Presynaptic Muscarinic Receptors Reduce Synaptic Depression and Facilitate its Recovery at Hippocampal GABAergic Synapses

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Hippocampal gamma oscillation, involved in cognitive processes, can be induced by muscarinic acetylcholine receptors activation and depends in large part on the activation of γ-aminobutyric acid-ergic (GABAergic) interneurons. The precise role of the modulatory action of muscarinic receptors on GABAergic transmission still remains unclear due to the great heterogeneity of observed effects. We have examined the presynaptic and postsynaptic mechanisms involved. Methacholine induces a down-regulation of evoked inhibitory postsynaptic currents (eIPSCs) not associated with the change of postsynaptic receptors. The significant decrease in the paired-pulse depression strongly suggested a presynaptic mechanism of action. We have used cumulative amplitude profile analysis to show that the impairment of eIPSCs is not related to a decreased size of the readily releasable pool, but rather depends on the reduced release probability by a down-modulation of voltage-gated calcium channels. The decreased neurotransmitter release probability only partially accounts for the dramatic reduction in the rate of synaptic depression evoked by short- and long-lasting tetanic stimuli. This effect is accompanied by a significant enhancement in the rate of recovery from synaptic depression that demonstrates the reinforcement of the synaptic recycling processes. These results show that muscarinic modulation of hippocampal GABAergic synapses confers a greater resistance to sustain periods of intense synaptic activity in the gamma frequency range.

Keywords: γ-aminobutyric acidergic synapses, hippocampal neurons, methacholine, muscarinic receptors

Introduction

Cholinergic projections from the basal forebrain to the cortex and hippocampus as well as some intrinsic cholinergic hippocampal interneurons play a critical role in cognitive function (Drachman and Leavitt et al. 1974; Cole and Nicoll 1983; Decker and McGaugh 1991; Fibiger et al. 1991; Dutar et al. 1995; Markowska et al. 1995). A particularly interesting problem relates to the interaction of cholinergic and γ-aminobutyric acidergic (GABAergic) systems (Arnaiz et al. 2008; González et al. 2011). Muscarinic acetylcholine receptors (mACHRs) are considered to be the primary receptors in cholinergic synaptic transmission involved in cognitive function (Beatty et al. 1986; Pavia et al. 1998). The blockades of mACHRs, or lesions of cholinergic septohippocampal projections, induce memory and attention deficits (Drachman and Leavitt 1974; Sutherland et al. 1982; Fibiger 1991; Callahan et al. 1993; Roldan et al. 1997). Whereas drugs that activate mACHRs are helpful in ameliorating the cognitive deficits in Alzheimer's disease (Whitehouse 1993). Cholinergic neurotransmission may lead to the concentration of acetylcholine in the mM range, at cholinergic terminals (Sarter and Parikh 2005). Termination of ACh activity is dependent on its dissociation from the receptor and the subsequent hydrolysis by acetylcholinesterase (AChE) that occurs in a few milliseconds (Anglister et al. 1994; Nair et al. 1994; Tai et al. 2003). Brain distribution of AChE includes both acetylcholine-releasing and cholinceptive neurons. It has been reported a pronounced activity in the striatum, and clearly detectable activity in the cortex and hippocampus (Anwar et al. 2012).

Network oscillations in the gamma frequency range (25–90 Hz), a characteristic feature of the awake brain during attention, have been proposed to provide a transient structure for various cognitive processes, selective attention (Fries et al. 2001), and consciousness (Llinas et al. 1998). Hippocampal gamma activity thought to be involved in memory processing (Jensen et al. 1996), can be induced by mAChR activation (Fisahn et al. 1998; Fellous and Sejnowski 2000), and depends in large part on the activation of GABAergic interneurons (Fisahn et al. 1998; Traub et al. 2000). Although the mechanisms whereby muscarinic receptors influence neuronal activity and cognitive processes are poorly understood, a wide variety of muscarinic effects have been identified: Activation of several second messenger cascades (McKinney 1993), postsynaptic modulation of ionic currents (Cole and Nicoll 1983; Toselli and Lux 1989; Krnjevic 1993), inhibition of neurotransmitter release (Raiteri et al. 1984; Pohorecky et al. 1988; Marchi and Raiteri 1989), enhancement of the N-methyl-D-aspartate glutamate receptors responsiveness (Markram and Segal 1990), and modulation of long time potentiation and long term depression (Blitzer et al. 1990; Markram and Segal 1990; Williams and Johnston 1990; Pang et al. 1993). For these reasons it is of particular interest to characterize in detail the impact of muscarinic receptor activation on GABAergic function in the hippocampus. The majority of the GABAergic neurons express mACHRs (Van del Zee and Luiten 1993) whose activation induce changes in resting potential and action potential firing (Widmer et al. 2006). GABAergic interneurons may synapse onto glutamatergic pyramidal cells, but also onto other interneurons (Gulyas et al. 1996; Freund and Gulyás 1997). So, drugs that modify the GABA release acting on interneuronal mACHRs will show dramatic effects on the network excitability.

In a recent paper, we have described a pharmacological study describing the mACHR subtypes involved in the modulation of spontaneous GABAergic transmission (González et al. 2011). In this study, we show that mACHR activation induces a powerful inhibition of evoked inhibitory postsynaptic currents.
(eIPSCs). This effect depends on a decrease in the neurotransmitter release probability as a consequence of the down-regulation of voltage-dependent calcium channels. As a direct result of the decrease in the release probability, the inhibitory synapses reduce the rate of depression evoked by short-lasting tetanic stimulation. This effect run in parallel with an increase in the steady-state postsynaptic currents during long-lasting tetanic stimulation alongside a faster recovery from synaptic depression; clearly demonstrating the reinforcement of the synaptic recycling processes. So thanks to the combination of a reduced GABA release efficiency and a decreased synaptic depression, muscarinic activation will switch GABAergic synapses into a new functional configuration, which is able to sustain efficiently and for a long time a high frequency synaptic activity in the gamma range.

