Muscarinic Suppression of Excitatory Synaptic Responses in Layer II of the Entorhinal Cortex

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ABSTRACT

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Shawnna G. Barrett

The entorhinal cortex is thought to play a role in mechanisms mediating sensory and mnemonic function, but the effects of acetylcholine on the strength of sensory cortical inputs to the entorhinal cortex are not well understood. We have previously shown that field excitatory postsynaptic potentials (fEPSPs) in the medial entorhinal cortex evoked by stimulation of the piriform cortex are suppressed during theta activity in behaving animals, and that cholinergic agonism suppresses fEPSPs in vivo. In addition, intracellular recordings from neurons in layer II of the entorhinal cortex also show a suppression of EPSPs in response to the cholinergic agonist carbachol. Here, we have used in vitro field potential recordings evoked by stimulation of layer I afferents to investigate the transmitter receptors that mediate the cholinergic suppression of synaptic responses in layer II of the medial entorhinal cortex. Ten-min bath-application of the cholinergic agonist carbachol (10 µM) potently suppressed the amplitude of fEPSPs. Carbachol also enhanced the paired-pulse facilitation ratio for EPSP amplitudes (30 ms interpulse interval), indicating that the cholinergic suppression is likely due to inhibition of transmitter release. Constant bath application of the M₂ receptor blocker methoctramine (5 µM) for 20 min prior to addition of carbachol did not prevent the cholinergic suppression, but application of the M₁ receptor blocker pirenzepine (1 µM) almost completely blocked the carbachol-induced suppression, indicating that the cholinergic suppression of excitatory synaptic responses in the entorhinal cortex is
dependent primarily on activation of $M_1$–like receptors. In addition to enhancements in neuronal excitability that follow cholinergic activation, therefore, cells in the superficial layers of the entorhinal cortex also display a suppression of excitatory synaptic input that is mediated mainly by $M_1$ muscarinic receptors.
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<tr>
<td>ACSF</td>
<td>artificial cerebral spinal fluid</td>
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<td>fEPSP</td>
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INTRODUCTION

Neurons in layer II of the entorhinal cortex receive synaptic inputs from neurons in multiple sensory areas including the perirhinal and piriform cortices, and they also provide the hippocampal formation with its largest cortical sensory input (Burwell and Amaral, 1998). The major inputs from sensory and associational cortices to layer II of the entorhinal cortex suggest that the entorhinal cortex plays a major role in the sensory functions of the medial temporal lobe. The strong interconnections between the entorhinal cortex and the hippocampal formation suggests that the entorhinal cortex may also play a major role in the mnemonic functions associated with the hippocampal formation (Witter et al., 1989). There has also been a strong, growing interest in the role of the entorhinal cortex in spatial navigation that has followed the discovery of “grid cells” in the dorsolateral portion of the medial entorhinal cortex; grid cells fire in a manner that is dependent on the animal’s spatial location, such that maximal firing occurs at points that may be described as the vertices of a grid of equilateral triangles (Moser and Moser, 2008). The activity of cells in the entorhinal cortex are strongly affected by neuromodulatory transmitters including dopamine (Caruana et al. 2006, 2007, 2008), norepinephrine (Lei et al., 2007), and acetylcholine (Hasselmo, 2006; Richter et al., 1999; Hamam et al., 2007) which in turn affects the function of the entorhinal cortex. Acetylcholine has strong excitatory effects on neuronal excitability and firing (Klink and Alonso, 1997b; Egorov et al., 2002), and also has a strong suppressive effect on the strength of excitatory inputs to layer II of the entorhinal cortex (Hamam et al., 2007; Richter et al., 1999), but the cholinergic receptors involved in the suppression of EPSPs within layer II of the medial entorhinal cortex has not been well characterized.
The entorhinal cortex generates theta-frequency (4-12Hz) electroencephalographic (EEG) activity that is dependent on cholinergic inputs from the medial septum (Mitchell and Ranck, 1980; Alonso and Garcia-Austt 1987a,b; Dickson et al., 1994, 1995, 2000). Theta activity occurs in the entorhinal cortex and hippocampus of the rat during active behaviors, while periods of large amplitude irregular EEG activity and desynchronized EEG activity are observed during behavioral immobility and automatic behaviors such as grooming (Bland, 1986; Bland and Colom, 1993; Bland, 2004; Hasselmo, 2006). “Type 1” theta activity occurs during behavioral activity and persists during the blockade of muscarinic cholinergic receptors, but “Type 2” theta activity which is observed during awake immobility, is dependent on cholinergic transmission and may co-occur during type 1 theta associated with movement (Kramis et al., 1975; Bland et al., 2006). Because theta activity occurs during movement and the active processing of sensory stimuli, it is possible that the rhythmic synchronous nature of neuronal activity during theta may contribute to sensory processing by enhancing transmission through the circuitry of the hippocampal formation (Bland and Oddie, 2001; Bland, 2004; Winson and Azbug, 1978). Theta activity may also contribute to mechanisms of learning and memory because the synchronization of synaptic activity associated with theta activity may enhance postsynaptic depolarization and promote activity-dependent synaptic plasticity (Buzsaki, 2002; Vertes, 2005). “Theta burst” stimulation protocols that provide intense synaptic stimulation at theta-frequency are highly effective at inducing long-term potentiation in the hippocampus and entorhinal cortex (Yun et al, 2000; Staubli and Lynch, 1987), and single stimulation pulses that are timed to arrive during either the peak or trough of the theta rhythm are sufficient to
induce long-term potentiation and depression, respectively (Auerbach and Segal, 1996). Theta is also likely to contribute to mechanisms of spatial navigation because theta occurs during behavioural activity and place-dependent cells in areas of the hippocampal region fire in a way that is dependent in part on the phase of theta activity (Taube, 1995, Jeffery and Hayman, 2004; Buzsaki, 2005).

