Presynaptic Inhibition of Excitatory Afferents to Hilar Mossy Cells

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INTRODUCTION

Hilar mossy cells are the only glutamatergic non-principal cells in the hippocampus or dentate gyrus (Soriano and Frotscher 1994). Unlike conventional hippocampal principal cells, hilar mossy cells project largely along the septotemporal axis of the hippocampus where they are thought to innervate both dentate granule cells and GABAergic interneurons (Amaral and Witter 1989; Buckmaster et al. 1992, 1996; Jackson and Scharfman 1996; Scharfman 1995; Schwartzkroin et al. 1990). They also have a strong contralateral projection, are extremely sensitive to excitotoxicity, and are strongly implicated in the etiology of temporal lobe epilepsy (Bekenstein and Lothman 1993; Buckmaster and Dudek 1997; Buckmaster and Jongen-Relo 1999; Buckmaster et al. 1996; Coulter 2004; Jefferys and Traub 1998; Ratzliff et al. 2002; Santhakumar et al. 2005; Sloviter 1991, 1994).

Mossy cells are believed to receive direct monosynaptic excitatory inputs from both dentate granule cells and CA3 pyramidal cells (Coulter 2004; Frotscher et al. 1991; Ishizuka et al. 1990; Penttonen et al. 1997; Ribak et al. 1985). They are thus largely driven by axon collaterals of some of the most heavily studied synaptic pathways in the CNS. For example, the mossy fiber (MF) pathway from dentate granule cells to CA3 pyramidal cells is well known for its tight spatial localization, strong frequency facilitation, clear sensitivity to metabotropic glutamate receptor (mGluR) agonists, possible role as a “detonator synapse,” developmental plasticity of transmitter phenotype, and unusual N-methyl-D-aspartate–independent and presynaptic form of long-term potentiation (for review see Henze et al. 2000; Nicoll and Schmitz 2005; Urban et al. 2001). Collateral projections of mossy fibers to interneuron targets in CA3 have also been extensively studied and, importantly, have shown both anatomical and functional differences when compared with mossy fiber inputs to CA3 pyramidal cells (Lawrence et al. 2004; Lei and McBain 2002; Pelkey et al. 2006; Toth et al. 2000). Nevertheless, at present, very little is known about either the physiology or pharmacology of collateral inputs from either mossy fibers or CA3 pyramidal cells to hilar mossy cells. This represents a notable gap in the literature in that the effect of these inputs on mossy cell function is likely to have profound consequences for both normal and abnormal hippocampal physiology.

One of the first studies to directly address these questions has recently demonstrated presynaptic plasticity of isolated mossy fiber inputs to mossy cells (Lysetskiy et al. 2005). The current study attempts to further efforts in this area by specifically examining both GABAergic and cholinergic mechanisms for presynaptic modulation of excitatory inputs to hilar mossy cells. Our results indicate a prominent role for γ-aminobutyric acid type B (GABA_B)–mediated inhibition of both MF and non-MF inputs to hilar mossy cells and also demonstrate both direct (non-MF) and indirect (MF) mechanisms for cholinergic inhibition. We also provide the first clear demonstration that hilar mossy cells express high-affinity GABA_A receptors capable of responding to changes in ambient GABA and present evidence that in this system, cholinergic changes in ambient GABA are tightly linked to action potential–dependent inhibitory neurotransmission.

METHODS

Hippocampal slice preparation

Male Sprague–Dawley rats, aged 18–25 days postnatal, were given an intraperitoneal injection of ketamine (80–100 mg/kg) and rapidly
were both obtained from Tocris Cookson (Ellisville, MO). Horizontal slices (~300 μm) were cut using a vibratome-sectioning system (Pelco, Redding, CA), incubated for 30 min in ACSF heated to 30–35°C, and finally equilibrated to room temperature. ACSF for sectioning and incubation was saturated with 95% O2–5% CO2 and contained (in mM): 124 NaCl, 2.5 KCl, 1.2 NaH2PO4, 1.5 MgSO4, 10 d-glucose, 1 CaCl2, and 25.9 NaHCO3. All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Florida and adhered to animal welfare guidelines issued by the National Institutes of Health.

Although we noted no age-related variability in our experimental results, mossy fiber projections to CA3 have been shown to undergo developmental changes in transmitter phenotype culminating around postnatal days 22–23 (Gutierrez 2003; Gutierrez et al. 2003). Thus some caution is warranted in generalizing the results of these studies to adults.

**Whole cell recording**

After incubation, slices were transferred to a recording chamber, maintained at 30°C, and perfused at a constant rate of 2 mL/min with ACSF. ACSF was saturated with 95% O2–5% CO2 and contained (in mM): 126 NaCl, 3 KCl, 1.2 NaH2PO4, 1.5 MgSO4, 11.8 d-glucose, 2.4 CaCl2, and 25.9 NaHCO3. Unless stated otherwise, ACSF also contained 50 μM picrotoxin [PTX, a γ-aminobutyric acid type A receptor (GABAAR) antagonist]. Slices were visualized by infrared differential interference contrast (IR DIC) microscopy using an Olympus BX51WI microscope. Whole cell voltage-clamp recordings were performed using micropipettes pulled from borosilicate glass using a Flaming/Brown electrode puller (Sutter P-97, Sutter Instrument, Novato, CA). Internal solutions contained (in mM): 140 CsMeSO4, 1 MgCl2, 3 NaCl, 0.2 Cs-EGTA, 10 HEPES, 4 Na2-ATP, 0.3 Na-GTP, and 5 QX-314 Cl (pH-adjusted to 7.3 using CsOH and volume-adjusted to an osmolarity of 290–315 mOsm). Before experimentation, sulforhodamine 101 was added (~63 μM) to the internal solution to allow for visualization of cells by fluorescence microscopy. Recording pipettes had a tip resistance of 3–6 MΩ. Typical access resistance during whole cell recording was between 10 and 40 MΩ and was generally uncompensated. Local application experiments were performed using a Picospripter III (General Valve, Fairfield, NJ) and generally used pipettes with slightly larger tip diameter. For the experiment presented in Fig. 4C (Glu spritz), a 10-ms spritz at approximately 20 psi was applied at 0.05 Hz using standard pipettes loaded with 50–200 μM glutamate. For experiments that involved local paired pulses the interstimulus interval was 60 ms.