Materials and Methods

Isolation and Culture of Rat Hippocampal Neurons

All experiments were carried out in accordance with the guidelines established by the National Council on Animal Care and were approved by the local Animal Care Committee of the Universidad Autónoma de Madrid. Pregnant Sprague-Dawley rats were killed by decapitation, and 18-day embryos were removed immediately by cesarean section. Hippocampi were dissected rapidly under a stereomicroscope and sterile conditions in cold (4 °C) phosphate buffer solution (PBS) containing (in mM): 137 NaCl, 2.7 KCl, 11.6 NaH2PO4, 1.47 KH2PO4, (pH 7.4, adjusted with NaOH). The tissue was digested with 0.5 mg/mL papain and 0.25 mg/mL DNase. The enzymes were dissolved in a Ca2+- and Mg2+-free PBS solution containing 1 mg/mL bovine serum albumin and 10 mM glucose at 37 °C for 20 min. The papain solution was replaced with 5 mL of Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Afterwards, the digested tissue was then gently dissociated by suction using a fire-polished glass to avoid cellular damage. The suspension was centrifuged for 4 min at 120 × g. The supernatant was removed, and the cells were resuspended in 5 mL of DMEM plus FBS and plated at a density of 60 000 cells/mL on 3.5 cm diameter Petri dishes (2 mL/dish) coated with poly-β-hydroxy (0.1 mg/mL). Cells were plated in DMEM supplemented with 10% FBS, 60 mg/mL L-glutamine, 100 μg/mL streptomycin, and 100 U/mL penicillin, and maintained in a 5% CO2 incubator at 37 °C. A day later, the medium was replaced by Neurobasal® medium deprived of serum to avoid fibroblast proliferation, and this neurobasal medium contained B27 supplement to facilitate hippocampal neuron survival in vitro. Under these conditions, the standard cell survival was 4 weeks, and the experiments were performed on neurons of 8–15 days in vitro (DIV) culture. The cell selection criteria for the recordings were: Adhesion to the substrate, soma diameters of 15–30 μm, neuronal shape without an evident of shrinkage or swelling, neurite extensions, and the absence of intracellular vacuoles (Baldelli et al. 2005).

Hippocampal autaptic neurons were prepared as described previously (Bekkers and Stevens 1991) but with slight modifications. Briefly, dissociated neurons were plated at very low density (20 000 cells/400 μL DMEM plus FBS) on microdots (40–60 μm in diameter) obtained by spraying a mixture of poly-β-hydroxy (0.1 mg/mL) and collagen (0.25 mg/mL) on 3.5 cm diameter dishes, which had been pre-treated with 0.1% agarose. After 2 h, the medium was exchanged with Neurobasal plus B27 supplement (2 mL). Both glial cells and single autaptic neurons were present under this culture condition. Electrophysiological experiments were performed on 8–15 DIV.

Current Recordings, Data Acquisition and Analysis of Postsynaptic GABAergic Currents, and Voltage-Dependent Calcium Currents

Spontaneous activity, GABAergic currents, and voltage-dependent calcium currents were studied using voltage-clamp recordings in the whole cell configuration of the patch-clamp technique (Hamill et al. 1981). Electrophysiological recordings were made with fire-polished electrodes (resistance 2–5 MΩ when filled with the intracellular solution) mounted on the headstage of an EPC-10 patch-clamp amplifier (HEKA Electronic, Lambrecht, Germany), allowing cancellation of capacitive transients and compensation of series resistance. Data were acquired with sample frequency ranging between 5 and 10 kHz and filtered at 1–2 kHz. Recordings with either leak currents >100 pA or series resistance >20 MΩ were discarded. Petri dishes containing the cells were placed on an experimental chamber mounted on the stage of a Nikon Eclipse TE2000 inverted microscope.

The cell being recorded was locally, rapidly, and continuously superfused with a Tyrode solution containing (in mM): 2 CaCl2, 140 NaCl, 4 KCl, 1 MgCl2, 10 glucose, 10 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)/NaOH (pH 7.4).

Evoked excitatory postsynaptic currents (eIPSCs) were recorded exclusively from autaptic glutamatergic cells (8–15 DIV), whereas eIPSC responses were derived either from autaptic GABAergic cells or from extracellular stimulation of GABAergic synapses in neuronal networks (14–21 DIV). Subsequent analyses did not reveal significant differences (data not shown) between the 2 eIPSC data sets. Autaptic postsynaptic currents were evoked by applying a brief depolarization (0.5 ms; +40 mV) from a holding potential of ~70 mV. Mean synaptic amplitudes (Fig. A1A, B1) were taken generally 2–3 min after establishing the whole-cell configuration. For extracellular synaptic stimulation in neuronal networks to take place, presynaptic stimuli were delivered through a glass pipette of 1 μm tip diameter filled with Tyrode solution and placed in contact with the soma of the GABAergic interneuron in a loose-seal configuration (Baldelli et al. 2002). Current pulses of 0.1 ms and variable amplitude (5–25 μA) delivered by an isolated pulse stimulator (model 2100; A-M System, Carlsbad, WA, USA) were required to induce monosynaptic eIPSCs with short latency (2–4 ms). Only responses with short latency (<4 ms) were considered. To ensure that only the synaptic contacts of the selected presynaptic neuron were stimulated by the extracellular stimulating pipette, we only used those eIPSCs that were completely lost after a few micrometer displacements of the stimulation pipette from the soma. Stimulation intensity was set at 1.5 times the threshold in all the experiments even though the evoked currents remained stable at stimulation intensities more than 2 times the threshold. The postsynaptic neurons were voltage-clamped at ~70 mV. The current artifact produced by the presynaptic extracellular stimulation was subtracted in all of the eIPSCs shown.

The internal solution used in miniature inhibitory postsynaptic currents (mIPSCs) and eIPSCs, respectively, contained (in mM): 100 Cs-methanesulfonate, 20 CsCl, 2 MgCl2, 5 ethylene glycol-bis(2-aminoethylether)-N,N,N’,N”-tetraacetic acid (EGTA), 4 Mg-ATP, 0.3 Na-GTP, 15 phosphocreatine, 10 QX-314, 10 HEPES/CsOH (pH 7.3); whereas the internal solution used in autaptic neuron recordings contained (in mM): 160 KCl, 10 EGTA, 5 Mg-ATP, 0.3 Na-GTP, 10 HEPES/CsOH (pH 7.3). Monosynaptic GABAergic eIPSCs were investigated in pairs of 14–21 DIV-cultured hippocampal neurons. mIPSCs were recorded using whole-cell configuration of the patch-clamp technique in neurons that were voltage-clamped at ~70 mV in the presence of tetrodotoxin (TTX, 1 μM) in order to block the propagation of spontaneous action potentials. D(-)-2-amino-5-phosphonovaleric acid (D-APV) (50 μM) and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (10 μM) were added to external solution to block the glutamatergic transmission. External solutions were rapidly exchanged using electronically driven miniature solenoid valves coupled with a multibarrel concentration-clamp device whose common outlet of which was placed at a distance of 100 μm from the cell to be patched. The flow rate was 1 mL/min and was regulated by gravity.