Cholinergic inputs to the entorhinal cortex and hippocampus are thought to contribute to the generation of theta activity in several ways. The medial septum contains both cholinergic and GABAergic neurons that project to the entorhinal cortex and hippocampus, and theta activity in these structures is known to be dependent in part on these inputs which can promote the depolarization and synchronization of principal neurons (Bland and Colom, 1993; Buzsaki, 2002; Klink and Alonso, 1997a; Cobb et al., 1995; Toth et al., 1997; Chapman and Lacaille, 1999a,b). Muscarinic receptor activation depolarizes the membrane potential of neurons in the hippocampus and entorhinal cortex (Benardo and Prince, 1982; Klink and Alonso, 1997b; McCormick and Prince, 1986), and this depolarization can lead to the generation of theta-frequency oscillations in membrane potential of principal neurons that are driven by an interaction of voltage-dependent conductances (Glasgow and Chapman, 2007; Glasgow and Chapman, 2008). In addition, the depolarization of local inhibitory interneurons by cholinergic inputs can also result in theta-frequency oscillations in the membrane potential of inhibitory neurons (Glasgow and Chapman, 2007). Because single inhibitory neurons can contact many principal neurons, the theta-frequency firing in inhibitory neurons can provide repetitive and synchronous inhibition of widespread populations of principal neurons, and this can reset the firing and oscillatory activity of principal cells to contribute to the synchronization of
theta-frequency (Glasgow and Chapman, 2007; Chapman and Lacaille, 1999a). Thus, cholinergic inputs to the entorhinal cortex may promote theta activity by depolarizing neurons and increasing the degree of synchronous firing among neurons.

In contrast to the excitatory effects of acetylcholine on membrane potential and neuronal excitability, activation of cholinergic inputs to both the hippocampus and entorhinal cortex generally result in a suppression of excitatory synaptic transmission. In the hippocampus and entorhinal cortex, increases in cholinergic tone during theta activity in freely-moving animals, and during application of cholinergic agonists in acute brain slices, is associated with a suppression of excitatory synaptic responses (Hamam et al., 2007; Hasselmo and Schnell, 1994; Leung, 1980; Wyble et al., 2000; Yun et al., 2000; Glasgow, 2011). Studies in behaving animals have shown that theta leads to a suppression of both the monosynaptic EPSP (Hargreaves et al., 1990) and the population spike evoked in the dentate gyrus by perforant path stimulation (Hargreaves et al., 1990; Buzsaki et al., 1981). Theta is also associated with a suppression of the EPSP (Segal, 1978; Leung, 1980; Herreras et al., 1988; Wyble et al., 2000) and population spike in the CA1 region (Leung, 1980; Herreras et al., 1988). In a study of fEPSPs evoked in the entorhinal cortex by stimulation of piriform cortex inputs, Hamam et al (2007) found that theta activity in behaving animals was associated with a suppression of entorhinal fEPSPs, and that the amplitude of entorhinal fEPSPs was also suppressed by systemic administration of the cholinergic agonist physostigmine. Hamam et al. (2007) also showed that the cholinergic agonist carbachol suppressed the amplitude of both field and intracellular EPSPs in layer II neurons of the medial entorhinal cortex. Several other studies of synaptic responses in the entorhinal cortex in vitro have also found that
carbachol results in a suppression of fEPSPs in layers III and V (Yun et al., 2000; Cheong et al., 2001) and suppresses fEPSPs in layer II of the medial entorhinal cortex evoked by layer V inputs (Richter et al., 1999).

The cholinergic suppression of EPSPs has usually been attributed to reductions of presynaptic transmitter release rather than to a reduced postsynaptic response to activation of receptors (Auerbach and Segal, 1996; Hasselmo and Schnell, 1994; Hounsgaard, 1978; Valentino and Dingledine, 1981). For example, the cholinergic suppression of EPSPs in layer II entorhinal neurons observed in the study of Hamam et al. (2007) was associated with an enhancement of the paired-pulse facilitation ratio which is indicative that the suppression is due to a reduction in presynaptic transmitter release; suppression of transmitter release can lead to increased paired-pulse facilitation due to an enhancement in the pool of readily releasable transmitter during the response to the second stimulation pulse. The most likely mechanism through which glutamate release may be suppressed is by actions on presynaptic voltage-gated calcium channels that gate transmitter release in response to action potential invasion of the terminal, and muscarinic cholinergic receptors have been reported to modulate voltage-gated calcium currents (Qian and Saggau, 1997; Toselli and Taglietti, 1995). Therefore, acetylcholine may suppress transmitter release by presynaptic inhibition of voltage-dependent calcium currents.

