J Physiol 587.21 (2009) pp 5139–5147

The Journal of Physiology

Activation of M2 muscarinic receptors leads to sustained suppression of hippocampal transmission in the medial prefrontal cortex

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Cholinergic innervation of the prefrontal cortex is critically involved in arousal, learning and memory. Dysfunction of muscarinic acetylcholine receptors and their downstream signalling pathways has been identified in mental retardation. To assess the role played by the muscarinic receptors at the hippocampal–frontal cortex synapses, an important relay in information storage, we used a newly developed frontal slice preparation in which hippocampal afferent fibres are preserved. Transient activation of muscarinic receptors by carbachol results in a long-lasting depression of synaptic efficacy at the hippocampal but not cortical pathways or local circuitry. On the basis of a combination of electrophysiological, pharmacological and anatomical results, this input-specific muscarinic modulation can be partially attributed to the M2 subtype of muscarinic receptors, possibly through a combination of pre- and postsynaptic mechanisms.

(Received 5 May 2009; accepted after revision 8 September 2009; first published online 14 September 2009)
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Abbreviations CCh, carbachol; APV, DL-aminophosphonovalerate; ERK, extracellular signal-regulated kinase; LTD, long-term depression; mAChR, muscarinic acetylcholine receptor; mPFC, medial prefrontal cortex; PFC, prefrontal cortex; PPF, paired pulse facilitation.

Introduction

The prefrontal cortex (PFC) receives and integrates information from multiple brain regions and its function is the target of many neuromodulators including acetylcholine. For example, cholinergic input to the PFC regulates attention (Robbins *et al.* 1989; Muir *et al.* 1992; Hasselmo, 1995), memory (Beninger *et al.* 1992; DeSousa *et al.* 1994), and anxiety induction processes (Berntson *et al.* 1998). Dysfunction of muscarinic acetylcholine receptors (mAChRs) and mAChR-mediated signalling pathways has been linked to Fragile X mental retardation (Volk *et al.* 2007), Alzheimer's disease (Fisher, 2008), and normal ageing processes (Joseph *et al.* 1993).

Cholinergic afferents to the prelimbic and infralimbic cortex, the major components of the medial prefrontal cortex (mPFC), rise primarily from the basal forebrain nucleus basalis (Gaykema *et al.* 1991). Among five mAChR subtypes, M1 and M2 receptors are the predominant form expressed in the mPFC. M1 receptors are mainly localized in postsynaptic compartments of glutamatergic synapses, whereas M2 receptors are found both pre- and postsynaptically (Volpicelli & Levey, 2004). Activation of mAChRs results in an array of effects involving different cellular mechanisms in various brain regions. These effects can be excitatory or inhibitory, mediated by M1, M2 and M4 receptors. For instance, M1 mAChR activation increases temporal summation of synaptic events in the prefrontal cortex by down-regulating Kir2 channels (Carr & Surmeier, 2007). However, in hippocampus (Scheiderer *et al.* 2006; Volk *et al.* 2007) and cortex (Kirkwood *et al.* 1999; Massey *et al.* 2001; McCoy & McMahon, 2007), the non-selective muscarinic agonist carbachol (CCh) induces long-term depression of synaptic transmission (CCh-LTD). This form of plasticity is mediated by M1 receptors and dependent on extracellular signal-regulated kinase (ERK) signalling pathways and protein synthesis mechanisms (McCoy & McMahon, 2007; Volk *et al.* 2007; McCoy *et al.* 2008; Scheiderer *et al.* 2008).

Among a number of glutamatergic afferents projecting to the mPFC, the input from the hippocampus is especially important. Previous work has shown that the CA1 and subiculum of ventral hippocampus send ipsilateral, unidirectional projections that terminate on neurons in the mPFC (Jay & Witter, 1991; Jay *et al.* 1992). The functional integrity of the hippocampal–mPFC network, and the flow of information between these two brain regions, are critical to the proper functioning of the mPFC

DOI: 10.1113/jphysiol.2009.174821

in memory formation (O'Donnell & Grace, 1995; Seamans *et al.* 1995; O'Donnell *et al.* 2002; Goto & O'Donnell, 2003).

Employing a newly developed frontal slice preparation on which hippocampal afferent fibres are preserved and can be selectively activated (Parent *et al.* 2009), we are equipped to test whether the synaptic properties of this pathway allow for input-specific regulation by neuronal activities and modulators. The goal of this study is to investigate the effects and temporal profile of the activation of mAChR on hippocampal–mPFC synapses, with an emphasis on agonist-induced synaptic long-term depression.

Methods

Preparation of frontal slices containing prelimbic cortex

The University of Minnesota Institutional Animal Care and Use Committee approved the use of animals for the studies described below. Experiments included in these studies comply with all polices and regulations, as detailed by Drummond (2009).