For voltage-dependent calcium current recordings, cells were dialyzed with an intracellular solution containing (in mM): 10 NaCl, 100 CsCl, 20 TEA-Cl, 5 Mg-ATP, 0.3 Na-GTP, 14 EGTA, 20 HEPES/CsOH (pH 7.3). Voltage-gated calcium currents (ICa) were recorded using 10 mM Ca2+ as the charge carrier. To represent the averaged time course of methacholine effect on ICa, the ICa variation among different cells was minimized by normalizing the amplitude current from each individual cell (ICa, max/ICa, min). Neurons were stimulated with 50-ms test pulses at 10 mV from a holding potential of ~70 mV.
Data acquisition was performed using PULSE programs (HEKA Electronic). The data analysis was performed with the MiniAnalysis program (Synaptosoft, Leonia, NJ, USA). All experiments were performed at room temperature (22–24°C).

**Paired-Pulse Recordings—Paired-Pulse and Train Stimulation**
Paired-pulse protocols consisted of 2 depolarizing pulses separated by variable interpulse intervals (IPIs) ranging from 10 to 2000 ms. For each cell, the mean paired-pulse ratio (PPR) at a given IPI was determined from the responses to at least 8 consecutive trials applied at 0.1 Hz. Moreover, a paired-pulse protocol (IPI 50 ms) was routinely applied in connection with train stimulation protocols. The response to the first stimulus ($I_1$) was used to follow the current amplitudes under baseline conditions and during recovery from train-induced variations. At the same time, the response to the second stimulus ($I_2$) allowed to calculate the PPR = $I_2/I_1$ and to infer possible changes in release probability. Recordings of recovery from train-induced depression where the current amplitude did not reach at least 80% of the baseline value within 180 s were routinely excluded from analysis. For the time course of PPR during recovery from train-induced depression, recordings displaying high variability in the baseline PPR (coefficient of variation >10%) were excluded from the data sets.

**Cumulative Amplitude Profile Analysis**
We used the cumulative amplitude profile analysis (Schneggenburger et al. 1999) to estimate the size of the readily releasable pool of...
aptic GABA receptors, we examined the effect of methacholine on GABAergic transmission, the Methacholine-Mediated Modulation of Inhibitory Postsynaptic GABAergic Receptors are not Involved in activation and decay kinetics were observed (Fig. 1A). The amplitude of the eIPSCs was dramatically decreased upon methacholine application (circles) in 11 neurons tested. The linear regression fitting by linear regression, in the control (filled circles) and on methacholine (empty circles) (n = 11). Data are means ± SEM. **P < 0.01.

Figure 2. Effect of mAChRs activation on GABA-evoked currents. (A) Time course of I Cl evoked by exogenous GABA (100 μM, 500-ms pulses) applied at 10-s intervals, in the control and during a 100-μM methacholine perfusion (top horizontal bar). Insets: original traces of GABA-evoked currents under the conditions described (n = 19). (B) GABA evoked currents amplitude versus holding potential relationship fitting by linear regression, in the control (filled circles) and on methacholine (empty circles) (n = 11). Data are means ± SEM. **P < 0.01.

Results

The Muscarinic Agonist, Methacholine, Selectively Down-Regulates eIPSCs, but not eEPSCs

To evaluate the effect of muscarinic modulation on GABAergic and glutamatergic transmission, we evoked postsynaptic currents in isolated hippocampal neurons forming autaptic contacts (Fig. 1A, B). IPSCs and eIPSCs were evoked at 10-s intervals, after a steady-state period under control conditions (Fig. 1A2–3, B2–3; “a”), the neurons were perfused with methacholine for 1 min (100 μM; Fig. 1A2–3, B2–3; “b”), and then the muscarinic agonist was washed out (Fig. 1A2–3, B2–3; “c”). The amplitude of the eIPSCs was dramatically decreased upon methacholine application (~64%), and the block was completely recovered after drug wash out (Fig. 1A2–3, “b” and “c”). Interestingly, methacholine application on glutamatergic autapses did not exert any effect (Fig. 1B3). A similar result was obtained evoking IPSCs by extracellular stimulation of GABAergic afferent fibers in hippocampal neurons cultured at low density. Also, under this condition, methacholine application induced a significant decrease in eIPSCs (Fig. 1C) alongside an increase in synaptic latency (Fig. 1F), while no changes in the activation and decay kinetics were observed (Fig. 1D, E).

Postsynaptic GABAergic Receptors are not Involved in the Methacholine-Mediated Modulation of Inhibitory Transmission

To evaluate a possible direct mACHR modulation on postsynaptic GABA A receptors, we examined the effect of methacholine on GABA A receptor-mediated Cl− currents (I Cl) activated by the application of exogenous GABA (100 μM, 500-ms pulses). GABA evoked a partially deactivating I Cl, completely blocked by the GABA A receptor antagonist bicuculline (40 μM; data not shown). Methacholine (100 μM) caused no significant modifications in the GABA A current amplitudes (Fig. 2A, 9.04 ± 0.5 and 8.95 ± 0.5 nA, under control conditions and after methacholine application, respectively; n = 19), in accordance with previous studies (Ma et al. 2003). Figure 2B shows the averaged current–voltage relationship in GABA-evoked Cl− currents, under control conditions (squares) and upon methacholine application (circles) in 11 neurons tested. The linear regression fits were superimposed, thereby confirming the absence of postsynaptic muscarinic modulation on Cl− current evoked by exogenous GABA.