Although the suppression of EPSPs by cholinergic receptor activation has usually been attributed to M₁ receptors, there have been several reports that provide some evidence for the involvement of M₂ receptors. The cholinergic suppression of excitatory synaptic transmission in layer V inputs to layer II of the entorhinal cortex is blocked by
the M₁ receptor antagonist pirenzepine (Richter et al., 1999), and M₁ receptors are also thought to mediate the cholinergic suppression of EPSPs in the CA1 region (Auerbach and Segal, 1996; Sheridan and Sutor, 1990). However, an early report by Dutar and Nicoll (1988) stated that gallamine, a muscarinic receptor antagonist with a higher affinity for M₂ versus M₁ receptor subtypes, effectively blocks the cholinergic suppression of EPSPs in the hippocampal CA1 region. In addition, although the cholinergic suppression of EPSPs in the CA1 region is markedly reduced in M₁ receptor knock-out mice, the presence of a small suppression in these mice suggests that other muscarinic receptor subtypes also mediate a suppression of EPSPs (Kremin et al., 2006). Additional work using knock-out mice has shown that the cholinergic suppression of EPSPs in the CA1 region is greatly reduced in mice lacking either the M₁ or M₄ receptor, suggesting that the M₄ receptor, which is part of the M₂-like receptor family, may contribute (Dasari and Gulledge, 2011).

In the present study, we used field potential recordings from acute brain slices maintained in vitro to examine the muscarinic receptors that mediate the suppression of fEPSPs in layer I inputs to layer II of the medial entorhinal cortex induced by the cholinergic agonist carbachol. While the suppression of synaptic responses in the entorhinal cortex has been well established and is likely due to actions on presynaptic mechanisms of transmitter release, (Yun et al 2000; Cheong et al 2001; Hamam et al, 2007) the dependence of the suppression of layer I inputs to layer II of the medial entorhinal cortex on muscarinic receptors has not been tested definitively, and the mechanisms of the suppression are not yet determined. Constant bath application of the M₁ and M₂ receptor antagonists pirenzepine and methoctramine were used to assess the
involvement of M₁ and M₂ receptors in the cholinergic suppression of synaptic transmission. In addition, responses to paired-pulse stimulation were used throughout to assess the dependence of the suppression of EPSPs on

In Vitro Slice Preparation

The methods for slice preparation have been described in detail in previous reports (Glasgow and Chapman, 2007; Glasgow and Chapman, 2008), and were conducted in accordance with the guidelines of the Canadian Council on Animal Care. Acute brain slices were obtained from 5 to 7-week old rats that were anesthetized using halothane and decapitated. The brain was extracted and submerged in ice-cold ACSF (4 °C) containing (in mM): 124 NaCl, 5 KCl, 1.25 NaH₂PO₄, 2 MgSO₄, 2 CaCl₂, 26 NaHCO₃, 10 dextrose, L-ascorbic acid (0.4 mM), uric acid (0.35 mM) and indomethecine (40 µM) saturated with 95% O₂ and 5% CO₂ (pH ~7.3; 300-310 mOsm). Horizontal slices (400 µM thick) were cut using a vibratome (WPI, Vibroslice NVSL), and allowed to recover in room temperature (~22°C) ACSF for ~1.5h. Individual slices were then transferred to a nylon net in a temperature-regulated gas-fluid interface chamber (Fine Science Tools). The upper surface of the slice was exposed to a humidified 95%/5% O₂/CO₂ atmosphere, and the chamber was perfused with oxygenated ACSF at a rate of 1.5 – 2.0 ml/min at 32 ± 0.5°C. There was a recovery period of 20 min before recordings.

Stimulation and Recording

Field potential recording electrodes were pulled from borosilicate glass (1.0 mm OD) using a horizontal puller (Sutter Instruments, P97), and were filled with ACSF (2-6 MΩ). Electrodes were positioned with the aid of a dissecting microscope (Leica, MS5)
and the field potential recording electrode was placed in layer I near the border of layer II at a depth of roughly 200µm below the surface of the slice. Synaptic responses were evoked with a fine concentric bipolar electrode (FHC) placed in layer I, 0.4 to .8 mm rostral to the recording electrode. Cathodal constant current pulses were delivered using a stimulus generator (WPI, Model A300) and a stimulus isolation unit (Model A360). Evoked fEPSPs were filtered and amplified (DC-3 kHz, Axon Instr., Axoclamp 2B) and digitized (20 kHz, Axon Inst., Digidata 1322A) for storage on computer hard disk using the pClamp 8.2 (Axon Instr.) software package. Stimulation intensities were adjusted to evoke fEPSPs with an amplitude of ~65-75% of the maximal response.