Coronal slices containing medial prefrontal cortex were prepared from 8- to 15-week-old mice following standard procedures (Parent *et al.* 2009). Briefly, animals were anaesthetized by a lethal dose mixture of ketamine and xylazine and perfused through the heart with ice-cold cutting solution containing (in mM): 240 sucrose, 2.5 KCl, 1.25 NaH_2PO_4, 25 $NaHCO_3$, 0.5 $CaCl_2$, and 7 $MgCl_2$. Prior to use, the cutting solution was saturated with 95% O_2–5% CO_2 and frozen. Both hemispheres were quickly removed and coronally sliced at 300–350 μm thickness using a HM 650V microtome (Microm International GmbH). After incubation in a holding chamber containing normal aCSF for at least 30 min at room temperature, slices were transferred into the recording chamber. The bath solution (aCSF) contained (in mM): 125 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 25 $NaHCO_3$, 2.0 $CaCl_2$, 1.0 $MgCl_2$, and 15 dextrose. All recordings were conducted at 31–33°C with a perfusion speed of 1.5–2 ml min^{-1}.

A Zeiss Axioskop 2 FS, fitted with ×40 water-immersion objective and differential interference contrast (DIC), was used to view slices. Light in the near infrared range (740 nm), in conjunction with a contrast-enhancing camera, was used to visualize individual neurons.

Electrophysiological recordings and synaptic stimulations

A Dagan 700A and an Axopatch 200B amplifier were used for whole-cell current- and voltage-clamp recordings, respectively. All recordings were made from neurons located in the prelimbic region of PFC, with recording pipettes (4–8 MΩ) containing (in mM): 120 potassium gluconate, 20 KCl, 10 Hepes, 0.2 EGTA, 2 Mg_2Cl, 4 Na_2ATP, 0.3 Tris-GTP, and 14 phosphocreatine (pH 7.25 with KOH). The hippocampal axonal bundle was stimulated electrically with a glass microelectrode (1–3 MΩ) filled with aCSF and controlled by a fine micromanipulator at a resolution of 1 μm. The distance between the recording and stimulus electrode was between 550 and 700 μm (Parent *et al.* 2009). Pulse generation and data acquisition were controlled with custom software written in the IGOR Pro environment. Test stimuli were delivered every 30 s unless otherwise stated. A hyperpolarizing current pulse was injected into the cell after the test stimulus to monitor the input resistance and series resistance throughout recording. Slope measurements of EPSPs were made from a line fitted to the rising phase of the EPSP. To measure paired pulse facilitation (PPF), two successive synaptic stimuli were delivered and EPSCs recorded. The PPF ratio was calculated as EPSC2/EPSC1 in amplitude.

Drugs

BAPTA tetrapotassium (Molecular Probes) was dissolved directly into the pipette solution. All other drugs were obtained from Tocris Bioscience. Carbachol, pirenzepine (M1 blocker), and CGP52432 were dissolved in distilled water, AF-DX 116 (M2 blocker), picrotoxin, nifedipine in DMSO, and DL-aminophosphonovalerate (APV) in NaOH. These drugs were diluted freshly from frozen stock aliquots. Because of the light-sensitivity of nifedipine, care was taken to minimize light exposure during these experiments.

Statistics

Data were expressed as mean ± S.E. Student's *t* test or ANOVA test was applied.

Results

Activation of mAChR induced long-term depression of synaptic efficacy in a pathway-specific manner

In a modified coronal slice preparation developed recently (Parent *et al.* 2009), we were able to identify hippocampal afferent fibres before they project into the prelimbic region of the mPFC (Fig. 1*A* and *B*). In response to electrical stimulation of these visually identified fibres, pyramidal neurons in layers 2/3 exhibited consistent, monosynaptic excitatory postsynaptic potentials (EPSPs) in whole-cell current clamp mode (Fig. 1*D*), when the resting membrane potential was constantly held at −70 mV. Bath application of the subtype-non-selective