Methacholine Enhances the Frequency of Miniature Inhibitory Postsynaptic Currents

The amplitude of postsynaptic currents is determined by the linear combination of a series of parameters, namely: The number of releasable SVs, the probability of release, the vesicular neurotransmitter content, and the postsynaptic receptor density. The study of the current activated by the application of exogenous GABA already showed that the GABA A postsynaptic receptor density was unaffected by methacholine treatment.

Miniature events are believed to be caused by the random release of single neurotransmitter packets, and variations in their amplitude are considered to be evidence of a modification of postsynaptic receptor properties; moreover, changes in their frequency suggest a modulation of the neurosecretion process. Here, we explored the possibility that the muscarinic
receptors might directly modulate the exocytotic machinery, thus enhancing neurotransmitter release without requiring propagated action potentials.

Figure 3A shows the original mIPSC traces obtained at −70 mV holding potentials and their reversible potentiation by the muscarinic agonist. Methacholine (100 μM), after a 2-s delay, elicited a sharp augmentation of mIPSCs that slowly returned to baseline upon washout. Superimposed averaged traces of miniature events under control conditions and during perfusion with methacholine showed no modification of the kinetic parameters (Fig. 3B; amplitude, 29.04 ± 1.1 pA; area, 146.7 ± 10 nC; slope10−90%, −15.74 ± 1 mV/ms; rise time10−90%, 1.68 ± 0.1 ms; decay time, 11.54 ± 0.5 ms; and half-width, 3.59 ± 0.1 ms). Neither the averaged area, nor the amplitude of miniature events, was affected by methacholine treatment (Fig. 3C). On the contrary, the frequency was increased from 1.4 ± 0.2 to 4.1 ± 0.7 Hz (n = 9, Fig. 3D,E). The absence of changes in the mIPSCs amplitude confirms the lack of postsynaptic modulation.

The increased mIPSCs frequency strongly suggests that muscarinic receptors located in presynaptic terminals are able to modulate the secretory machinery. Since this effect occurs in the absence of firing activity and action potential-dependent Ca2+ influx, we conclude that activation of presynaptic muscarinic receptors can affect the spontaneous neurotransmitter release (miniature events) by Ca2+ mobilization from the endoplasmic reticulum through a Gq protein/phospholipase C (PLC) pathway (Bonner 1989), or by inducing a moderate membrane depolarization (Gonzalez et al. 2011), that enhances presynaptic Ca2+ concentration thereby increasing spontaneous vesicular fusion.

**Methacholine Reduced the Paired-Pulse Depression**

We hypothesized that presynaptic muscarinic receptors play a role in the decrease of eIPSC. To confirm this hypothesis, we next examined the possibility of a change in the paired-pulse depression (PPD).

Among the multiple mechanisms known to contribute to changes in the PPR (PPR = I2/I1) at short IPIs, 2 processes are of major importance (Zucker and Regehr 2002): 1) Residual calcium from the first pulse causes an increase in the SVs release probability (Pt) during a second pulse applied at short intervals; 2) SV depletion generated by the first pulse leads to a decrease in the number of readily releasable vesicles for the second pulse. IPSC responses were characterized by PPD (PPR <1), mainly due to a high basal Pt in inhibitory synapses, thus favoring vesicle depletion over residual calcium.

Repetitive paired eIPSCs (IPI = 100 ms) applied at 10-s intervals were recorded under control conditions (a), during application of methacholine (b), and during the recovery after drug washout (c) (Fig. 4A). Note that methacholine induced a dramatic decrement in the amplitude of the IPSCs evoked by the first pulse that was coupled with a lower reduction in the IPSC evoked by the second pulse. The time course of the PPR clearly shows that inhibitory synapses are characterized by a pronounced PPD, significantly reduced by methacholine application (Fig. 4B). Indeed PPR, characterized by a small initial value (0.65) under control conditions (a), was increased to close 1 in a reversible manner by methacholine application.

The PPR was analyzed in detail applying a series of paired-pulse stimuli with IPIs ranging from 10 to 2000 ms (Fig. 4C, D), under control condition and under methacholine treatment. The current traces used in the analysis were averaged from 8 consecutive responses repeated every 10 s. PPR at short IPIs (Fig. 4D, inset) was clearly enhanced by methacholine treatment. In control neurons, we found that PPD had large initial values (88% at Δt = 20 ms) that were significantly diminished at all intervals tested during methacholine treatment (60% at Δt = 20 ms). The recovery from PPD exhibited 2 distinct kinetic components, namely, a fast component that was similar in control and methacholine-treated neurons (τfast = 46 ± 7 and 39 ± 5 ms, respectively), and a slow component that was significantly faster for methacholine-treated neurons than for the control ones (τslow = 158 ± 95 and 382 ± 30 ms, respectively). Note that the decrease in the methacholine-induced eIPSC amplitude is accompanied by a reduction in the synaptic depression evoked by a pair of presynaptic

**Figure 3.** Frequency of miniature inhibitory postsynaptic currents was enhanced by methacholine. (A) Representative recording of mIPSCs under control conditions and in the presence of methacholine for the time indicated by the top horizontal bar. (B) Averaged traces of mIPSCs under control conditions and during methacholine application. (C) Averaged data of mIPSC amplitude (top panel) and area (bottom panel). The box plot represents the median and the quartiles (the lower quartile is the 25th percentile and the upper quartile is the 75th percentile), with a line from the lower quartile to the minimum point and another line from the upper quartile to the maximum point. The points represent the outlier data not included in the statistical analysis. (D) Averaged data of the frequency of quantal events for mIPSCs recorded under the conditions described. Note the enhancement during the methacholine application. Data are mean ± SEM of 11 neurons. **P < 0.01. (E) Frequency histogram of quantal events for mIPSCs recorded in the conditions indicated. Note the enhancement during the methacholine application at short interevents intervals (MCho, black columns).
stimuli. Such result demonstrates a presynaptic mechanism of action and strongly suggests that the effects on the eIPSC amplitude could be due to a decreased release probability.

**Methacholine Reduced the Vesicular Release Probability, but not the Size of the Readily Releasable Pool of Synaptic Vesicles**

To investigate in detail the presynaptic mechanism underlying the modulation exert on eIPSCs by muscarinic receptors, we further evaluated the effects of methacholine on the size of the RRP and the probability of SVs release from the RRP ($P_r$). This analysis assumes that synaptic depression evoked by a tetanic stimulation (1 s at 40 Hz) reaches a steady-state phase characterized by equilibrium between released and recycled vesicles (Schneggenburger et al. 1999). As shown in Figure 5A, a significant depression of eIPSCs became immediately apparent during the stimulation train (1 s at 40 Hz) in both control and methacholine-treated neurons.