To characterize the cholinergic suppression of fEPSPs, synaptic responses were evoked every 20 sec to establish a stable baseline of at least 10-20 min, followed by 10-min constant bath application of the cholinergic agonist carbachol (CCh, 10 µM), and a washout period in normal ACSF for 20 min. The muscarinic receptor subtypes that mediate the cholinergic suppression of fEPSPs were assessed using the M₁–like receptor blocker pirenzepine dihydrochloride (1 µM) and the M₂–like receptor blocker methoctramine (5 µM). Following a stable baseline in normal ACSF, the antagonist was bath applied for 20 min prior to addition of carbachol for 10 min. All drugs were stored as frozen stock solutions and added to ACSF just prior to recordings. All chemicals were obtained from Sigma (St. Louis, MO, USA) except for pirenzipine which was purchased from Ascent Scientific (Princeton, NJ, USA).

The cholinergic suppression of fEPSPs in the entorhinal cortex has been previously found to be associated with an enhancement in the paired-pulse facilitation ratio (Hamam et al., 2007), suggesting that the suppression is due to a reduction in
presynaptic transmitter release (Manabe et al., 1993; Zucker and Regehr, 2002). Changes in paired-pulse facilitation associated with drug application were also monitored here to replicate and verify this result, and to determine if paired-pulse facilitation is modulated by muscarinic receptor antagonists. Tests were conducted using a 30 ms interpulse interval that is known to induce a strong paired-pulse facilitation (Chapman and Racine, 1997b; Kourrich and Chapman, 2003; Hamam et al. 2007).

Data Analysis

Averages of five consecutive evoked field potential responses were obtained for graphical display, and the amplitudes of synaptic potentials recorded before and after drug treatment were measured using the pClamp 8.2 software package (Axon Instr.) and expressed as the mean ± SEM. To assess the effects of cholinergic drugs on fEPSP amplitudes, repeated measures ANOVAs were used to analyse changes in average fEPSP amplitude between the initial 10 min period in normal ACSF, the last 5 min of application of antagonists (for pirenzepine and methoctramine tests), the first 5 min following carbachol application, and the last 5 min of the follow-up period. The effect of adding carbachol to normal ACSF on fEPSP amplitudes was tested initially using a one-way repeated measures ANOVA for the factor Time (normal ACSF, carbachol, wash). The effect of the muscarinic antagonists alone on amplitude of fEPSPs was assessed using a two-way Time (normal ACSF vs. antagonist) by Drug (pirenzepine vs. methoctramine) ANOVA. The effects of muscarinic receptor blockers on the suppression of fEPSPs due to carbachol was assessed using a Drug Group (normal ACSF, pirenzepine, methoctramine) by Time (baseline, carbachol, wash) ANOVA, and the significant interaction effect was investigated using Neuman-Keuls post-hoc tests. To
determine if there was a statistically significant difference in the size of the suppression effects induced by carbachol in normal ACSF versus in the presence of either methoctramine or pirenzepine, more restricted ANOVAs were conducted to assess the significance of the 2x2 interaction between Time (baseline, carbachol) and Drug Group (normal ACSF versus pirenzepine, or normal ACSF versus methoctramine).

Paired-pulse facilitation was quantified by calculating a ratio in which the amplitude of responses evoked by the second of two pulses was expressed as a percentage of responses to the first stimulation pulses (Hamam et al., 2007). Paired-pulse facilitation ratios were compared between baseline, drug, and washout conditions for each drug group using one-way repeated measures ANOVAs of Time (baseline, carbachol, wash). A one-way between-subjects ANOVA was also used to determine if there was a statistically significant difference in the amount of paired-pulse facilitation during baseline recordings in each of the three drug groups. Significant effects were investigated using Neuman-Keuls post-hoc tests.

RESULTS

Stimulation of layer I resulted in large negative-going synaptic field potential components recorded in layer II of the medial entorhinal cortex (e.g., Figure 1A). The effect of cholinergic receptor activation on the evoked responses was assessed using constant bath application of the cholinergic receptor agonist carbachol (10 µM). Following a delay as the drug entered the recording chamber, bath application of carbachol resulted in a strong and reversible suppression of excitatory synaptic transmission in layer I inputs to layer II of the medial entorhinal cortex (Fig. 1). The amplitude of evoked synaptic responses was rapidly suppressed during the 10 min bath-
application to $58.86 \pm 10.18\%$ of baseline levels ($-0.35 \pm 0.04$ versus $-0.65 \pm 0.06$ mV), and reversed after 20 min of washout in normal ACSF ($\text{F}_{2,17} = 7.36$, $p = .006$, N-K, $p = .042$). The suppression of fEPSPs reversed as carbachol was washed off in normal ACSF, and the responses increased to $130.54 \pm 14.25\%$ of baseline values at the end of the follow-up period (N-K, $p = .132$).