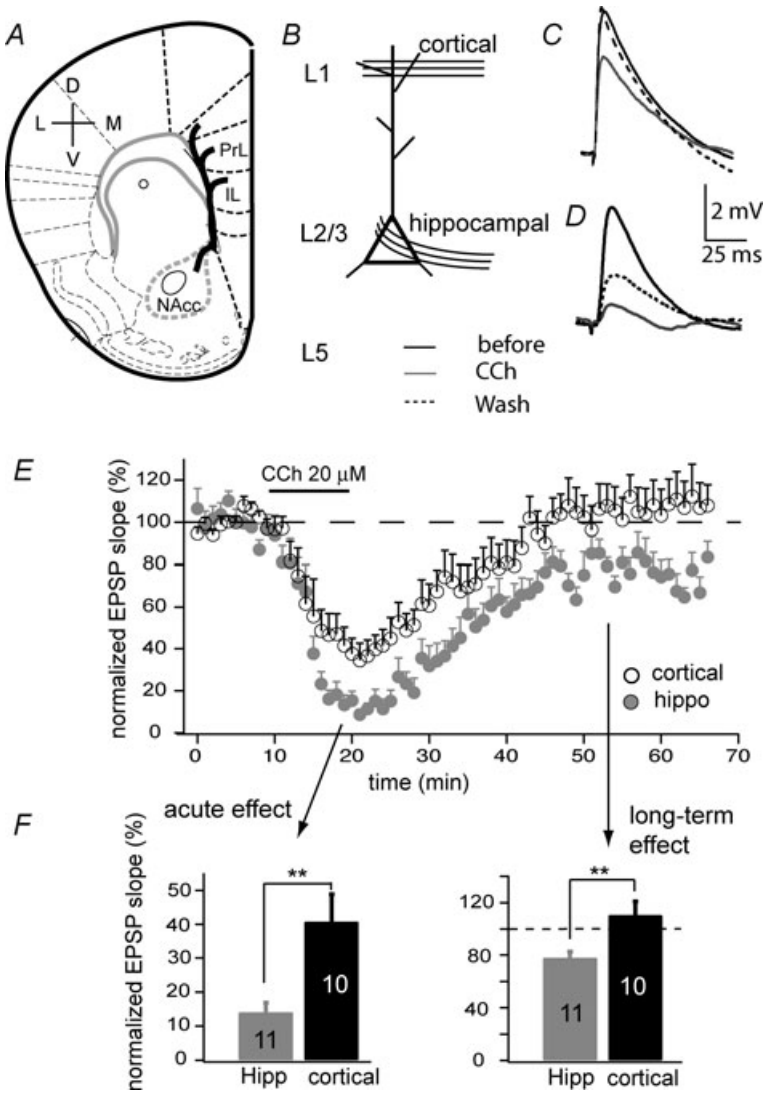

Figure 1. Acute and long-term suppression of EPSPs in the mPFC
A, schematic diagram showing a coronal slice preparation on which hippocampal projections to mPFC were preserved. The bold curvy line indicates the projection trajectory. PrL: prelimbic; IL: infralimbic region. *B*, recording configuration highlighting layer 2/3 (L2/3) pyramidal neurons that receive both cortical inputs at layer 1 (L1) and hippocampal afferents. Selective stimulation of these pathways resulted in input-specific EPSPs (*C* and *D*). *C*, representative cortical EPSPs before, during and 30 min after application of 20 μM carbachol (CCh). *D*, representative hippocampal EPSPs before, during and 30 min after application of 20 μM CCh. *E*, comparison of the time course of CCh-induced EPSP suppression at the cortical and hippocampal pathways. *F*, EPSP suppression at different phases (**$P < 0.01$).

muscarinic agonist carbachol (CCh, 20 μM) for 10 min suppressed the EPSP slope to $17.2 \pm 3.3\%$ of control ($n = 11$). Following washout of the agonist, EPSPs recovered partially; 40–45 min after removing CCh, late phase synaptic responses were $76.6 \pm 5.7\%$ of the original (Fig. 1*D* and *E*). Since layer 5 pyramidal neurons also receive hippocampal inputs, we tested how the hippocampal–layer 5 pathway responded to CCh. Similar to layer 2/3 terminating fibres, hippocampal fibres terminating on pyramidal neurons in layer 5 exhibited acute reduction (to $10.1 \pm 3.4\%$) in response to CCh and long-lasting depression (to $75.9 \pm 5.7\%$, $n = 4$) when CCh was washed out. We focused on layer 2/3 neurons for the rest of the studies.

This ~25% long-term depression of the hippocampal synapses in mPFC, induced by a brief CCh exposure, showed a similarity in kinetics and amplitude to CCh-LTD as observed in other cortical regions (McCoy & McMahon, 2007). In addition to synaptic changes, CCh application also depolarized membrane potential by 5–10 mV and increased firing frequency in response to a current injection of 700 ms (data not shown), consistent with previous reports of the muscarinic modulation of membrane excitability (Carr & Surmeier, 2007). Unlike the long-term suppression of synaptic responses, effects on intrinsic membrane properties were reversible.

In addition to pyramidal neurons, muscarinic receptors are expressed in interneurons (Hajos *et al.* 1998). Although the waveform of synaptic responses recorded in the absence of GABA receptor blockers did not show obvious contributions from inhibitory postsynaptic responses (IPSPs), we cannot rule out the possibility that the long-lasting suppression of the synaptic events is due to the indirect effects of CCh on GABAergic transmission. To test this possibility, we next performed a similar experiment in the presence of $GABA_A$ and $GABA_B$

receptor blockers picrotoxin (20–50 μM) and CGP52432 (3 μM). Under this condition, transient exposure to CCh still suppressed EPSPs to 69.0 ± 6.0% of baseline responses ($n = 5$, $P < 0.05$). The kinetics and degree of CCh-LTD showed no significant difference from previous observations when no blockers were included ($P = 0.4$). These results suggest that inhibitory activity does not contribute directly to the formation of CCh-LTD at hippocampal–mPFC glutamatergic synapses.