Mossy fiber inputs to mossy cells were identified by four separate criteria: rise time; latency; frequency facilitation; and sensitivity to DCG-IV, a potent group II mGluR agonist (Kamiya et al. 1996; Ohishi et al. 1995; Yoshino et al. 1996; also see Fig. 1, B and C). Of these, sensitivity to DCG-IV was the major criterion. Acceptable responses exhibited a minimum of 80% block by DCG-IV. The only exception to that standard was for cells that were treated with DCG-IV before full recovery from baclofen. In those cases, a 50% block by DCG-IV was considered acceptable. Although DCG-IV was typically applied by bath at a concentration of 1 μM at the end of each experiment, in some cases, 1 μM DCG-IV was delivered before further experimentation to the surface of a slice just above a patched cell by local application (10 s at ~20 psi; e.g., Fig. 4).

In addition to sensitivity to DCG-IV, we also considered rise time, latency, and frequency facilitation observed in response to a five-pulse train of stimuli delivered at 16.67 Hz as diagnostic criteria helpful in identifying mossy fiber inputs. In general, mossy fibers exhibited faster rise times, shorter latencies (e.g., 1.17 ± 0.09 ms, n = 19, vs. 1.86 ± 0.25 ms, n = 7, P < 0.003), and more robust frequency facilitation than non-MF inputs that failed the DCG-IV test or that were isolated in the continual presence of DCG-IV. In every cell, individual sweeps that lacked a smooth rising phase, showed multiple peaks, or were contaminated by sEPSCs were manually removed from analysis.

**Independent measurement of phasic and tonic currents from continuous voltage-clamp recordings of sIPSCs**

Changes in spontaneous inhibitory postsynaptic currents (sIPSCs; phasic current) are most often measured using traditional event detection. Changes in tonic current, on the other hand, are most often measured as differences in holding current (Ihold). However, such measurements must be carefully obtained to prevent contamination by sIPSCs (Prenosil et al. 2006; Scimemi et al. 2005). Alternatively tonic currents have been characterized using noise analysis (Brickley et al. 1996; Michedlishvili and Kapur 2006) or by fitting a Gaussian curve to an all-points histogram of sampled data (Glykys and Mody 2006a; Glykys et al. 2006; Petrini et al. 2004; Wall and Usowicz 1997). Unfortunately, these techniques generally fail to simultaneously describe changes in both tonic and phasic currents. For the current study, we developed custom software in OriginC that accomplishes that goal. Our method is directly based on that described in a recent report from Glykys and Mody (2006b).

In brief, continuous time series data were recorded at 20 kHz, downsampled to 5 kHz, and analyzed in 5-s intervals. For each
consecutive interval, an amplitude histogram was constructed from all 25,000 data points. A nonlinear least-squares fit (NLSF) was then performed on a portion of each amplitude histogram using a Gaussian function of the form

$$y = \frac{A}{w \sqrt{\pi/2}} e^{-x - x_0^2/w}$$  \hspace{1cm} (f)

where A is the area under the normal curve, w is the width at half-peak amplitude, and x0 is the x value at the center of the curve. Importantly, the portion of the amplitude histogram used for NLSF fitting excluded all histogram bins left of center that had fewer than half the number of counts in the largest bin. Because physiological noise in the absence of spontaneous IPSCs is expected to be normally distributed, the portion of the amplitude histogram used for NLSF fitting excluded all histogram bins left of center that had fewer than half the number of counts in the largest bin. Therefore this sum was considered to represent total phasic current per interval.

Glyksy and Mody tested this technique against real and simulated sIPSCs and reported it to be sensitive to minor (~15%) changes in sIPSC parameters. We verified our own code in two ways. First we demonstrated that adding a standing current to all points selectively modulates tonic current, whereas doubling the amplitude of all points >2 SD from the mean selectively modulates phasic current (data not shown). Second, we plotted phasic and tonic currents analyzed in 1-s intervals on top of raw sIPSCs as in Fig. 2. These plots emphasize the ability of the tonic current to accurately identify Inon that is tonic irrespective of sIPSC frequency and also demonstrate that phasic current clearly increases with event frequency, showing sensitivity to as little as one event.