The inhibitory responses evoked by the stimulation train were plotted as cumulative amplitude profiles in Figure 5B. In both experimental groups, the cumulative profile of repeated eIPSCs showed a rapid initial rise followed by a slower linear increase at later pulses. The linear increase at later stages of train stimulation was clearly slower under control conditions. The linear fitting of the steady-state phase was evaluated by calculating the best linear fit including the maximum number of data points starting from the last point; in our case, the last 25 data points were fitted by linear regression. Assuming that the slow linear rise is attributable to the equilibrium between the release-induced depletion and the constant replenishment of the RRP, back extrapolation of the linear portion to time 0 yields a rough estimation of the total release minus the total replenishment, corresponding to the size of the RRP (Schneggenburger et al. 1999). As shown in Figure 5C, the RRP was not modified by methacholine ($3636 ± 414$ and $3574 ± 776$ pA in control and methacholine-treated neurons, respectively; $n = 11$) (left panel); however, methacholine decreased the mean amplitude of the first IPSC in the stimulation train ($1347 ± 331$ pA in control condition). The SV release probability ($P_r$), calculated as the ratio between the first eIPSC ($I_1$) and RRP, was significantly decreased by methacholine ($0.35 ± 0.04$ from $0.53 ± 0.04$ in control neurons, Fig. 5D, middle panel). To estimate the number of vesicles ($N_{syn}$) forming the RRP, we divided the size of the RRP by the mean size of the quantal event (i.e., the mean amplitude of mIPSCs obtained under the same experimental conditions, $29.04 ± 1$ pA). $N_{syn}$ was not modified by methacholine ($131 ± 14$ and $126 ± 27$ in control and treated neurons, respectively; Fig. 5D, right panel).

**Figure 4.** Effect of mAChRs activation on the PPD. (A) Representative eIPSCs recorded in response to paired stimuli separated by 100 ms and repeated every 10 s, in the control (a), during application of methacholine (b), and after washout (c). (B) Time course of PPR ($I_2/I_1$) over consecutive responses, as indicated in A. Note that methacholine, applied as indicated by the top horizontal bar, enhanced the PPR that recover to control values after methacholine washout. (C) eIPSCs recorded in the control (left) and during methacholine perfusion (right), in response to paired stimuli separated by the indicated IPIs (Δt). (D) The mean PPD was plotted as a function of the IPR (20, 30, 40, 50, 80, 200, 400, 800, and 2000 ms) and fitted by an exponential function (control, filled circles and methacholine, empty circles). Data are mean ± SEM of 15–16 neurons evaluated.
Down-Modulation of Voltage-Gated Calcium Channels Elicited by Methacholine

Acetylcholine modulates several ionic conductances via muscarinic receptors (Guerineau et al. 1995) and inhibits the activation of voltage-gated calcium channels (VGCCs) (Toselli and Lux 1989; Sahara and Westbrook 1993). A down-modulation of VGCC may in turn decrease the neurotransmitter release, thus defining the mechanism underlying the decrease in the probability of release that we have previously shown here. So, we have evaluated the effect of methacholine on somatic voltage-dependent calcium currents ($I_{\text{Ca}}$).

The mean averaged somatic $I_{\text{Ca}}$ amounted to $2.8 \pm 0.2$ nA and, after 60 s of methacholine application, reached a steady-state level of depression at $1.35 \pm 0.1$ nA. Inhibition of somatic $I_{\text{Ca}}$ was measured for each individual neuron at the end of a 100-s perfusion period with methacholine; down-modulation amounted to $55.7 \pm 5.1\%$ ($n = 8$) and was partially recovered after drug washout (Fig. 6A). Under methacholine treatment, the residual $I_{\text{Ca}}$ showed a lower current inactivation ($t_{\text{inactivation}} 14.86 \pm 0.2$ and $8.68 \pm 0.1$ ms, in control and methacholine-treated neurons, respectively; Fig. 6A, inset). Moreover, the amplitude–voltage relationship recorded shows than under the presence of methacholine, a $-10$ mV shifts of the $I/V$ plot (Fig. 6B). Methacholine inhibited $I_{\text{Ca}}$ at all the potentials tested, especially at the more depolarized values, whenever the N- and P/Q-type calcium channels were predominant. These results suggested that the blockage exerted by muscarinic receptors mainly affects the fast inactivating N- and P/Q-type calcium channels, which are the main calcium channels involved in the GABA release of hippocampal neurons (Baldelli et al. 2005). This result suggested that the blockage exerted by muscarinic receptors mainly affects the fast inactivating N- and P/Q-type calcium channels, as previously reported (Jeong and Wurster 1997; Liu et al. 2002).

When neurons were pretreated with the mAChR antagonist atropine (González-Rubio et al. 2006), methacholine did not induce any change on $I_{\text{Ca}}$ (Fig. 6C). These results demonstrate that the inhibition of VGCCs by methacholine was mediated by the activation of mAChRs, excluding the possibility of a direct action of methacholine on the VGCCs.

We next dissected pharmacologically the somatic calcium current measured and their individual sensitivity to methacholine. Whole-cell currents were measured from a holding potential of $-70$ mV. Using neurons of 10–14 DIV and test pulses to $0$ mV in $10$ mM Ca$^{2+}$, we observed 4 distinct high-threshold Ca$^{2+}$ currents on the basis of their sensitivity to $3\mu$M nifedipine (L-type), $1\mu$M ω-conotoxin GVIA (N-type), and $2\mu$M ω-agatoxin IV A (P/Q-type). As has been previously reported (Baldelli et al. 2000, 2002); the contribution of the different calcium channel subtypes to the total somatic current is: N-type 22%, P/Q-type 12%, L-type 44%, and 21% of residual current (R-type). These somatic Ca$^{2+}$ channels subtypes showed a different sensitivity to methacholine. After application of the muscarinic agonist, L-type was depressed by 3.2 ± 5%, N-type 33.5 ± 7%, P/Q-type 44.7 ± 3%, and R-type 12.6 ± 2% (Fig. 6D, $n = 6–12$).