In this slice preparation, we were also able to selectively activate fibres from other cortical regions by positioning the stimulus electrode in mPFC layer 1 (Fig. 1*B*). Laminar stimulation in other layers within the mPFC may activate a mix of local network and extrinsic inputs. We first examined separately, and then combined, the muscarinic modulation data for these two conditions because results were similar. Specifically, CCh application elicited a quick reduction of EPSPs (to 37.4 ± 7.0%, $n = 10$; Fig. 1*C* and *E*). However, unlike the long-lasting depression induced at the hippocampal pathway, EPSPs evoked by laminar stimulation recovered completely within 30 min of CCh washout (Fig. 1*E* and *F*).

Taken together, the hippocampal–mPFC synapses exhibit input-specific LTD that is induced by activation of muscarinic receptors but not dependent on inhibitory activity.

Pathway-specific depression was accompanied by presynaptic changes

Hippocampal inputs projecting to either layer 2/3 or layer 5 of the mPFC exhibited CCh-induced LTD. One common feature shared by these two pathways is their presynaptic origin. To address whether any presynaptic mechanisms were involved in the synaptic suppression, we recorded synaptic currents, in voltage clamp mode, in response to two consecutive stimulations with an interval of 40 ms (Fig. 2*B* and *C*). During the brief 10 min exposure to CCh, EPSCs were heavily suppressed, making it unreliable to measure the paired pulse facilitation (PPF) ratio. We thus evaluated the PPF ratio prior to CCh application and 40 min after CCh removal (Fig. 2*A*).

EPSCs from the hippocampal pathway generally exhibited a PPF ratio of 1.6 ± 0.1 ($n = 5$). EPSCs recovered but not completely 30–45 min after CCh was washed out, consistent with what we have observed in EPSP recordings. Furthermore, the decreased EPSCs were accompanied by an increased PPF ratio (2.0 ± 0.1, $P < 0.05$; Fig. 2*D*). EPSCs recorded from activation of non-hippocampal pathways, i.e. laminar stimulation, did not show changes in the PPF ratio ($n = 6$; Fig. 2*E*). An increased PPF associated with the sustained phase at the hippocampal–mPFC pathway suggests that presynaptic mechanisms may contribute to the CCh-LTD.

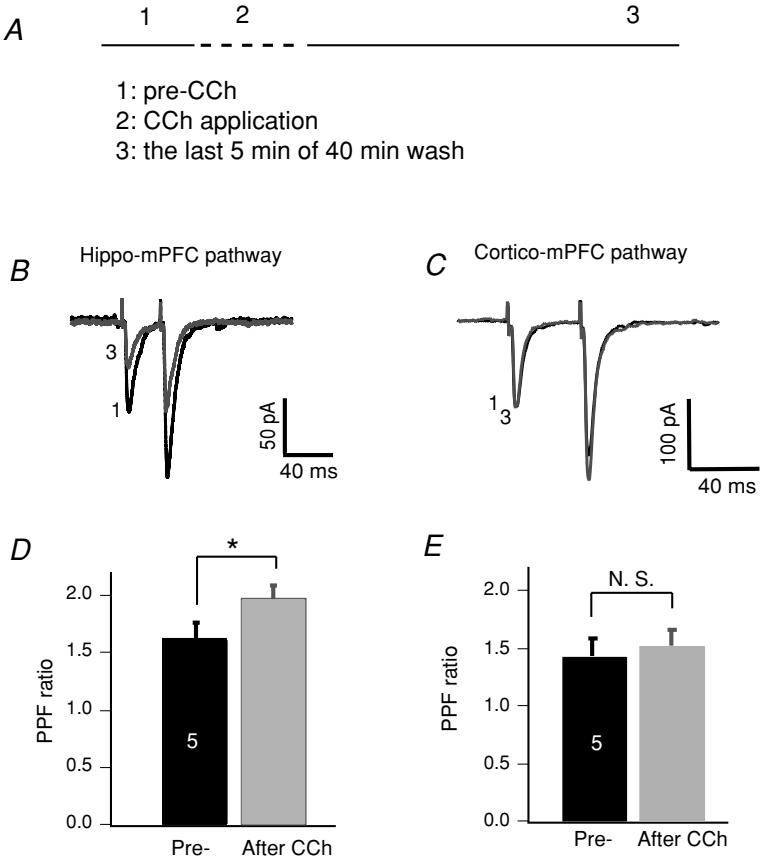

Figure 2. Changes in PPF ratio associated with the hippocampal pathway
A, experimental procedure showing three phases: pre-CCh, CCh application and CCh washout. *B*, averaged EPSC traces in response to two consecutive stimulations of the hippocampal–mPFC pathway, taken from phases 1 and 3, were superimposed. Note that the first EPSC remained suppressed after CCh was washed out. *C*, traces of EPSCs in response to stimulation of cortical pathways. Note that the first EPSC was completely recovered from CCh-induced acute depression. *D*, comparison of the PPF ratio of hippocampal synapses before CCh application and after CCh washout (**$P < 0.01$). The PPF ratio was calculated as EPSC2/EPSC1. *E*, comparison of the PPF ratio of cortical/local circuitry synapses before CCh application and after CCh washout (N. S.: non-significant).