**Results**

Presynaptic GABA\textsubscript{B} receptors are expressed on mossy fiber inputs to mossy cells

Miniature excitatory postsynaptic currents (mEPSCs) were recorded in the presence of 1 \( \mu \text{M} \) tetrodotoxin (TTX) from hilar mossy cells voltage clamped at -70 mV with a CsMeSO\textsubscript{3}-based internal solution. Under these conditions, bath application of (R)-4-amino-3-(4-chlorophenyl)butanoic acid (baclofen), a GABA\textsubscript{B} agonist, caused a significant reduction in mEPSC frequency (to 65.9 ± 4.64% of baseline, \( n = 9, P < 0.0001 \); Fig. 3C) without affecting mEPSC amplitude (99.6 ± 0.05; Fig. 3D). This effect of baclofen was completely abolished by pretreatment with CGP 52432, a GABA\textsubscript{B} receptor antagonist (frequency: 99.2 ± 12.07%, \( P > 0.05 \), Fig. 3C; amplitude: 99.1 ± 6.11%, \( P > 0.05 \), Fig. 3D; \( n = 8 \)). Interestingly, baclofen-induced inhibition of mEPSC frequency was absent when the experiment was repeated in ACSF that contained 2.5 mM Mg\textsuperscript{2+}, 1 mM Ca\textsuperscript{2+}, and 200 \( \mu \text{M} \) Cd\textsuperscript{2+}, conditions designed to further reduce the probability of observing calcium-dependent mEPSCs (frequency: 99.3 ± 12.37%, \( P > 0.05 \); amplitude: 100.3 ± 4.95%, \( P > 0.05 \); \( n = 6 \); data not shown). These findings clearly suggested that activation of presynaptic GABA\textsubscript{B} receptors can reduce...
transmission from excitatory afferents to hilar mossy cells, but did little to distinguish which specific glutamatergic inputs were sensitive. To address this question more directly we used minimal stimulation techniques, coupled with bath or local application of the mGluR group II agonist DCG-IV to isolate mossy fiber inputs to hilar mossy cells (see METHODS). We found that bath application of baclofen reduced the amplitude of EPSCs evoked by minimal stimulation of identified mossy fiber inputs to 9.9 ± 1.37% of baseline (n = 14, P < 0.0001; Fig. 4B). Consistent with a presynaptic site of action, baclofen also increased the failure rate of meEPSCs (see METHODS) from 11.3 ± 3.02 to 75.9 ± 4.97% and increased the coefficient of

FIG. 2. Simultaneous measurement of phasic and tonic currents. A: in the absence of spontaneous activity, tonic current (dashed lines) accurately reflects the holding current (I _hold_), whereas phasic current (dotted lines) calculated as described in METHODS is near 0. For simplicity, phasic current is plotted in all panels as an offset from tonic current. B: phasic current shows excellent sensitivity to spontaneous activity. In this panel, a single event produces a phasic current of 7.3 pA when calculated over a 1-s interval. C: phasic current clearly increases with the level of spontaneous activity, whereas tonic current continues to accurately report I _hold_ even during periods of very high event frequency. Legend and scales apply to all panels. Data are spontaneous inhibitory postsynaptic currents (sIPSCs) taken from baseline (A and B), and early carbachol (CCh) period (C) of cell shown in Fig. 8A.

FIG. 3. (RS)-4-Amino-3-(4-chlorophenyl)butanoic acid (baclofen) reduces miniature excitatory postsynaptic current (mEPSC) frequency without affecting amplitude. A: cumulative probability histogram (CPH) for a sample cell. Baclofen (10 μM) significantly increased the interevent interval (IEI) of mEPSCs [baseline IEI: 179.5 ± 4.31 ms; baclofen IEI: 328.2 ± 10.92 ms; P < 0.001, Kolmogorov–Smirnov (K-S) test] with only a negligible change in amplitude (baseline amplitude: 43.3 ± 0.89 pA; baclofen amplitude: 41.4 ± 1.22 pA; data not shown). B: CPH for a sample cell pretreated with 5 μM [3-[(3,4-dichlorophenyl)methyl][amino]propyl(diethoxymethyl)phosphinic acid (CGP 52432). Baclofen had no significant effect on mEPSC interevent interval (P = 0.06, K-S test) or amplitude (P = 0.05, K-S test; data not shown). Inset: consecutive 2-s intervals during either the baseline or after 6 min of baclofen wash-in. C: summary graph of baclofen’s effect on frequency. Baclofen significantly reduced mEPSC frequency (34.1 ± 6.44%, n = 9, P < 0.0001), whereas pretreatment with 5 μM CGP 52432 completely blocked this effect (0.8 ± 12.07%, n = 8, P > 0.05). D: summary graph of baclofen’s effect on amplitude. Baclofen did not significantly affect the amplitude in either control conditions (0.4 ± 4.26% reduction from baseline, n = 9, P > 0.05) nor did pretreatment with 5 μM CGP 52432 (9.9 ± 6.11% reduction from baseline, n = 8, P > 0.05).
bacclofen had no significant effect on mossy cell responses to local application of exogenous glutamate (50–200 μM, 96.4 ± 9.7% of baseline, n = 10; summarized in Fig. 4B).

