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Monosynaptic GABAergic Signaling from Dentate to CA3 with a Pharmacological and Physiological Profile Typical of Mossy Fiber Synapses

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Summary

Mossy fibers are the sole excitatory projection from dentate gyrus granule cells to the hippocampus, where they release glutamate, dynorphin, and zinc. In addition, mossy fiber terminals show intense immunoreactivity for the inhibitory neurotransmitter GABA. Fast inhibitory transmission at mossy fiber synapses, however, has not previously been reported. Here, we show that electrical or chemical stimuli that recruit dentate granule cells elicit monosynaptic $GABA_A$ receptor–mediated synaptic signals in CA3 pyramidal neurons. These inhibitory signals satisfy the criteria that distinguish mossy fiber–CA3 synapses: high sensitivity to metabotropic glutamate receptor agonists, facilitation during repetitive stimulation, and NMDA receptor–independent long-term potentiation. GABAergic transmission from the dentate gyrus to CA3 has major implications not only for information flow into the hippocampus but also for developmental and pathological processes involving the hippocampus.

Introduction

The coexistence of glutamate, dynorphin, and zinc, among other substances, in mossy fiber terminals distinguishes the axons of dentate granule cells as a highly unusual projection in the mammalian brain (McGinty et al., 1983; Assaf and Chung, 1984; Howell et al., 1984; Henze et al., 2000). Of these transmitters, only glutamate is known to open ionotropic receptors in postsynaptic CA3 pyramidal neurons and hilar interneurons. Dynorphin modulates mossy fiber signaling via presynaptic receptors (Weisskopf et al., 1993), while zinc may have multiple effects on $GABA_A$ and glutamate receptors (Westbrook and Mayer, 1987; Bresink et al., 1996; Buhl et al., 1996; Vogt et al., 2000) as well as glutamate transporters (Spiridon et al., 1998). Thus, mossy fibers are primarily excitatory but with an unusual capacity for modulation via indirect effects of coreleased dynorphin and zinc on pre- and postsynaptic receptors and transporters.

Mossy fibers also show intense immunoreactivity for GABA (Sandler and Smith, 1991; Sloviter et al., 1996) and have also been reported to contain the GABA synthetic enzyme GAD67 (Schwarzer and Sperk, 1995; Sloviter et al., 1996). These observations raise the possibility that mossy fibers, uniquely in the CNS, could release both GABA and glutamate (see also Taupin et al., 1994). Given that the mossy fiber tract is a major input to the hippocampus proper, GABA release would have an extensive impact for models of information flow in mesial temporal structures and for the role of mossy fibers in epilepsy and other CNS diseases. Because of the ramifications of this conclusion, what further evidence exists that mossy fibers can signal via the release of GABA at synapses on CA3 pyramidal neurons?

One element associated with GABAergic transmission is apparently missing: the vesicular GABA transporter VGAT1 has not been seen in hippocampal mossy fiber terminals (Chaudhry et al., 1998). VGAT1 is not, however, necessary for GABAergic transmission, because the same authors failed to observe VGAT1 in GABA-containing inhibitory terminals in the lateral superior olive. Thus there must be other mechanisms to package GABA into vesicles, such as alternative vesicular transporters or even enzymatic transamination of vesicular glutamate.

A further element necessary for fast ionotropic GABAergic transmission (although not metabotropic signaling) is the presence of postsynaptic $GABA_A$ receptors. At least one $GABA_A$ receptor subunit (γ_3) is relatively selectively expressed in the mossy fiber termination zone (stratum lucidum) of the CA3 hippocampal subfield (Sperk et al., 1997). This issue has not, however, been examined at an ultrastructural level with antibodies against different subunits.

Ultimately, the most direct evidence for GABAergic transmission would be an electrophysiological demonstration that inhibitory postsynaptic currents (IPSCs) can be elicited at mossy fiber synapses. An unambiguous demonstration that GABAergic IPSCs occur at mossy fiber synapses would require paired recordings from identified pairs of granule cells and postsynaptic neurons. However, it has been estimated that a single granule cell has less than a 0.005% probability of making a synaptic contact with an individual CA3 pyramidal neuron (Amaral et al., 1990). Although many more synapses are made with interneurons (Acsady et al., 1998), the projection frequency is still too low to make this approach feasible. Due to this problem, specific pharmacological and physiological criteria have been developed to identify mossy fiber synapses activated by extracellular stimulation of granule cells or their axons.

We have applied stimuli designed to recruit granule cells and recorded from postsynaptic CA3 pyramidal neurons in vitro. Do such stimuli elicit monosynaptic GABAergic signals? A positive answer does not guarantee that the responses are indeed mediated by mossy fibers, because electrical stimuli in the dentate gyrus might also recruit axon collaterals of GABAergic interneurons, some of which have been shown to project into the granule cell layer (Lubke et al., 1998; Sik et al., 1997). Recruitment of such interneurons can, however, be minimized by activating presynaptic neurons via den-

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dritic receptors in stratum moleculare (Weisskopf and Nicoll, 1995).

A more compelling test that GABAergic signals arise from mossy fibers would be to show that they have several highly unusual characteristics of mossy fiber synapses, established from recordings of glutamatergic excitatory postsynaptic currents or potentials (EPSCs or EPSPs). The conventional "signature" of CA3–mossy fiber synapses is 3-fold: profound sensitivity to group II (Kamiya et al., 1996) and, in guinea pig, group III (Yamamoto et al., 1983, Lanthorn et al., 1984) metabotropic glutamate receptor agonists; marked facilitation in response to modest increases in stimulation frequency (Regehr et al., 1994), and NMDA receptor–independent long-term potentiation (LTP) (Harris and Cotman, 1986). One or more of these phenomena have frequently been used as criteria to identify mossy fiber EPSPs and EPSCs (e.g., Scanziani et al., 1997; Maccaferri et al., 1998). If GABAergic signals elicited by stimulating in the dentate gyrus satisfy all three criteria, then the results would provide strong evidence that mossy fibers can indeed signal via the release of GABA, in addition to glutamate, dynorphin, and zinc.