The role of M2 AChR in synaptic depression of hippocampal–mPFC pathway

Previous work has demonstrated that M1 AChR mediates CCh-induced LTD in the hippocampus (Scheiderer *et al.* 2006) and cortex (Kirkwood *et al.* 1999; Massey *et al.* 2001), possibly through postsynaptic mechanisms. With the data presented above suggesting the involvement of presynaptic mechanisms, we wondered which subtype(s) of muscarinic receptors contribute to CCh-induced LTD in the hippocampal–mPFC pathway.

To this end, we combined CCh with receptor subtype-selective antagonists to dissect mechanisms underlying acute *vs.* long-lasting depression induced by CCh (Fig. 3). In the presence of M1-selective blocker pirenzepine (2 μM), the CCh-induced acute reduction in EPSP slope was attenuated from the CCh-only condition but still significant (to $48.4 \pm 6.8\%$ of baseline transmission, $P < 0.05$, $n = 7$). However, pirenzepine did not appear to block or attenuate CCh-LTD. Under this condition, synaptic responses in the late phase remained suppressed to $69.0 \pm 5.4\%$ of baseline ($n = 7$, $P < 0.05$) and the degree of suppression was comparable to that in the absence of pirenzepine ($P = 0.36$). On the contrary, the M2-selective blocker AF-DX 116 (2 μM) completely blocked CCh-induced LTD. Specifically, in the presence of AF-DX 116, CCh acutely reduced the EPSP slope to $44.7 \pm 5.6\%$ of baseline response but EPSPs were completely recovered to $110.6 \pm 11.7\%$ after CCh was removed ($n = 7$). M1 and M2 blockers together completely eliminated both the acute and the long-term effects of CCh on EPSPs (Fig. 3).

It has been suggested that pirenzepine at 2 μM may block M4 receptors in addition to M1 (Dorje *et al.* 1991; Marino *et al.* 1998). We thus tested whether a lower concentration would show different effects on the acute depression induced by CCh. Co-application of CCh and 75 nM pirenzepine reduced the EPSP slope to $24.5 \pm 3.8\%$ of baseline within 15 min ($n = 3$). This degree of acute synaptic suppression was indistinguishable from that induced by CCh alone ($P = 0.37$), but significantly larger than that caused by the combination of CCh and 2 μM pirenzepine ($P < 0.05$). The difference in attenuating acute suppression between the two concentrations may indicate the possible involvement of M4.