Presynaptic GABA<sub>B</sub> receptors on mossy fiber inputs to mossy cells are both sensitive to ambient GABA and tonically active under control conditions

We found that the amplitude of mossy fiber–mediated meEPSCs recorded from hilar mossy cells in the presence of 50 μM PTX was reduced by bath application of the GABA uptake inhibitor 1-[2-[[diphenylmethylene]iminooxy]ethyl]-1,2,5,6-tetrahydro-3-pyridinecarboxylic acid hydrochloride (NO-711) to 40.85 ± 11.49% of baseline levels (n = 6, P < 0.03; Fig. 5B). Further, in seven of nine separate experiments, we found that bath application of 10 μM CGP 52432, also in the presence of 50 μM PTX, increased the amplitude of mossy fiber–mediated meEPSCs to 254.7 ± 33.5% of baseline (n = 7, P < 0.005; Fig. 5B). In both cases, apparent GABA<sub>B</sub>-mediated modulation of meEPSC amplitude appeared to be presynaptic in nature. Specifically, concurrent with NO-711–mediated inhibition of meEPSCs we observed a clear increase in failure rate (from 8.5 ± 4.4 to 22.9 ± 6.43%, n = 6, P = 0.03; Fig. 5C) and a similar increase in CV (from 0.74 ± 0.08 to 1.04 ± 0.11, n = 6, P < 0.01; Fig. 5D). Conversely, CGP 52432–mediated increases in meEPSC amplitude were accompanied by a clear decrease in both failure rate (from 33.3 ± 8.39 to 7.0 ± 3.1%, n = 7, P < 0.01; Fig. 6C) and CV (from 0.99 ± 0.09 to 0.64 ± 0.06, n = 7, P < 0.01; Fig. 6D). Note that in two of nine cases bath application of CGP produced much milder increases in amplitude (to 110 ± 2.90% of baseline) without consistent changes in failure rate and CV.

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**Fig. 4.** Baclofen blocks meEPSCs from isolated mossy fiber terminals by presynaptic γ-aminobutyric acid type B receptors (GABA<sub>B</sub>Rs). A: effect of 10 μM baclofen in a representative cell. A 10-s local application of 1 μM DCG-IV completely blocked meEPSCs (1.8 ± 0.7% of baseline). After recovery from local application of DCG-IV, bath application of 10 μM baclofen reduced meEPSC amplitude to 10.4 ± 1.0% of baseline. During the baclofen washout, meEPSC amplitude showed considerable recovery (to 68.3 ± 7.8% of baseline). Insets: individual traces from the relevant periods are shown in gray, with the average trace overlaid in black. B: summary graph comparing baclofen’s effect on MF meEPSCs and exogenous glutamate spritz. Cells treated with DCG-IV either before or after baclofen were pooled and showed significant inhibition by the GABA<sub>B</sub> agonist (90.1 ± 3.7%, n = 14, P < 0.001). By contrast, baclofen failed to inhibit exogenously applied glutamate (50–200 μM; 3.6 ± 9.7% block, n = 10).

**Fig. 5.** Presynaptic GABA<sub>B</sub>Rs on MF terminals respond to increases in ambient GABA. A: single-cell recording shows that bath application of 1 μM 1-[2-[[diphenylmethylene]iminooxy]ethyl]-1,2,5,6-tetrahydro-3-pyridinecarboxylic acid hydrochloride (NO-711), a potent GABA uptake inhibitor, reversibly depressed average meEPSC amplitude, strongly suggesting that ambient GABA directly inhibits mossy fiber inputs. Insets: individual traces from the relevant periods are shown in gray, with the average trace overlaid in black. B: summary graph of effects of NO-711 and DCG-IV on meEPSCs. In 6 cells, NO-711 significantly inhibited meEPSC amplitude (to 40.9 ± 11.49% of baseline, P < 0.03), whereas subsequent DCG-IV treatment further reduced the amplitude (to 13.2 ± 2.01% of baseline, P < 0.01). Error bars indicate SE. C and D: summary plots for the effect of NO-711 on failure rate and coefficient of variation (CV). Connected points represent data from a single cell. In 6 cells, NO-711 significantly increased the average failure rate (from 8.5 ± 4.4 to 22.9 ± 6.43%, n = 6, P = 0.03) and CV (from 0.74 ± 0.08 to 1.04 ± 0.11, n = 6, P < 0.01).
Bath application of muscarinic agonists produces GABA\(_{\text{B}}\)-receptor–mediated inhibition of mossy fiber inputs to mossy cells

We next tested the hypothesis that putative presynaptic muscarinic receptors on mossy fiber inputs to mossy cells could provide an additional mechanism for presynaptic inhibition of mossy fiber transmission. Consistent with this hypothesis, we initially found that in 11 of 12 cells tested, bath application of muscarinic agonists [e.g., 3 \(\mu\)M carbachol (CCh) or 5 \(\mu\)M muscarine] reduced mossy fiber–mediated meEPSCs recorded from hilar mossy cells to 52.0 ± 5.69% of baseline (\(n = 11\), \(P < 0.01\); data not shown; one cell showed no significant change). This effect was accompanied by a significant increase in the CV from 0.52 ± 0.06 to 0.63 ± 0.07 (\(n = 11\), \(P = 0.05\); data not shown). To provide further evidence that the observed muscarinic effect was presynaptic, we demonstrated that bath application of 3 \(\mu\)M CCh failed to inhibit responses to locally applied exogenous glutamate (100.1 ± 6.74% of baseline, \(n = 9\), \(P > 0.5\); data not shown). However, for six cells in which application of a muscarinic agonist reduced the amplitude of mossy fiber–mediated meEPSCs to 55.9 ± 6.81%, subsequent application of CGP 52432 completely reversed the effect (e.g., to 108 ± 11.6% of baseline, \(n = 6\), \(P < 0.01\); Fig. 7B). This result is more consistent with an indirect mechanism of muscarinic inhibition [i.e., one that does not involve activation of muscarinic acetylcholine receptors (mAChRs) directly on the terminal]; however, because previous experiments indicated that mossy fiber inputs to hilar mossy cells are subject to tonic inhibition by ambient GABA, we also tested the ability of muscarinic agonists to inhibit mossy fiber transmission to mossy cells in slices pretreated with CGP 52432. Under those conditions, no muscarinic inhibition of mossy fiber transmission was observed and, in fact, a slight but statistically insignificant increase in meEPSC ampli-