Attending than $I_{\text{Ca}}$ recorded above are mainly due to somatic VGCC and to evaluate the methacholine down-modulation on presinaptic VGCC, we have measured the contribution of the calcium channels subtypes to the GABAergic synaptic transmission though the recording of eIPSCs in
control conditions and after treatment with methacholine. eIPSCs were recorded sequentially applying to each neuron the selective Ca\(^{2+}\) channels blockers: Nifedipine (3 μM), ω-conotoxin GVIA (1 μM), and ω-agatoxin IVA (2 μM)

Figure 7 shows the normalized time course of the eIPSC amplitude of 2 representative neurons, maintained under control conditions (black circle) or exposed to methacholine (100 μM, white circle). This approach allowed us to estimate the mean percentage contribution of each Ca\(^{2+}\) channel subtypes to eIPSCs (Fig. 7C). The blocking effect of nifedipine on the eIPSCs was weak in both control and treated neurons (11.8 ± 3% and 17.7 ± 3%, respectively). In control condition, GABAergic release was mainly controlled by a mixed population of N-, P/Q-, and R-type channels (27.7 ± 2%, 31.5 ± 3%, and 28.8 ± 3%, respectively). In methacholine-treated neurons, the contribution of N- and P/Q-type channels to eIPSCs was dramatically reduced (13.8 ± 5% and 9.1 ± 2%, respectively), and the role of N- and P/Q-type channels was mainly assumed by the R-type (59.3 ± 5%). These results allow to conclude that, as already observed for somatic Ca\(^{2+}\) current, also for the presynaptic Ca\(^{2+}\) channels, the N- and P/Q-types represent the specific target of muscarinic down-modulation, while the R-type resulted to be insensitive to methacholine.

**Methacholine Decreased Synaptic Depression Induced by Short- and Long-Lasting Tetanic Stimuli at GABAergic Synapses**

To evaluate whether the activation of muscarinic receptors may modulate vesicular recycling processes of GABAergic terminals, we studied the effect of methacholine on the time course of synaptic depression and recovery from depression induced by various patterns of repetitive stimulation.

Synaptic depression in GABAergic synapses was initially evaluated from the eIPSC responses to a short stimulation at a high frequency fully comprise within the gamma range oscillations (1 s at 40 Hz) (Fig. 8A). Both control and methacholine-treated inhibitory neurons showed a rapid decrease in the eIPSC amplitude during the first pulses of the stimulation train, followed by a progressive decay into a quasi-stationary level. However, methacholine significantly slowed down the rate of the fast decrease and increased the current amplitude at the steady-state level. Double exponential fitting of the averaged data resulted in the following values: For the time constant of fast decay \(\tau_{fast}\) (10.7 ± 2 ms for control; 26.5 ± 8 ms for methacholine; \(P<0.05\), unpaired Student's t-test), for the time constant of slow decay \(\tau_{slow}\) (182 ± 34 ms for control; 451 ± 134 ms for methacholine; \(P<0.05\), unpaired Student's t-test), and for the steady-state eIPSC \(I_{ss}\) (2.4 ± 0.8% for control; 9.5 ± 3% for methacholine; \(P<0.05\), unpaired Student's t-test) (Fig. 8B). Under control conditions, the brief tetanic stimulation (1 s at 40 Hz) of GABAergic afferent fibers induced a post-tetanic depression (PTD) of the eIPSC amplitude, possibly due to a transient decrease of the \(P_r\) or of the RRP size (Jensen et al. 1999).
In control neurons, a fast- and short-lasting PTD (around −25%) was observed 10 s after the stimulation train, and then eIPSC recovered their control values with a time constant of 35.87 ± 11.6 s. However, the application of methacholine prevents the PPD exerted by the short tetanic stimulation from taking place (78.5 ± 10% and 107.4 ± 4% in control and methacholine-treated neurons, respectively, Fig. 8D).

Additionally, the PTD was not accompanied by any change in the PPR (Fig. 7E), suggesting that this form of short-term plasticity cannot be ascribed to a reduced P$_r$.

Afterwards, we induced depression of single eIPSCs, using long-lasting repetitive stimulation (75 s at 8 Hz) of the presynaptic GABAergic fibers (Fig. 9). In control neurons, the eIPSCs showed a progressive decay during long-lasting stimulation train, therefore, at the last part of the train, the residual current was only 13% in relation to the initial value (a maximum depression of 86.5%, Fig. 9B). Methacholine treatment, reduced the rate of decay and the maximum synaptic depression (66.5%) induced by the long-lasting stimulation, described by a double exponential function ($\tau_{fast} = 0.65 \pm 0.2$ s in the control; $0.71 \pm 0.3$ s on methacholine; $P > 0.05$, unpaired Student’s t-test; and $\tau_{slow} = 21.55 \pm 2.4$ s in the control; 25.10 ± 3.5 s on methacholine; $P > 0.05$, unpaired Student’s t-test). $I_{exc} = 13\%$ and 33%, in the control in methacholine-treated neurons, respectively ($n = 7$; Fig. 9C).

The recovery from synaptic depression obtained returning the stimulation frequency to 0.1 Hz, (Fig. 9D) was already complete 120 s after the end of the stimulation train and could be well described by a single exponential function in both control and treated cells. However, upon methacholine application, the rate of recovery increased, therefore eIPSC recovered its prestimulation level earlier than in control neurons ($\tau = 41.9 \pm 3$ and 28.7 ± 3 s, in the control and methacholine-treated neurons, respectively, $n = 11$; Fig. 9D, inset).

In summary, methacholine reduced the rate of synaptic depression and the maximum synaptic depression induced by the short- and long-lasting stimulation trains. In parallel, methacholine induced a faster recovery from synaptic depression. These results could in part be explained by the reduction in the probability of release induced by the activation of mAChRs but, as well, methacholine may increase the SVs recycling efficiency during intense repetitive stimulation. In this way, mAChR activation allows inhibitory synapses to keep pace with periods of intense synaptic activity.

