cholinergic hypofunction without apparent neurotoxicity (20). The specific impairment in muscarinic regulation of GABA transmission, but not of intrinsic firing, in APP transgenic mice suggests that not all of the cholinergic functions are altered by Aβ. Given the significance of PFC GABAergic inhibition in working memory (19), the alteration of muscarinic regulation of GABAergic signaling in APP transgenic mice could contribute to the deficit in cognition and memory associated with AD.

Earlier observations reported that exposure cortical cultures to Aβ peptides reduces carbachol-stimulated GTPase activity, inositol phosphate accumulation, and intracellular Ca^{2+} increase (64). It prompted us to hypothesize that one potential mechanism for the loss of muscarinic modulation of GABA transmission in the mutant APP-overexpressing mice is the impairment of m1/G-protein-mediated signal transduction by elevated levels of Aβ. In agreement with this, we found that mAChRs regulate GABA transmission partly through a PKC-dependent mechanism, and mAChRs fail to activate PKC in APP transgenic mice. It suggests that the Aβ-induced disruption of muscarinic activation of PKC contributes to the impaired muscarinic regulation of GABA signaling. Interestingly, previous studies have shown that activation of PKC by stimulation of m1 receptors markedly inhibits the production and release of Aβ in cell lines (66–68). The significant attenuation of muscarinic activation of PKC in APP transgenic mice provides a mechanism for preventing the m1/PKC-mediated down-regulation of amyloidogenic Aβ generation. The loop between Aβ and ACh could lead to enhanced Aβ production and exacerbated cholinergic deficiencies in AD.

Taken together, the central finding of this study is that activation of mAChRs enhances GABA transmission in PFC pyramidal neurons, and the m1-mediated, PKC-dependent modulation of GABA signaling is impaired in neurons from APP transgenic mice, which is attributable to the loss of muscarinic activation of PKC in these mutants. This finding provides a possible connection between amyloid burden and cholinergic dysfunction in AD. Elucidation of the functional abnormalities in the transgenic mouse models of AD may offer valuable insights into the pathogenesis of the disease and potential therapeutic interventions.

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