FIG. 6. Mossy fiber inputs to mossy cells are subject to tonic inhibition by ambient GABA, mediated through GABA\(_{\text{B}}\)Rs. A: effects of 10 \(\mu\)M CGP 52432 and 1 \(\mu\)M DCG-IV in a representative cell. CGP 52432 significantly increased the average meEPSC amplitude (to 211.4 ± 14.29% of baseline) and decreased the failure rate (0 failures in CGP 52432). Subsequent bath application of DCG-IV reduced meEPSC amplitude by 95.6 ± 1.9% and significantly increased the failure rate (to 75.5%), suggesting the responses resulted from mossy fiber stimulation. Insets: individual traces from the relevant periods are shown in gray, with the average trace overlaid in black. B: summary graph of the effect of CGP 52432 on meEPSCs. In 7 of 9 cells, CGP 52432 significantly increased meEPSC amplitude (to 254.7 ± 33.5% of baseline, \(P < 0.005\)), whereas DCG-IV almost completely blocked all meEPSCs (10.8 ± 2.0% of baseline, \(P < 0.005\)). Error bars indicate SE. C and D: summary plots for the effect of CGP 52432 on failure rate and CV. Connected points represent data from a single cell. CGP 52432 significantly reduced the average meEPSC failure rate (from 33.3 ± 8.39 to 7.0 ± 3.1%, \(n = 7\), \(P < 0.01\)) and CV (from 0.89 ± 0.09 to 0.58 ± 0.06, \(n = 7\), \(P < 0.01\)).

FIG. 7. Muscarinic inhibition of mossy fibers is reversed by a selective GABA\(_{\text{B}}\)R antagonist. A: single-cell recording shows that bath application of 5 \(\mu\)M muscarine decreased meEPSC amplitude. This effect was reversed by 10 \(\mu\)M CGP 52432, implying that muscarinic receptors indirectly inhibit neurotransmitter release from mossy fibers. Insets: individual traces from the relevant periods are shown in gray, with the average trace overlaid in black. B: in 6 cells, muscarinic agonists (5 \(\mu\)M muscarine or 3 \(\mu\)M CCh) reduced the average meEPSC amplitude (to 55.9 ± 6.81% of baseline), whereas 10 \(\mu\)M CGP 52432 reversed this effect (to 108 ± 11.6% of baseline, \(P < 0.01\)). Bath application of 1 \(\mu\)M DCG-IV significantly reduced meEPSC amplitude (to 14.7 ± 1.86% of baseline, \(P < 0.05\)).
Bath application of muscarinic agonists produces increases in ambient GABA that are strongly correlated with increases in spontaneous IPSCs

The data described earlier clearly implicated presynaptic GABA_B receptors on mossy fiber terminals to mossy cells in muscarinic inhibition of glutamatergic transmission. These observations raised two interesting questions. First, can hypothetical changes in ambient GABA produced by bath application of muscarinic agonists be detected by additional means that are not dependent on mossy fibers? Second, if bath application of muscarinic agonists does indeed increase levels of ambient GABA, what is the mechanism? We were able to address both of these questions simultaneously using a recently developed analytical technique that allows independent quantification of both tonic and phasic GABA_A-receptor–mediated currents during a period of high spontaneous activity (see METHODS). In brief, mossy cells were voltage clamped at −70 mV using an internal solution that contained approximately 60 mM chloride, ionotropic glutamate receptors were blocked by bath application of 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo-[f]quinoxaline-7-sulfonamide disodium (NBQX, 10 μM) and DL-2-amino-5-phosphonovaleric acid (APV, 40 μM), and spontaneous IPSCs mediated by synaptic activation of postsynaptic GABA_A receptors were observed as inward currents. After a 10-min baseline period, 3 μM CCh was bath applied, exactly as in previous experiments with meEPSCs (see Fig. 8A for sample recording). In six of ten cells tested, conventional event analysis showed clear and sustained increases in sIPSC frequency and, in some cases, clear increases in sIPSC amplitude as well (data not shown). These changes were collectively reflected as an increase in “phasic current” of 4.29 ± 1.68 pA/5-s interval (n = 6, P = 0.05; Fig. 8C). This represented an average increase in phasic current to 767.4 ± 280.9% of baseline. Importantly, the CCh-induced changes in phasic current were also accompanied by increases in “tonic current” to 118.0 ± 5.74% of baseline, corresponding to an average

![Figure 8](http://jn.physiology.org/)