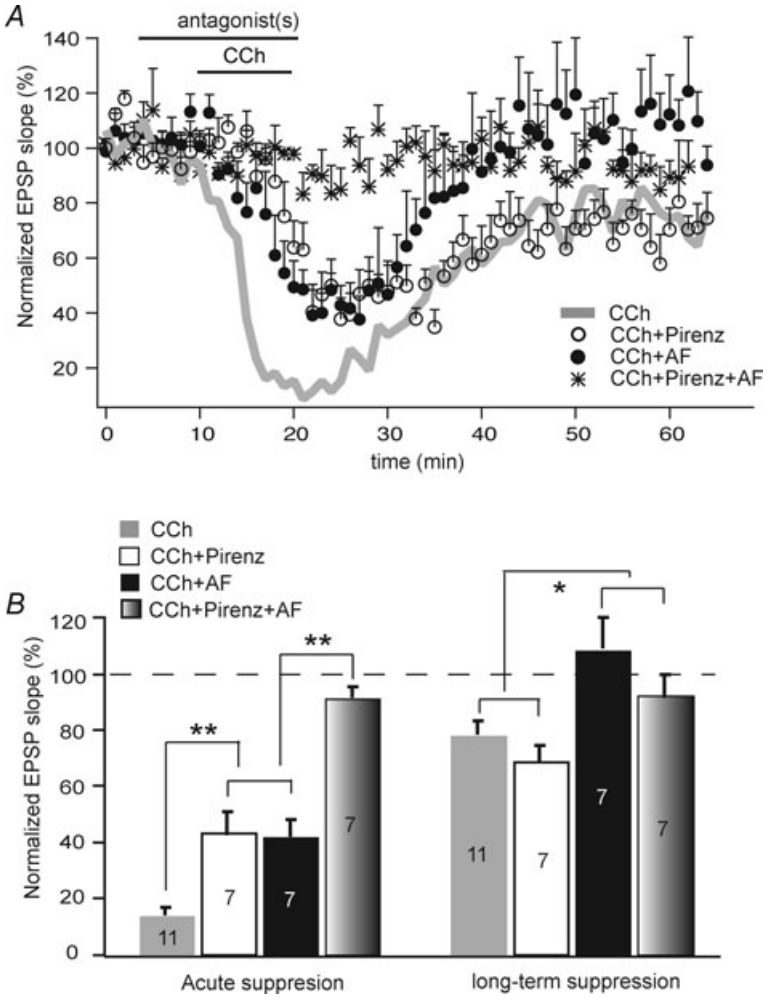

Figure 3. Involvement of different subtypes of mAChR in various phases of CCh-induced synaptic suppression of the hippocampal pathway
A, time course of changes in EPSP slope of the hippocampal synapses under various conditions: CCh alone, CCh + M1 antagonist pirenzepine (2 μM), CCh + M2 antagonist AF-DX 116 (2 μM), and CCh + M1 + M2 antagonists. *B*, summarized EPSP reduction in both acute and sustained phases (*$P < 0.05$; **$P < 0.01$).

Taken together, these data suggest that multiple muscarinic receptors may contribute to the acute suppression of synaptic transmission; however, M2 receptors appear solely responsible for CCh-induced long-term depression.

Synaptic activity is required for CCh-LTD during mAChR activation

Is activation of M2 the only mechanism that is required to establish CCh-induced LTD? One intriguing phenomenon attracted our attention while pursuing the mechanisms of CCh-mediated LTD. We noticed that certain levels of synaptic activity during CCh application at the hippocampal–mPFC pathway were required to induce LTD.

As stated in Methods, we normally delivered electrical stimulation to the hippocampal afferent fibres every 30 s (i.e. 0.033 Hz) to obtain EPSPs throughout the course of an experiment. To assess the role of activity in CCh-LTD, we next halted electrical stimulation only during the 10 min of CCh application. We recorded EPSPs immediately before and after application; the degree of acute reduction in EPSP slope, in the absence of concurrent electrical stimulation, was similar to that of the normal condition. Contrarily, LTD was completely prevented by the lack of concurrent stimulation. The EPSP slope after CCh washout was not different from the pre-drug level (98.6 ± 5.5, $n = 5$, $P > 0.05$; Fig. 4*A*).

How does concurrent stimulation contribute to CCh-LTD? Electrical stimulation during CCh application evokes glutamate release that then activates NMDA and AMPA receptors. Furthermore, AMPARs mediate EPSPs in dendrites that activate L-type Ca^{2+} channels, the low-threshold VGCCs (Magee & Johnston, 1995). Thus, we questioned whether blockade of NMDA receptors (NMDARs) or L-type Ca^{2+} channels would prevent CCh-induced LTD despite the presence of concurrent stimulation.

In the presence of the NMDAR blocker APV (50 μM), EPSP was still suppressed by CCh to $33.6 \pm 5.5\%$ and remained at $73.4 \pm 9.6\%$ ($n = 5$) after CCh was washed out, indicating little involvement of NMDAR (Fig. 4*B*). The L-type Ca^{2+} channel blocker nifedipine at 10 μM did not alter the degree of acute synaptic depression by CCh ($38.6 \pm 5.9\%$). However, the long-term suppression after CCh washout was completely blocked, i.e. EPSP was recovered to $101.0 \pm 9.8\%$ of control level ($n = 6$) in the presence of nifedipine (Fig. 4*C*). These results suggest that Ca^{2+} influx through L-type Ca^{2+} channels, but not NMDARs, is required to establish the long-term suppressive effects of CCh.

Since the neuronal responses suppressed by CCh were mainly subthreshold individual EPSPs, with no involvement of action potentials throughout the recording, we hypothesized that the amount of Ca^{2+} influx through L-type channels may be moderate, thus a moderate concentration of BAPTA may be able to chelate intracellular Ca^{2+} changes. We included 5 mM BAPTA in the pipette solution and then induced CCh-LTD. While the acute suppression of hippocampal transmission persisted, $101.0 \pm 7.4\%$ recovery of EPSP was observed in the late phase ($n = 5$), indicating that chelating intracellular Ca^{2+} with BAPTA blocked the CCh-LTD.

Discussion

The present results reveal input-specific modulation of hippocampal synapses in response to muscarinic receptor activation in the mPFC region. Specifically, these synapses exhibit acute and long-term depression in the presence of, and after the removal of, mAChR agonists, respectively. While multiple muscarinic receptors may be involved in the acute phase, only M2 receptors appear to mediate the long-term suppressive effect. On the contrary, synapses that originate from cortical projections or the local circuitry are modulated by muscarinic activation only in an acute way.