The contribution of muscarinic receptors to the suppression of fEPSPs was assessed by adding either the M$_2$ receptor antagonist methoctramine, or the M$_1$ receptor antagonist pirenzepine to the bathing medium prior to addition of carbachol. Adding 1 µM methoctramine to the bath for 20 min prior to addition of carbachol failed to block the suppression of fEPSPs evoked by carbachol ($n = 7$, Figure 2A). Responses were reduced from $-0.63 \pm 0.07$ in the presence of methoctramine to $-0.43 \pm 0.05$ mV with the addition of carbachol. Further, the addition of pirenzepine to the bath prior to carbachol resulted in a smaller reduction in the amplitude of fEPSPs ($n = 7$, Figure 2B). Responses were reduced from $-0.75 \pm 0.07$ in the presence of pirenzepine to $-0.71 \pm 0.08$ mV with the addition of carbachol. The block of the cholinergic suppression by pirenzepine, but not methoctramine was reflected in a significant interaction of Time with Drug Group ($\text{F}_{4, 34} = 4.18$, $p = .007$) associated with significant fEPSP suppression in carbachol versus normal ACSF, and in carbachol versus methoctramine (N-K, $p = .001$, and $p = .035$, respectively) but no significant cholinergic suppression in pirenzepine ($p = .598$). The suppression of fEPSPs returned toward baseline levels in methoctramine and there was no significant difference between the amplitudes of fEPSPs during the baseline and washout periods ($p = .356$).

To determine whether the size of the suppression from baseline to carbachol
differed between the antagonists and ACSF, two 2x2 ANOVAs were conducted. Although the size of the suppression did not differ significantly between the slices bathed with carbachol alone and methoctramine ($F_{1,11}=0.80, p=.391$), there was a significantly smaller suppression induced by carbachol during application of pirenzepine versus in normal ACSF ($F_{1,11}=7.27, p=.021$). Therefore there was a significant reduction in the size of the suppression in the presence of pirenzepine, but no significant difference in the size of the suppression in the presence of methoctramine (Figure 2C).

Comparison of the average amplitudes in normal ACSF in the presence of antagonists showed that responses were larger during application of the antagonists compared to normal ACSF. Responses were increased to 125.3 ± 5.6% of baseline values in pirenzepine, and increased to 124.4 ± 6.8% of baseline values in methoctramine. An ANOVA comparing amplitudes in normal ACSF and antagonists for the pirenzepine and methoctramine conditions showed a significant main effect of antagonists ($F_{1,12}=44.74, p<.001$), but the size of the increase does not depend on which antagonists was applied ($F_{1,12}=0.729, p=.410$).

**Paired-Pulse Facilitation**

Paired-pulse facilitation tests were used to determine if the suppression of fEPSPs was due primarily to reduced transmitter release or changes in postsynaptic mechanisms (Creager et al., 1980; Giocomo and Hasselmo, 2007). The effects of cholinergic receptor activation on paired-pulse facilitation were assessed using the 30 ms interpulse interval that induces maximal facilitation effects in the entorhinal cortex (Hamam et al, 2007), and similar to the findings of Hamam et al (2007) the suppression of fEPSPs induced by application of carbachol in normal ACSF was associated with an increase in paired-pulse
facilitation ratio (Figure 3). The addition of carbachol to normal ACSF resulted in a significant increase in the paired-pulse facilitation ratio from $96.6 \pm 3.1\%$ to $122.92 \pm 8.9\%$ ($F_{2, 10}=4.39, p=.043; \text{N-K, p=.043}$) which was reversible during wash with normal ACSF ($\text{N-K, p=.522}$), suggesting that the suppression of fEPSPs is due to a suppression of presynaptic transmitter release.