**FIG. 8.** CCh induced increases in sIPSCs produce significant increases in GABA_A-mediated tonic inhibition of hilar mossy cells. A: sample current trace from a mossy cell treated with CCh and picrotoxin (PTX). Shortly after the initial CCh (3 μM) application, a sudden, dramatic increase in both sIPSC frequency and amplitude was observed. Effect on sIPSCs was accompanied by a negative shift in the I_hold. Both phenomena were reversed by application of 50 μM PTX. Note: data clipped to allow for visualization of changes in both phasic and tonic currents. B: effects of CCh and PTX on tonic (black dots) and phasic (white dots) currents in the same sample cell calculated as described in METHODS. Bath application of 3 μM CCh significantly increased both tonic and phasic currents (by 15.81 and 3.3 pA, respectively; P < 0.001 in both cases), whereas 50 μM PTX significantly reversed these effects (P < 0.001 in both cases). C: summary graph of effects of CCh and PTX on phasic (white bars) and tonic (black bars) currents. In 6 of 10 cells, CCh significantly increased the phasic current (by 23.2 ± 4.61 pA from baseline, P < 0.005), whereas PTX reversed this effect (−0.65 ± 0.27 pA from baseline, P > 0.05). CCh also significantly increased the tonic current (by 23.2 ± 4.61 pA from baseline, P < 0.005), whereas PTX reversed this increase (−4.91 ± 4.76 pA from baseline, P > 0.05). Error bars indicate SE. D: average changes in tonic current were plotted against changes in phasic current for each condition and fit by linear regression. Calculated slope is 5.48 with R^2 = 0.996, P = 0.002, indicating a strong correlation between phasic and tonic currents across all 3 experimental conditions.
increase in tonic current of $23.2 \pm 4.61 \text{ pA (} n = 6, P < 0.005; \text{ Fig. 8C).}$

Several lines of evidence suggest that the changes in tonic current observed in this manner are directly caused by CCh-mediated action potential-dependent increases in ambient GABA, and subsequent activation of high affinity GABA$_A$ receptors expressed by hilar mossy cells. For example, in these same six cells we found that subsequent application of $50 \mu M$ PTX restored tonic current to $97.6 \pm 3.14\%$ of baseline levels (Fig. 8C), and often reduced phasic current to below baseline levels (e.g., to $53.7 \pm 23.5\%$ of baseline, $n = 6, P = 0.11$; Fig. 8C). Further, an analysis of mean change in both phasic and tonic currents [expressed in picoamperes (pA)/interval] across all three experimental conditions revealed an extremely strong correlation between these currents ($R^2 = 0.996$, slope = 5.48, $P = 0.002$; Fig. 8D). Consistent with that result, we also found that in the four of ten cells that failed to show sustained increases is sIPSC frequency or phasic current after bath application of CCh, no significant effect on tonic current was observed (103 $\pm 5.31\%$ of baseline, $P = 0.53$).

To explicitly eliminate possible contributions of postsynaptic mACHRs to observed CCh-mediated increases in tonic current, we also demonstrated that bath application of CCh fails to change $I_{\text{hold}}$ measured by conventional means under experimental conditions designed to eliminate most forms of glutamatergic and GABAergic transmission (e.g., in the presence of $10 \mu M$ NBQX, $40 \mu M$ APV, $10 \mu M$ CGP 52432, and $50 \mu M$ PTX, $1 \mu M$ TTX, and in ACSF that contained low calcium and high Mg$^{2+}$ as described in METHODS). Specifically, $I_{\text{hold}}$ after bath application of CCh under those conditions was $100 \pm 0.75\%$ of baseline ($n = 3, P = 0.71$; data not shown). Finally, repeating this experiment in the absence of PTX also failed to restore CCh-mediated effects on holding current in four cells tested, suggesting action potential–independent mechanisms likely do not contribute substantially to CCh-mediated changes in ambient GABA (data not shown).

Non-mossy fiber–mediated excitatory inputs to mossy cells are directly inhibited by both GABA$_A$ receptors and mACHRs

The nature of the experiments presented in Figs. 4–7 is such that many runs were completed on isolated inputs that subsequently did not meet the criteria described in METHODS to be classified as mossy fibers. It is thus potentially informative to compare the results of these putative non-MF inputs to those described earlier. Such analysis of these parallel experiments suggested several similarities and one prominent difference. Specifically, like MF inputs to mossy cells, putative non-MF inputs were strongly inhibited by baclofen (to $18.2 \pm 3.4\%$ of baseline, $n = 7, P < 0.01$; data not shown) and, in two of four cases, showed clear increases in amplitude after bath application of CGP 52432 (e.g., to $379 \pm 72.3\%$ of baseline; data not shown; two cells failed to show robust increases). Further, in 23 of 26 cells, bath application of muscarinic agonists reduced meEPSC amplitude to $47.0 \pm 4.68\%$ of baseline. However, strikingly unlike MF inputs, putative non-MF inputs to mossy cells failed to demonstrate CGP 52432–mediated reversal of muscarinic inhibition. Specifically, we tested the ability of CGP 52432 to reverse inhibition produced by muscarinic agonists in nine cells where meEPSCs failed to meet criteria to be categorized as mossy fibers. In those nine cells, amplitude of the meEPSCs after application of muscarinic agonists was $40.6 \pm 7.55\%$ of baseline; this value remained virtually unchanged (at $40.9 \pm 10.8\%$ of baseline; data not shown) after bath application of CGP 52432.