What are the mechanisms of long-term muscarinic suppression (CCh-LTD) at the hippocampal–mPFC synapses? We present several lines of evidence suggesting that CCh-LTD is due to a reduction of presynaptic transmitter release via the M2 receptor subtype. First, CCh-LTD was not associated with any changes to input resistance, despite a reversible membrane depolarization, indicating that the suppression has synaptic relevance. Second, although the nearly complete elimination of EPSCs during CCh application prevented the measurement of PPF ratios in the acute phase, PPF measured during the late phase was increased. This observation supports the hypothesis that reduction of hippocampal synaptic responses is probably mediated, at least in part, by reduced presynaptic transmitter release. Finally, complete blockade of the acute suppression of hippocampal EPSP by the combination of subtype-specific antagonists demonstrates the involvement of multiple receptors including M1, M2 and M4 in immediate effects of CCh. However, only application of the M2 antagonist completely abolished the long-term muscarinic suppression, pointing to a central role for M2 receptors.

CCh-LTD has been observed at various synapses in the cortex, including cortex–cortex connections (Massey *et al.* 2001) and layer 4–layer 2/3 synapses (Kirkwood *et al.* 1999; McCoy & McMahon, 2007). However, in the mPFC region, we failed to induce CCh-LTD at synapses originating from cortical projections or the local circuitry. We speculate that a possible lack of presynaptic M2 at these synapses prevents CCh-LTD.

How does a presynaptic M2 receptor regulate neurotransmitter release? M2 receptors have been reported to localize at both cholinergic and glutamatergic presynaptic terminals (Rouse *et al.* 2000; Volpicelli & Levey, 2004). Activation of M2 autoreceptors, which preferentially couple with G_i type of G-proteins, increases the frequency of presynaptic K^+ channel openings (Caulfield *et al.* 1993), inhibits Ca^{2+} channels (Segal, 1989; Bernheim *et al.* 1992; Caulfield *et al.* 1993), and interferes with the presynaptic release machinery by direct protein–protein interactions (Linial *et al.* 1997). All these actions lead to decreased presynaptic release of acetylcholine. The role of M2 receptors localized on non-cholinergic terminals is not well understood due to the difficulty of accessing a specific afferent. Regardless, immunostaining reveals that M2 receptors are present on both excitatory and inhibitory terminals in the hippocampus (Rouse *et al.* 2000). Stimulation of these receptors inhibits glutamate release from hippocampal synaptosomes (Marchi & Raiteri, 1989). An *in vivo* study in which field EPSPs were recorded in the entorhinal cortex in response to stimulation of the piriform cortex (Hamam *et al.* 2007), also supports the notion that neurotransmission can be suppressed by presynaptic cholinergic mechanisms. Although both cholinergic and non-cholinergic synapses can be inhibited presynaptically, it remains speculative whether these effects share the same mechanisms at both types of terminals. Furthermore, we do not understand which signalling pathways, located presynaptically or downstream of M2 receptors, are required to cause long-lasting