Changes in paired-pulse facilitation associated with application of carbachol were also assessed during constant bath application of pirenzepine and methoctramine (Figure 3B). In presence of pirenzepine, in which there was no significant reduction in fEPSPs associated with application of carbachol, there was also no significant change in paired-pulse facilitation ratios following the addition of carbachol ($105.2 \pm 3.9\%$ to $113.4 \pm 4.4\%$; $F_{2,12}=3.37, p=.069$). Consistent with the enhanced paired-pulse facilitation ratios observed during the cholinergic suppression of fEPSPs in normal ACSF, the suppression of fEPSPs observed following the addition of carbachol during constant bath application of methoctramine was also associated with an enhancement of the paired-pulse facilitation ratio, and paired-pulse facilitation was increased from $113.3 \pm 14.8\%$ to $136.8 \pm 15.9\%$ ($F_{2,12}=4.67, p=.032; \text{N-K, p=0.025}$). Note although there was a larger mean in paired-pulse facilitation in methoctramine alone, there was no significant difference in paired-pulse facilitation between the three groups ($F_{2,17}=.76, p=.483$).
amplitude of evoked fEPSPs in the entorhinal cortex *in vitro*. A. Averaged fEPSP recordings (10 sweeps) evoked by stimulation of layer I of the medial entorhinal cortex are greatly attenuated by 10 min bath-application of 10 µM CCh. Responses returned to baseline levels after washout. B. The mean amplitude of fEPSPs among the six slices tested were significantly reduced by carbachol (black bar). Bars represent ± the SEM.
Figure 2. The cholinergic suppression of the amplitude of fEPSPs in the entorhinal cortex is dependent on activation of M₁-like, but not M₂-like, muscarinic receptors. A. Traces in A₁ show averaged representative fEPSPs recorded during constant bath-application of ACSF, the addition of M₂-like receptor antagonist, methoctramine (Methoc, 1 μM), the addition of 10 μM carbachol (CCh), followed by the wash off of carbachol. Group data in A₂ show that the application of the M₂ receptor antagonist methoctramine for 20 min prior to the addition of carbachol failed to block the cholinergic suppression of fEPSPs evoked in control ACSF. B. Sample traces and group data in B₁ and B₂ show that pirenzepine, a M₁ receptor antagonist, was effective in blocking the suppression effect of charbachol. Conventions are as in panel A. C. The histogram shows group averages of fEPSP amplitudes recorded during the first 10 min of the recording period in normal ACSF, the 5 min period prior to the addition of carbachol, the first 5 min after carbachol application, and the last 5 min of the wash period. Asterisks indicate a significant cholinergic suppression of fEPSPs relative to the baseline period for slices tested in normal ACSF or during the antagonist period for slices in the antagonist groups (*, p < 0.05; **, p < 0.01).
Figure 3. The cholinergic suppression of fEPSPs was associated with enhanced paired-pulse facilitation, suggesting that the suppression is expressed presynaptically. A. Representative averaged responses (10 sweeps) to a pair of superficial stimulation pulses separated by an interpulse interval of 30 ms in control ACSF and carbachol (10 μM). Traces from normal ACSF and carbachol have been superimposed and scaled to the amplitude of the first response in control ACSF to assess changes in the amount of paired-pulse facilitation. Note the larger scale amplitude of the second response recorded in carbachol (arrow). B. Group averages of paired-pulse facilitation ratio show a significant enhancement for groups tested in normal ACSF or methoctramine (*p<0.05) but when the suppression due to carbachol was blocked by the presence of pirenzepine, there was no increase in paired-pulse facilitation.
DISCUSSION

Previous work has shown that fEPSPs in layers III and V of the entorhinal cortex are suppressed in vitro by the cholinergic agonist carbachol (Yun et al., 2000; Cheong et al., 2001), and that carbachol also suppresses fEPSPs in layer II of the medial entorhinal cortex evoked by layer V inputs in vitro (Richter et al., 1999). The present study has investigated the muscarinic receptors that mediate the suppression of synaptic responses in layer I inputs to layer II of the medial entorhinal cortex using field potential recordings in acute brain slices. Application of cholinergic agonist carbachol resulted in a marked suppression in fEPSPs that was blocked by the M₁ receptor antagonist pirenzepine but not by the M₂ receptor antagonist methoctramine, indicating that the cholinergic suppression of synaptic responses is mediated by activation of M₁-like receptors. Further, increases in paired-pulse facilitation during application of carbachol suggest that the suppression of synaptic transmission is mediated by pre-synaptic mechanisms that lead to a reduction in neurotransmitter release.

Functional Significance of the Cholinergic Suppression

The medial septum provides cholinergic inputs to the entorhinal cortex and other structures in the hippocampal region during periods of theta EEG activity that are associated with behavioural arousal and exploration (Alonso and Kohler, 1984), and the current results indicate that cholinergic inputs to the entorhinal cortex during theta activity are likely to suppress the responsivity of the entorhinal cortex to inputs from sensory cortices during behaviours associated with theta activity. Behaviours associated with theta EEG activity include movement and active exploration of the environment that are associated with processing of sensory information (Bland, 1986; Buzsaki, 2002), and
the entorhinal cortex is a major temporal lobe structure that receives large converging input pathways from multiple cortical areas including the piriform, perirhinal and postrhinal cortices that carry a great deal of sensory information (Burwell and Amaral, 1998). Although the overall amount of synaptic input to the entorhinal cortex may, therefore, be expected to increase during theta related behavior, the cholinergic suppression of fEPSPs in the entorhinal cortex observed here and by others (Hamam et al., 2007; Hasselmo and Schnell, 1994; Leung, 1980; Wyble et al., 2000; Yun et al., 2000) suggests that the impact of these synaptic inputs are weakened during theta activity. The functional impact of the reduced synaptic strength is unclear, however, because it is has not been determined how the frequency of firing among the synaptic inputs to the entorhinal cortex is affected by theta-related behaviours, and the concurrent muscarinic receptor-mediated depolarization of entorhinal cortex neurons (Alonso and Garcia-Austt, 1987a) may make these neurons more likely to fire in response to synaptic inputs that are received.