One potential criticism of this analysis is that putative non-MF inputs in these cases often still showed significant sensitivity to DCG-IV. For example, meEPSCs in the nine cells described earlier that lacked CGP 52432–mediated reversal of muscarinic inhibition were still reduced to $47.8 \pm 5.9\%$ of baseline after bath application DCG-IV. Perforant path inputs to the dentate gyrus have been reported to have intermediate sensitivity to DCG-IV (Dietrich et al. 2002; Kew et al. 2002; Kilbride et al. 1998), although this could also simply indicate that some of our minimally evoked responses had mixed MF and non-MF origin. Therefore we also tested the ability of 3–10 $\mu M$ CCh to inhibit meEPSC amplitudes recorded from mossy cells in slices pretreated with both DCG-IV and CGP 52432. Again, in sharp contrast to the results reported earlier for MF inputs, we found that robust inhibition of non-MF inputs was still apparent under those conditions. Specifically, bath application of 3–10 $\mu M$ CCh reduced the amplitude of non-MF–mediated meEPSCs to $55.4 \pm 8.49\%$ of baseline ($n = 12, P < 0.01$; Fig. 9B). This effect was also accompanied by a significant increase in failure rate (from $18.8 \pm 4.25$ to $38.8 \pm 7.32\%$, $n = 12, P < 0.01$) and an average increase in CV (from $0.88 \pm 0.06$ to $1.07 \pm 0.10$, $n = 12, P = 0.12$). Finally, we also noted that bath application of baclofen reduced the amplitude of non-MF–mediated EPSCs in slices pretreated with DCG-IV to $30.8 \pm 4.1\%$ of baseline (Fig. 9B), also concurrent with a significant increase in both failure rate (from $19.0 \pm 7.26$ to $67.0 \pm 5.71\%$) and CV (from $0.7$ to $1.1$, $n = 7, P < 0.01$ in both cases). Collectively, these results strongly suggest that the majority of all glutamatergic inputs to mossy cells express presynaptic GABA$_A$ receptors that are subject to tonic inhibition by ambient GABA, but that only non-MF inputs also express presynaptic mACHRs capable of directly inhibiting glutamatergic transmission at the terminal.

Origin of non-MF inputs

In addition to MF inputs from dentate granule cells, existing data suggest that mossy cells may receive glutamatergic input from both the perforant path and from CA3 pyramidal cells (Ishizuka et al. 1990; Kohler 1985; Muller and Mis geld 1991; Scharffman 1991, 1994b). Our experiments with minimally evoked EPSCs were designed, first and foremost, to isolate MF-mediated inputs to mossy cells. As such, they are not particularly useful for distinguishing between various potential sources of non-MF responses. To address this question more directly, we performed a final series of experiments designed to selectively activate excitatory inputs to hilar mossy cells originating either from the CA3 pyramidal cell layer or from the perforant path. Putative inputs from CA3 pyramidal cells to hilar mossy cells were isolated by stimulating the CA3 pyramidal cell layer with a bipolar stimulator (0.1-ms duration, generally <100 $\mu A$) in the presence of both DCG-IV and CGP 52432. In eight of ten cases CCh produced an atropine-sensitive reduction in
GABA<sub>B</sub> inhibition of miniature EPSCs

We found that bath application of baclofen produces a CGP 52432-sensitive reduction in the frequency of mEPSCs recorded from hilar mossy cells without changing their amplitude, strongly suggesting a presynaptic site of action. Although this represents the first report of GABA<sub>B</sub>-mediated inhibition of excitatory inputs to hilar mossy cells, presynaptic inhibition mediated by GABA<sub>B</sub> receptors is quite common in the CNS (for review see Bowery 1993). At various glutamatergic terminals, both calcium-dependent (for review, see Chen and Regehr 2003; Dittman and Regehr 1997; Ishikawa et al. 2005; Misgeld et al. 1995; Sakaba and Neher 2003; Takahashi et al. 1998) and calcium-independent (Capogna et al. 1996; Dittman and Regehr 1996; Jarolimek and Misgeld 1992; Kolaj et al. 2002) GABA<sub>B</sub> receptors have been shown to inhibit neurotransmitter release.

In sharp contrast, we found that stimulation of the middle to outer perforant path (also in the presence of DCG-IV and CGP 52432) failed to produce detectable EPSCs in hilar mossy cells in approximately 75% of attempts, even when stimulus intensity surpassed 200 μA. This is not likely to indicate that perforant path inputs to mossy cells have high sensitivity to DCG-IV, first because previous work suggested perforant path inputs have intermediate sensitivity to DCG-IV and, second, because identical stimulation frequently produced detectable EPSCs in dentate granule cells (data not shown). Further, even when small EPSCs were detected in hilar mossy cells subsequent to stimulation of the perforant path, only minimal sensitivity to CCh was observed (10.4 ± 0.57%, n = 3, P = 0.003; data not shown). Cumulatively, these experiments suggest that the minimally evoked EPSCs of non-MF origin described earlier are more likely to represent afferent input from CA3 pyramidal cells than from the perforant path.
mossy cells express presynaptic GABAB receptors, are subject to tonic inhibition by ambient GABA (Hirata et al. 1992; Ruiz et al. 2003; Vogt and Nicoll 1999; Vogt and Regehr 2001). Similarly, although an early study of cholinergic modulation of MF inputs to CA3 pyramidal cells concluded that they were subject to direct inhibition by presynaptic mAChRs (Williams and Johnston 1990), a more recent study revealed an indirect mechanism dependent on GABA\textsubscript{B} receptors that is consistent with our findings in the hilus (Vogt and Regehr 2001). One aspect of our study not generally available in work on other MF terminal zones is the implication that levels of ambient GABA around MF terminals in the hilus are tightly coupled to action potential–dependent release of GABA. This conclusion is consistent with studies in developing and mature cerebellum that showed a clear link between sIPSCs and tonic currents mediated by ambient GABA (Brickley et al. 1996; Carta et al. 2004; Kaneda et al. 1995; Wall and Usowicz 1997). It is also consistent with cell culture studies of both cerebellar and hippocampal neurons that show TTX-mediated reductions in tonic GABAergic currents (Leao et al. 2000; Petrini et al. 2004) and with in vitro work in hippocampus directly implicating KA-induced increases in sIPSCs with GABA\textsubscript{A}-mediated changes in holding current (Frerking et al. 1999). Nevertheless, it is clear that action potential–independent mechanisms for regulating ambient GABA have also been implicated in some studies (Rossi et al. 2003; Wall and Usowicz 1997).