**Discussion**

In the present study, we have characterized the effects of mAChRs activation on the eIPSCs. GABAergic postsynaptic currents were significantly reduced by an application of methacholine. Glutamatergic postsynaptic currents were not affected. The reduction in the eIPSC was not mediated by the modulation of postsynaptic GABA$_A$ receptors. Cumulative amplitude analysis of eIPSC revealed a mainly presynaptic mechanism of action based on a decreased release probability without any change in the RRP size. We showed that the strong inhibition of VGCC accounts for the decreased P$_r$. The reduction in the P$_r$ probably explains the multiple changes in the short-term plasticity properties of the inhibitory synapses, namely, the reduction in PPD and synaptic depression.
induced by short stimulation trains. However, the sharp decrease in synaptic depression induced by a long-lasting repetitive stimulation and the increase in the rate of recovery from depression cannot be completely explained by a reduced $P_r$. Nevertheless, these results suggest that activation of mAChRs can also affect the efficiency of synaptic recycling processes to an important degree.

**The mAChRs Selectively Modulate eIPSCs, but have no Effects on eEPSCs**

In GABAergic interneurons, we observed that methacholine induced a fast and large decrement in the amplitude of eIPSCs in contrast to autaptic glutamatergic neurons in which methacholine did not exert any effect. The effect exerted by muscarinic activation on synaptic transmission is a cause of controversy, going from studies that report a potentiation of EPSCs (Benardo and Price 1982; Cole and Nicoll 1983; Fernández de Sevilla and Buño 2010) to others that find an increase in spontaneous inhibitory post synaptic currents (Zhong et al. 2003) that may be associated with an enhancement of $I_{GABA}$ (Ma et al. 2003) or an after depolarization that evoke action potentials (McQuiston and Madison 1999); in any case, never mediated by excitatory pathways (Pitler and Alger 1992; Widmer et al. 2006). Some authors show that mAChRs enhance the excitability by decreasing the release of GABA (Salgado et al. 2007). Additionally, inhibition of EPSCs has been associated with activation of presynaptic (Scanziani et al. 1995) or postsynaptic mAChRs (Seeger and Alzheimer 2001). It has also been published that mAChRs increase the frequency and amplitude of spontaneous IPSCs and inhibit electrically evoked GABA release (Behrends and Bruggencate 1993). These discrepancies may be associated with ontogenetic modifications of neuronal excitability and developmental changes in neurotransmitter receptors at different brain stages of maturation (Aronica et al. 2011).

**mAChRs Modulate eIPSCs Through a Presynaptic Mechanisms of Action**

Some authors have reported a postsynaptic action of mAChRs that enhance GABA$_A$ receptor functions on pyramidal neurons through a protein kinase C (PKC)-dependent signaling (Ma et al. 2003). We have reported that methacholine did not affect $Cl^-$ currents directly activated by the application of exogenous GABA; however, we must consider that these $Cl^-$ currents may be partially associated with nonsynaptic GABA receptors, which may be different than these synaptically located. Moreover, the lack of change in mIPSCs amplitude confirmed the absence of the modification of postsynaptic receptor properties. And, no modification of mIPSCs has been reported when mAChRs decreased IPSCs and increased PPR (Salgado et al. methacholine, empty circles). (B) The time course of synaptic depression was fitted to a biexponential curve: $I = (A_{fast}e^{-t/\tau_{fast}} + A_{slow}e^{-t/\tau_{slow}}) + Iss$. Plot histograms showing averaged $\tau_{fast}$, $\tau_{slow}$, and Iss for control (empty bars) and methacholine-treated (filled bars) neurons. (C) Representative eIPSCs elicited at 0.1 Hz, before and after a brief tetanization. (D) Mean normalized eIPSC amplitude (0.1 Hz) observed in control (filled circles) and methacholine-treated (empty circles) neurons undergoing a short stimulation train (arrowhead, 1 s at 40 Hz). Post-tetanic single eIPSCs elicited at 0.1 Hz were normalized to the pretetanic eIPSC level. (E) Averaged PPR obtained immediately before (b) and after (a) the short tetanic stimulation. Note that tetanization of the presynaptic neuron does not modify the PPR. Data are mean ± SEM of paired data (n = 10).
Besides, the reduction in synaptic PPD that accompanied the decrease in the eIPSC amplitude induced by methacholine is strongly suggested of a presynaptic mechanism.

We further used the cumulative amplitude analysis to investigate this presynaptic mechanism in depth. So, we observed that methacholine did not change the RRP size in spite of inducing a 50% decrement of the Pr. Interestingly, a detailed analysis of the mACHr activation effects on the quantal parameters of hippocampal inhibitory transmission was lacking. Only in striatal glutamatergic neurons has recently been shown (Higley et al., in 2009) that mACHr activation induced a decrease in both the probability of release and the concentration of glutamate in the synaptic cleft, thus reducing the duration of synaptic potentials and limiting the temporal summation of afferent inputs.

**Inhibition of VGCC by Presynaptic mACHRs**

Activation of mACHRs affects individual neurons by influencing several types of ionic currents: The M-current (Adams et al. 1982), the K⁺ “leak” current (Madison et al. 1987), the slow Ca²⁺-activated K⁺ current (Cole and Nicoll 1983), SK-channels (Giessel and Sabatini 2010), the Ca²⁺-activated nonselective cation current (Guerineau et al. 1995), and VGCC (Howe and Surmeier 1995). Both, excitatory and inhibitory presynaptic mechanisms evoked by mACHr have been shown to be associated with afterdepolarization in the interneurons (McQuinton and Madison 1999) and inhibition of VGCCs (Salgado et al. 2007). It has also been reported that the presynaptic mACHr reduction in eEPSCs in the hippocampus is not mediated by inhibition of presynaptic Ca²⁺ channels, but rather by a direct interference at some point in the neurotransmitter release process subsequent to calcium influx (Scanziani et al. 1995).