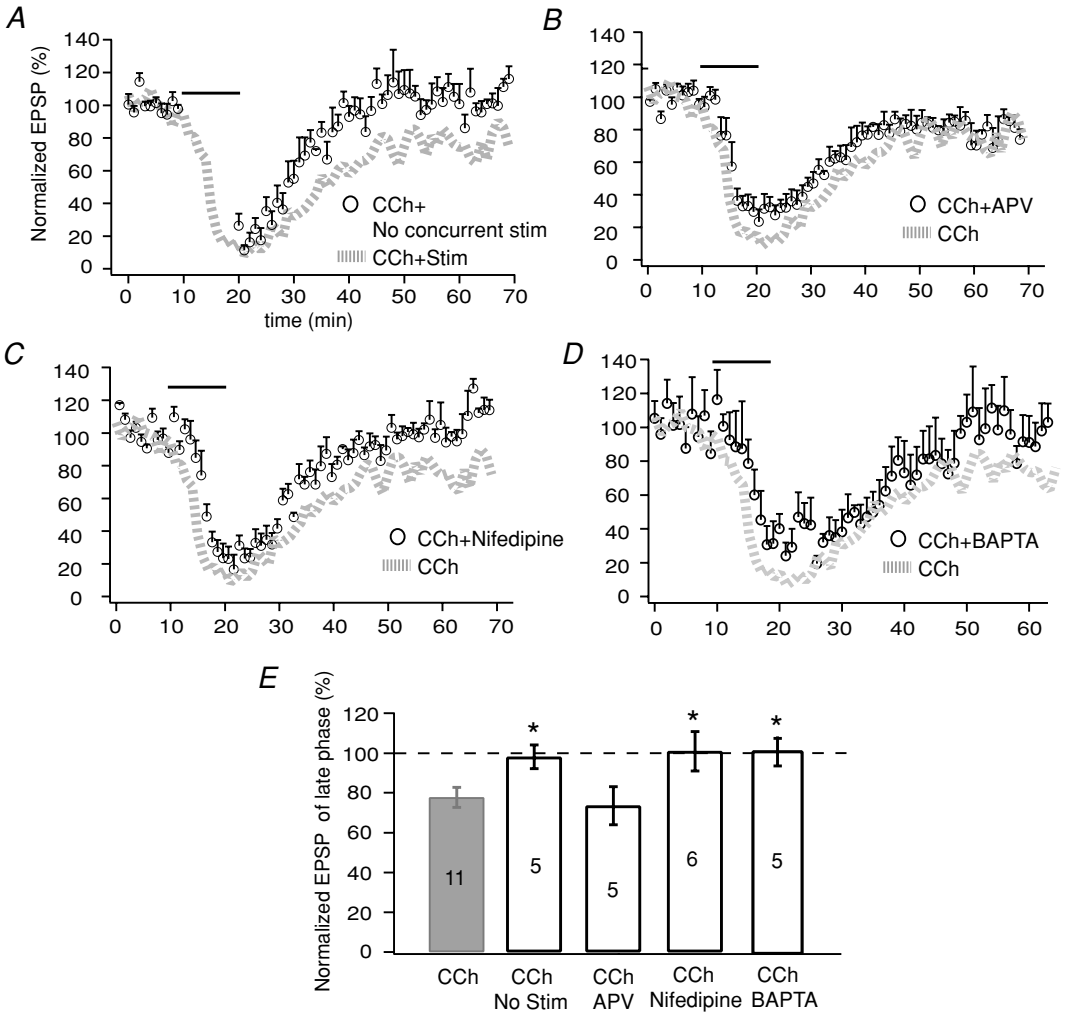

Figure 4. Involvement of synaptic activation in CCh-LTD
A, comparison of CCh effects between two conditions: with and without concurrent synaptic stimulation during 10 min CCh application. *B*, time course of EPSP suppression when APV (50 μM) was co-applied with CCh. *C*, time course of EPSP suppression when nifedipine (10 μM) was co-applied with CCh. *D*, time course of EPSP suppression by CCh when BAPTA (5 μM) was included in recording pipettes throughout the experiment. *E*, summarized data showing the effects of concurrent stimulation, APV, nifedipine, or intracellular BAPTA on CCh-induced LTD. Statistical comparisons were made between each experimental condition and CCh alone (*$P < 0.05$).

depression that goes beyond the initial activation of M2.

While presynaptic cholinergic mechanisms are clearly involved, to some extent, in the induction of CCh-LTD, we do not know what other players or signalling pathways are involved in the expression and maintenance of long-lasting depression at the hippocampal–mPFC pathway. Previous work reveals the activity dependence of CCh-LTD in other brain regions (Kirkwood *et al.* 1999; McCoy & McMahon, 2007); we suspect this is also the case for hippocampal–mPFC synapses. In our experiments, stimulation was normally delivered to the hippocampal afferents at 0.033 Hz. One phenomenon worthy of noting is that when no concurrent stimulation of the afferent fibres was present during the CCh application, the acute suppressive effect of CCh persisted but not the long-lasting one. What mechanism does this observation reveal? It appears that activation of other synaptic components (presynaptic, postsynaptic, or both) is required to achieve CCh-LTD. Our data further suggest that Ca^{2+} influx through L-type Ca^{2+} channels, but not NMDA receptors, represent a postsynaptic component that mediates the observed effects of CCh. In both the visual cortex (McCoy & McMahon, 2007) and the hippocampus (Volk *et al.* 2007; Scheiderer *et al.* 2008), postsynaptic M1 receptor-dependent LTD appears to require ERK1/2 activation and protein synthesis, possibly leading to enhanced endocytosis of cell surface glutamate receptors (Volk *et al.* 2007). It would be of interest to further identify downstream signalling pathways that would eventually lead to and maintain the long-lasting suppression of hippocampal synaptic transmission in mPFC.

The requirement of two simultaneously active mechanisms, i.e. presynaptic acetylcholine binding to M2 receptors and activation of postsynaptic L-type Ca^{2+} channels, suggests the dependence of CCh-LTD on coincidental events. In other words, release of ACh and subsequent binding to presynaptic M2 receptors is not sufficient to achieve sustained reduction of synaptic strength, unless the synapse is also active during the ACh exposure. The significance of M2-mediated selective long-term suppression of hippocampal input to the mPFC and the behavioural consequences of these findings remain to be further investigated.

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Author contributions

L.W. conceived, designed and performed experiments, analysed and interpreted data, and approved the final version of the manuscript. L.-L.Y. conceived and designed experiments, analysed and interpreted data, drafted the manuscript, and approved the final version of the manuscript. All experiments were performed at the University of Minnesota, USA.

Acknowledgements

We thank Marc Parent for technical assistance, and Mu Sun, Paulo Kofuji, Esam El-Fakahany, and Amber Lockridge for comments on the manuscript. This work was supported by the National Institutes of Health (NS049129).