With respect to non-MF inputs to hilar mossy cells, our results mirror previous studies on the terminal zones for CA3 pyramidal cell axons in indicating direct presynaptic inhibition mediated by both presynaptic GABA\textsubscript{B} receptors and mAChRs (De Sevilla and Buno 2003; De Sevilla et al. 2002; Houmgaard 1978; Lei and McBain 2003; Vogt and Regehr 2001). This raises the interesting question of whether the majority of our non-MF–mediated inputs in fact represent collateral projections from CA3 pyramidal cells to hilar mossy cells. On the one hand, event latency analysis was consistent with that hypothesis in indicating that the non-MF inputs we observed, like previously identified collateral inputs from CA3 (Amaral 1978; Frotscher et al. 1991; Scharfman 1994a; Schwartzkroin et al. 1990), are likely to synapse on more distal dendrites of hilar mossy cells than MF inputs. On the other hand, our experiments with mEPSCs were not directly designed to distinguish putative CA3 inputs from other types of non-MF–mediated responses. Therefore to address that question more directly, we examined responses in mossy cells that were evoked by direct stimulation (with a bipolar stimulator) of the CA3 pyramidal cell layer and the perforant path. Our results indicated that, like our minimally evoked non-MF–mediated population, EPSCs evoked in mossy cells by direct stimulation of CA3 are inhibited by bath application of CCh, even in the presence of DCG-IV and CGP 52432. Although these experiments were done in the absence of PTX, increases in ambient GABA mediated by NO-711 failed to produce comparable inhibition of these CA3-evoked EPSCs. In sharp contrast, most mossy cells failed to show detectable responses to stimulation of the perforant path under identical conditions, although granule cells were still responsive. Further, those mossy cells that did exhibit small EPSCs in response to perforant path stimulation lacked robust inhibition by CCh. Cumulatively these data suggest that our non-MF–mediated and minimally evoked EPSCs are indeed more likely mediated by collateral inputs from CA3 than from the perforant path. Nevertheless it must be noted that we cannot as yet explicitly rule out a contribution of mossy cell inputs to other mossy cells, although previous work has suggested that granule cells and molecular layer interneurons are likely the primary targets of mossy cell axons (Wenzel et al. 1997).

Direct and indirect inhibition of minimally evoked EPSCs

The minimal stimulation techniques we used to isolate mossy fiber versus non-mossy fiber inputs to hilar mossy cells are very similar to those used by other labs and are based on well-recognized features of mossy fiber transmission (e.g., see Cossart et al. 2002; Henze et al. 2000; Lysetskiy et al. 2005; Toth et al. 2000; Walker et al. 2001). Our experiments with minimally evoked EPSCs clearly indicated that MF inputs to mossy cells express presynaptic GABA\textsubscript{B} receptors, are subject to tonic inhibition by ambient GABA, lack presynaptic muscarinic receptors, and yet can be indirectly inhibited by CCh-induced action potential–dependent increases in ambient GABA. This result complements a very recent report of CCh-mediated effects on mossy cell gain (Kerr and Capogna 2007) and it is also generally consistent with previous work on MF inputs to CA3 pyramidal cells. For example, MF inputs to CA3 pyramidal cells also express GABA\textsubscript{A} receptors that are mediates by both GABA\textsubscript{B} and GABA\textsubscript{A} receptors (Hirata et al. 1992; Ruiz et al. 2003; Vogt and Nicoll 1999; Vogt and Regehr 2001). Similarly, although an early study of cholinergic modulation of MF inputs to CA3 pyramidal cells concluded that they were subject to direct inhibition by presynaptic mAChRs (Williams and Johnston 1990), a more recent study revealed an indirect mechanism...
A final interesting question with regard to our non-MF-mediated minimally evoked EPSCs is why they lacked any evidence of indirect inhibition mediated by GABA_B-receptor activation. Given our results with mossy fibers, and the clear expression of GABA_B receptors on non-MF terminals, at least a partial reversal of mAChR-mediated inhibition of non-MFs might have been expected. Because changes in ambient GABA responsible for indirect inhibition of MF inputs are highly action potential dependent, we expect that spatial factors most likely account for this apparent discrepancy. If that were the case, we would predict that synapses from mAChR-expressing interneurons are likely to terminate on more proximal dendrites of hilar mossy cells, where they would be closer to MF than to non-MF inputs. Nevertheless, based on present data, we cannot explicitly rule out the possibility that presynaptic GABA_B receptors on non-MFs have lower sensitivity to changes in ambient GABA than those on MFs, or the possibility that GABA_B- and mAChR-dependent inhibition of non-MFs share a common presynaptic signaling pathway and thus can fully or partially occlude one another.

In summary, our results have examined several specific mechanisms that modulate glutamatergic transmission to hilar mossy cells and have identified clear differences between two primary types of excitatory afferents. We believe that further studies into specific mechanisms that regulate mossy cell excitability will be necessary to develop a more complete understanding of hippocampal function and dysfunction ranging all the way from memory consolidation to epileptogenesis.

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