Inhibition of VGCC by muscarinic agonists has been reported in several types of neurons (Mathie et al. 1992; Allen and Brown 1993; Liu et al. 2002). It has mainly been targeted on non-L-type channels (Jeong and Wurster 1997; Delmas et al. 1998; Stewart et al. 1999; Liu et al. 2002) through activation of PI3K/Ca²⁺-independent and PLC/Ca²⁺-dependent PKC (Salgado et al. 2007). N- and P/Q-types are the main calcium channels involved in the GABA release of hippocampal neurons (Baldelli et al. 2005). In our study, we have showed that somatic N- and P/Q-types are more sensitive than L- and R-types to muscarinic modulation. This result was confirmed when we measured the contribution of the different presynaptic high voltage activated calcium channel subtypes to the eIPSCs in control conditions and after treatment with methacholine. We observed that methacholine selectively exerted its inhibitory action on N- and P/Q-type Ca²⁺ channels, whereas R-type, due to its insensitivity to mACHRs...
activation, became the main source Ca$^{2+}$ driving GABA release in methacholine-treated cells.

It has been reported that this inhibition is mediated by activation of pertussis toxin-sensitive Gi-proteins (Toselli and Lux 1989; Jeong and Wurster 1997), involving m2 and m4 mAChR subtypes (Allen and Brown 1993; Liu et al. 2002). The inhibition of VGCC is exerted by the direct binding of Gi-βγ subunits to the calcium channels (Campbell et al. 1995; Herlitz et al. 1996; Ikeda 1996). It has been reported that strong depolarizing prepulses (Hernández-Guijo et al. 1998), or high frequency stimulation (Brody and Yue 2000), relieve the binding of Gi-βγ subunits to the VGCC (Brody and Yue 2000) allowing the recovery of Ca$^{2+}$ currents from the condition of initial inhibition due to mAChRs activation. It is particularly interesting to mention here that this voltage-dependent recovery of N- and P/Q-type Ca$^{2+}$ conductance could contribute to the reduced synaptic depression observed in GABAergic synapses treated with methacholine.

mAChRs Reduced Synaptic Depression and Favored its Recovery in GABAergic Synapses

The application of methacholine prevents the depression exerted by brief tetanic stimulation. This effect seems to be mainly related to the reduction in the $P_r$ that in turn decreases the rate of SVs depletion during short tetanic stimulation. Additionally, we have also observed that when presynaptic GABAergic neurons were stimulated with long-lasting trains, methacholine reduced the rate of synaptic depression and increased the remaining steady-state eIPSC in the last part of the train. This effect cannot be entirely explained by the reduction in $P_r$, which is a consequence of the decreased calcium influx. Indeed, if depletion of the RRP size is the main mechanism underlying synaptic depression induced by short tetanic stimulation, this mechanism would represent a minor contribution when a stimulation train was applied for >1 min (Fig. 8). Hence, the amount of synaptic depression would mainly be dependent on the efficiency of the recycling processes active at the presynaptic terminal (Zucker and Regehr 2002). Moreover, when the stimulation frequency was returned to a lower frequency, methacholine would increase the rate of recovery from depression induced by both short- (Fig 8D) and long-lasting (Fig 9D) stimuli. This represents another clear indication that activation of mAChRs increases the efficiency of SVs recycling, following the period of intense repetitive activity. Thus, mAChRs play a crucial role during high frequency network oscillation, supporting inhibitory synapses to keep pace with these periods of intense activity.

The strong effect of methacholine on the SVs recycling processes helped us to reconcile an apparent inconsistency between the strong inhibition of the eIPSCs and the increase of the mIPSCs frequency exerted by methacholine. Indeed, despite the general suppression of voltage-gated Ca$^{2+}$ currents, muscarinic activation paradoxically increases intracellular Ca$^{2+}$ accumulations in the dendrites and spines by means of both K$^+$ conductance blockade and calcium release from intracellular stores (Muller and Connor 1991; Beier and Barish 2000). Probably, the enhanced mIPSC frequency has little weight on the strength of inhibitory inputs when compared with the strong reduction in the amplitude of the eIPSC. On the contrary, it has been shown that the increase of basal intracellular Ca$^{2+}$ significantly speed up the recycling processes (Marks and McMahon 1998; Beutner et al. 2001), furnishing an additional mechanism to better sustain high frequency and persistent synaptic activity. So, it is possible that the enhancement of miniatures frequency represents a side effect of the methacholine-mediated increase of basal Ca$^{2+}$ that together with the strong reduction in $P_r$, by inhibition of VGCC, is functional to the reduction in synaptic depression.

Conclusion

In summary, our results show that muscarinic modulation of GABAergic transmission in the hippocampus exerts a double action: A decrease in the amplitude of evoked GABA release due to a reduction in the SVs release probability combined with a reinforcement of the synaptic recycling processes. These effects dramatically affect the short-term plasticity properties of inhibitory synapses and switch the synaptic dynamics into a completely different functional configuration.

Therefore, a significant consequence of synaptic dynamics is that synapses can act as filters: Synapses with a low initial probability of release can function as high-pass filters, whereas synapses with a high initial probability of release can act as low-pass filters (Abbot and Regehr 2004). Several examples of low-pass filtering have been described at a number of inhibitory synapses in the central nervous system, including those in the hippocampus (Poncer et al. 2000; Hefft et al. 2002), and neocortex (Gupta et al. 2000). Our findings demonstrate that, under control conditions, inhibitory neurons show a fast and pronounced synaptic depression, typical of a low-pass filtering. Remarkably interesting is the fact that, by lowering GABA release probability and enhancing SVs recycling, methacholine strongly reduced the synaptic depression of the hippocampal inhibitory synapses, thereby changing their filtering properties and enhancing their cutoff frequency. These properties are of particular interest in the light of the basic synaptic mechanisms underlying hippocampal gamma oscillations. Indeed, hippocampal gamma oscillations (25–90 Hz) are induced by mAChRs agonists, which mimic cholinergic input from the septum and are completely blocked by GABA$\alpha$ receptor antagonists (Bartos et al. 2007). This emphasizes the significance of muscarinic modulation of GABAergic synapses in the generation of the gamma oscillations.

Notes

We acknowledge the support provided to the present study through a Ramon y Cajal contract signed to J.M. Hernández-Guijo, the Instituto de Salud Carlos III grant PI1080227 for J.M.H.-G., and the Italian Ministry of Health-Progetto Giovani, Compagnia di San Paolo-Torino, and GGP09066-Telethon-Italy support to P.B. We thank the support of J.C.G., MICINN fellow, and the continuous support given to our studies by “Fundación Teófilo Hernando.” Conflict of Interest: None declared.

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