An overall suppression of synaptic transmission may also serve to enhance the processing of select sensory input patterns by increasing the signal to noise ratio for the strongest incoming sensory inputs; a reduction of synaptic noise associated with a general suppression of synaptic inputs may enhance the relative strength of the strongest or most salient sensory signals. It has also been proposed that the cholinergic suppression of synaptic transmission among local, intrinsic synaptic connections within the piriform cortex and in the hippocampal formation may prevent new incoming sensory inputs from causing the reactivation of old representations and associations that are held in memory by those intrinsic synaptic connections (Hasselmo and McGaughy, 2004). Thus, the
suppression of synaptic transmission within the entorhinal cortex may contribute to a reduction in the interference between new sensory inputs and the reactivation of representations that are associated with those inputs to the entorhinal cortex.

The cholinergic suppression of synaptic transmission observed here may make the relative timing of synaptic inputs with ongoing theta-frequency EEG activity particularly important with respect to both the effective transmission of synaptic inputs to the entorhinal cortex, and the possible induction of endogenous forms of long-term potentiation. Rhythmic oscillations in membrane potential associated with theta activity are thought to strongly affect the efficacy of synaptic inputs in a manner that depends on the relative phase of the incoming sensory input and the phase of theta, such that inputs that arrive on the hyperpolarized phase of theta may be ineffective in inducing firing while synaptic inputs arriving at the depolarized phase of theta may be more effective (Singer, 1993). Hamam et al. (2007) found that the amplitude of field potential responses in layer II of the entorhinal cortex evoked by piriform cortex stimulation were larger during the trough of theta associated with cellular depolarization, than during the rising phase associated with cellular hyperpolarization. A similar phase-dependent modulation of the strength of synaptic inputs during active exploration could modulate the salience of sensory inputs to the entorhinal cortex. Similarly, the timing with which synaptic inputs arrive relative to ongoing theta activity may also affect the induction of long-lasting changes in synaptic strength, such that synchronous synaptic inputs that arrive during the depolarizing phase of theta activity are most likely to result in levels of postsynaptic depolarization that can contribute to long-term synaptic strengthening (Staubli and Lynch, 1987; Pavlides et al., 1988; Huerta and Lisman, 1995; Chapman and Racine, 1993).
In this way, a sufficient level of synchronized synaptic activity associated with theta activity may promote the induction of learning-related synaptic plasticity, even during a cholinergic suppression of excitatory synaptic transmission.

**Muscarinic Receptors that Mediate the Suppression of EPSPs**

Several previous studies have demonstrated suppression of synaptic responses induced by muscarinic receptor activation in the hippocampal region (Glasgow, 2011; Benardo and Prince, 1982; McCormick and Prince, 1986). The cholinergic suppression of EPSPs has been observed in the entorhinal cortex in the current study as well as by others (Hamam et al., 2007; Richter et al., 1999, Cheong et al., 2001; Yun et al., 2000). It has been a common finding that M<sub>1</sub> receptors mediate the suppression effect in the hippocampus (Hasselmo and McGaughy, 2004; Dasari and Gulledge, 2011; Kremin et al., 2006), and similarly, the suppression effects observed in various pathways of the entorhinal cortex have also been primarily linked to M<sub>1</sub> receptors. Yun et al. (2000) found a reduction in fEPSPs that is dependent on muscarinic receptor activation in layer II inputs to layer III of the entorhinal cortex. Similarly, cholinergic receptor activation leads to a suppression of fEPSPs in layer V evoked by local activation that is blocked by the muscarinic receptor antagonist atropine (Yun et al., 2000). In layer V to II of the medial entorhinal cortex, and in layer II inputs to layer II of the lateral entorhinal cortex, it has been found that application of the M<sub>1</sub> receptor antagonist pirenzepine reduces the cholinergic suppression of fEPSPs (Richter et al., 1999). The present study has examined responses evoked by inputs to layer II from layer I in the medial entorhinal cortex and has also found that pirenzepine blocks the suppression of fEPSPs induced by carbachol.
Pirenzepine is most likely to have resulted in a block of the cholinergic suppression through actions on M₁ receptors. The M₁-like family of receptors includes the M₁, M₃, and M₅ receptor subtypes, while the M₂-like family includes M₂ and M₄ receptors (Dutar and Nicoll, 1988; Caulfield and Birdsall, 1998). Pirenzepine has a strong binding affinity for M₁ receptors, but it can also block the M₂-like M₄ receptors (Dorje et al., 1991). However it is most likely that the block of the cholinergic suppression by pirenzepine is due to a block of the activity of M₁ receptors because higher doses of pirenzepine are required to obtain strong effects on M₄ receptors, and there was not a significant block of the cholinergic suppression in the presence of the M₂ receptor blocker methoctramine, which is known to block both M₂ and M₄ receptors. Therefore, M₄ receptors are not likely to contribute significantly to the suppression effects observed here.

The amplitudes of baseline responses in normal ACSF were increased when either pirenzepine or methoctramine was added to the bathing medium (Figure 2). A similar effect was observed by Glasgow (2011) following application of pirenzepine and 5 uM methoctramine. The increase observed by Glasgow following application of pirenzepine was attributed to a block of a suppressive effect of endogenously released acetylcholine on synaptic responses. A block of M₁ receptors may also contribute to the facilitation effect observed here in pirenzepine, but the size of the increase was similar in both pirenzepine and methoctramine suggesting that both effects may be due in part to slow spontaneous increases in the amplitude of recordings associated with recovery of slices.

The washoff of carbachol in normal ACSF was associated with a reversal of the suppression of fEPSPs, and the amplitude of fEPSPs also rose significantly above baseline levels (Figure 1B). Low concentrations of carbachol can induce a delayed
potentiation of synaptic responses in the hippocampus and entorhinal cortex termed muscarinic long-term potentiation (Auerbach and Segal, 1994; Auerbach and Segal, 1996; Yun et al., 2000). Although such an effect might contribute to the delayed increase in fEPSP amplitudes observed here, a substantial increase was only observed in three of the six cells tested, and a tendency for responses to increase over time was also observed in other recording conditions. The current data, therefore, do not provide strong evidence for a delayed potentiation of responses such as that observed by Auerbach and Segal (1994).

Mechanisms of the Cholinergic Suppression of fEPSPs

Changes in postsynaptic conductances associated with cholinergic activation are unlikely to have contributed substantially to the suppression of fEPSPs observed in the entorhinal cortex. Cholinergic activation of entorhinal layer II neurons can have multiple effects on ionic conductances that can lead to membrane potential depolarization including enhancement of a mixed cationic current (Klink and Alonso, 1997a; Shalinsky et al., 2002). However, a lowering of input resistance is unlikely to have contributed substantially to the reduction in fEPSPs observed here because carbachol has relatively minor effects on input resistance in entorhinal neurons (Klink and Alonso, 1997a; Hamam et al., 2007). Further, although the depolarization of membrane potential induced by carbachol could be expected to reduce EPSP amplitude by reducing the driving force on the EPSP, it has been previously shown that repolarizing entorhinal neurons to the baseline resting potential during bath application of carbachol does not significantly affect the amplitude of intracellular EPSPs.

The changes in paired-pulse facilitation observed here suggest that the suppression
of fEPSPs was due to an attenuation of transmitter release. The enhancement of paired-pulse facilitation was reversible following washoff of carbachol. An increase in paired-pulse facilitation ratio provides evidence that the suppression of fEPSPs is due to a reduction in transmitter release, because reduced transmitter release results in a larger readily releasable pool of transmitter that is available in response to the second stimulation pulse (Valentino and Dingledine, 1981). Increases in paired pulse facilitation were consistently observed in the present study in association with a suppression of fEPSP amplitude when carbachol was applied in either normal ACSF or in the presence of methoctramine. Further, no change in paired-pulse facilitation was observed in the presence of pirenzepine in which there was also no suppression of fEPSP amplitude. Similar increases in paired-pulse facilitation associated with the cholinergic suppression of EPSPs have also been observed in other pathways within the entorhinal cortex (Richter et al., 1999), suggesting that the suppression of transmitter release may be a common mechanism for the muscarinic suppression of EPSPs in the entorhinal cortex.

The mechanisms by which activation of M₁ receptors can suppress fEPSPs by suppressing transmitter release in the entorhinal cortex have yet to be determined. One possibility is that acetylcholine may act on presynaptic M₁ receptors to reduce transmitter release (Sheridan and Sutor, 1990; Kremin et al., 2006). Future research may also benefit from assessing the possible contribution of endocannabinoid signaling. The M₁ family of muscarinic receptors that include the M₁, M₃ and M₅ receptors act through G_Q/11 proteins, whereas the M₂ family, that includes both M₂ and M₄ receptors act via G_i/o proteins (Dutar P, Nicoll RA., 1988; Caulfield and Birdsall, 1998). Muscarinic M₁ receptor-mediated activation of G_Q/11 proteins leads to activation of phospholipase C (PLC) and
the production of IP$_3$ and diacylglycerol (DAG) signaling molecules. In addition, it has also recently been found that DAG may lead to synthesis of the potent endocannabinoid 2-AG. This molecule can act as a retrograde signal that can diffuse out of the postsynaptic cell to activate CB1R receptors on the presynaptic terminal (Wilson and Nicoll, 2001), and it is known that activation of these receptors can lead to reduced transmitter release in hippocampal neurons by reducing activation of voltage dependent calcium channels that control transmitter release (Twitchell et al., 1997). It has been well established that CB1R receptors are present in the entorhinal cortex (Liu et al., 2003), and activation of CB1R and 2R receptors is also well known to result in a suppression of inhibitory synaptic transmission (Morgan et al., 2008) but the contribution of cannabinoid receptors to modulation of excitatory transmission is less clear. The dependence on the suppression of EPSPs on the activation of PLC could be tested by assessing the cholinergic suppression of EPSPs in the cell in which a blocker of PLC is diffused into the postsynaptic cell via a patch clamp recording pipette (e.g., Lin et al., 2004). Further, the effects of CB1R activation on spontaneous and evoked EPSPs, and the necessity of CB1R activation for the cholinergic suppression of EPSPs, could be tested using specific agonists and antagonists for CB1 receptors (Morgan et al., 2008).
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