Results

Dentate Gyrus Stimulation Evokes a Nonglutamatergic Signal in CA3

We initially identified mossy fiber projections in guinea pig hippocampal slices by recording field EPSPs with an extracellular electrode positioned in stratum lucidum. In order to minimize the risk of recruitment of interneurons, we stimulated granule cells with an electrode positioned in stratum granulosum of the dentate gyrus rather than in stratum lucidum (Figure 1A). Mossy fiber–mediated field EPSPs were identified by a >2.5-fold increase in amplitude upon switching the stimulation frequency from 0.05 Hz to 1 Hz (Regehr et al., 1994). Once this criterion was met, we obtained a whole-cell voltage-clamp recording from a CA3 pyramidal neuron close to the original recording site, with a pipette containing a CsCl-based solution. We adjusted the intensity of the stratum granulosum stimulus to elicit a large inward postsynaptic current (PSC) when the CA3 neuron was held at −60mV. This PSC contained both monosynaptic and polysynaptic components. Perfusion of the NMDA receptor antagonist DL-2-amino-5-phosphonovalerate (APV; 100 μM) and the AMPA/kainate receptor antagonist 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo [f]quinoxaline-7-sulphonamide disodium (NBQX; 10 μM) reduced but did not abolish the postsynaptic response (Figure 1B). The residual PSC represented ~5% of the initial amplitude (range: 1%–25%), implying that the original PSC was mainly mediated by ionotropic glutamate receptors.

Under identical recording conditions, AMPA, kainate, and NMDA receptor–mediated EPSCs recorded either in pyramidal neurons or in interneurons are completely blocked by 100 μM APV and 10 μM NBQX (Min et al., 1998, 1999; Semyanov and Kullmann, 2000). Thus, in the presence of these antagonists, interneurons cannot be recruited to firing threshold via glutamate receptors. The residual PSC therefore represents a monosynaptic nonglutamatergic signal. This is further supported by the finding that its latency was no different from the latency of the synaptic current elicited in the absence of glutamate receptor antagonists; the mean latency difference before and after addition of NBQX and APV (± SEM) was 0.04 ± 0.16 ms ($n = 10$; paired t test, $p = 0.8$).

Although the initial PSC elicited in the absence of glutamate blockers showed relatively little trial-to-trial fluctuation (CV < 20%), the residual current recorded in APV and NBQX was generally accompanied by intermittent failures of transmission (Figure 1C). This implies that the quantal content was smaller than for the initial mainly glutamatergic signal. The residual PSC had an average amplitude 80 ± 20 pA and a 20%–80% rise time of 6.3 ± 1.0 ms.

Nonglutamatergic PSCs Show Pharmacological and Physiological Features of Mossy Fiber Synapses

We asked whether the residual PSC shows the unusual pharmacological sensitivity and stimulation frequency dependence of mossy fiber field EPSPs.

The use of guinea pig hippocampal slices permitted us to exploit the high sensitivity of mossy fiber synapses to low concentrations of the group III metabotropic glutamate agonist L(+)-2-amino-4-phosphonobutyric acid (L-AP4) (Yamamoto et al., 1983), which is not seen in rat (Lanthorn et al., 1984). These receptors show a highly specific distribution at different synapses in the hippocampus (Shigemoto et al., 1996, 1997). In the continued presence of NBQX and APV, we tested the effect of applying L-AP4 (10 μM). PSCs were decreased to 49% ± 6% of control ($n = 15$; $p < 10^{-6}$); washout = 89% ± 13% (Figures 1C and 1F). The high sensitivity of the residual PSCs to L-AP4 is in marked contrast to the lack of effect of higher concentrations on interneuron-mediated synaptic signals in pyramidal neurons (Scanziani et al., 1998; Semyanov and Kullmann, 2000). We reexamined the effect of L-AP4 on interneuron–CA3 pyramidal neuron synapses by positioning a second stimulating electrode in stratum radiatum (Figure 1A). L-AP4 (10 μM) had no effect on IPSCs elicited via this electrode (Figure 1D). In six neurons tested, the IPSC amplitude in the presence of L-AP4 was 107% ± 18% of control ($p > 0.5$; washout = 96% ± 10%). L-AP4 thus selectively depresses dentate stratum granulosum–evoked PSCs (Figure 1F), compatible with a mossy fiber origin.

In the same experiments, we examined the frequency dependence of dentate stratum granulosum–evoked PSCs to establish whether these responses show the pronounced facilitation with modest increases in frequency exhibited by mossy fiber signals (Regehr et al., 1994). Increasing the stimulation frequency from 0.05 Hz to 1 Hz caused a facilitation to 271% ± 86% of control (mean increase in amplitude 11–20 stimuli after stepping the frequency; $n = 14$; Wilcoxon signed rank, $p < 0.05$; Figure 1E). The average size of the frequency-dependent facilitation met the criterion initially used to identify mossy fibers (see above) and was very similar to that reported for glutamatergic PSCs (Salin et al., 1996). Again, because little is known about the frequency dependence of nonglutamatergic transmission over similar stimulation rates, we delivered the same increment in frequency via the second electrode in CA3

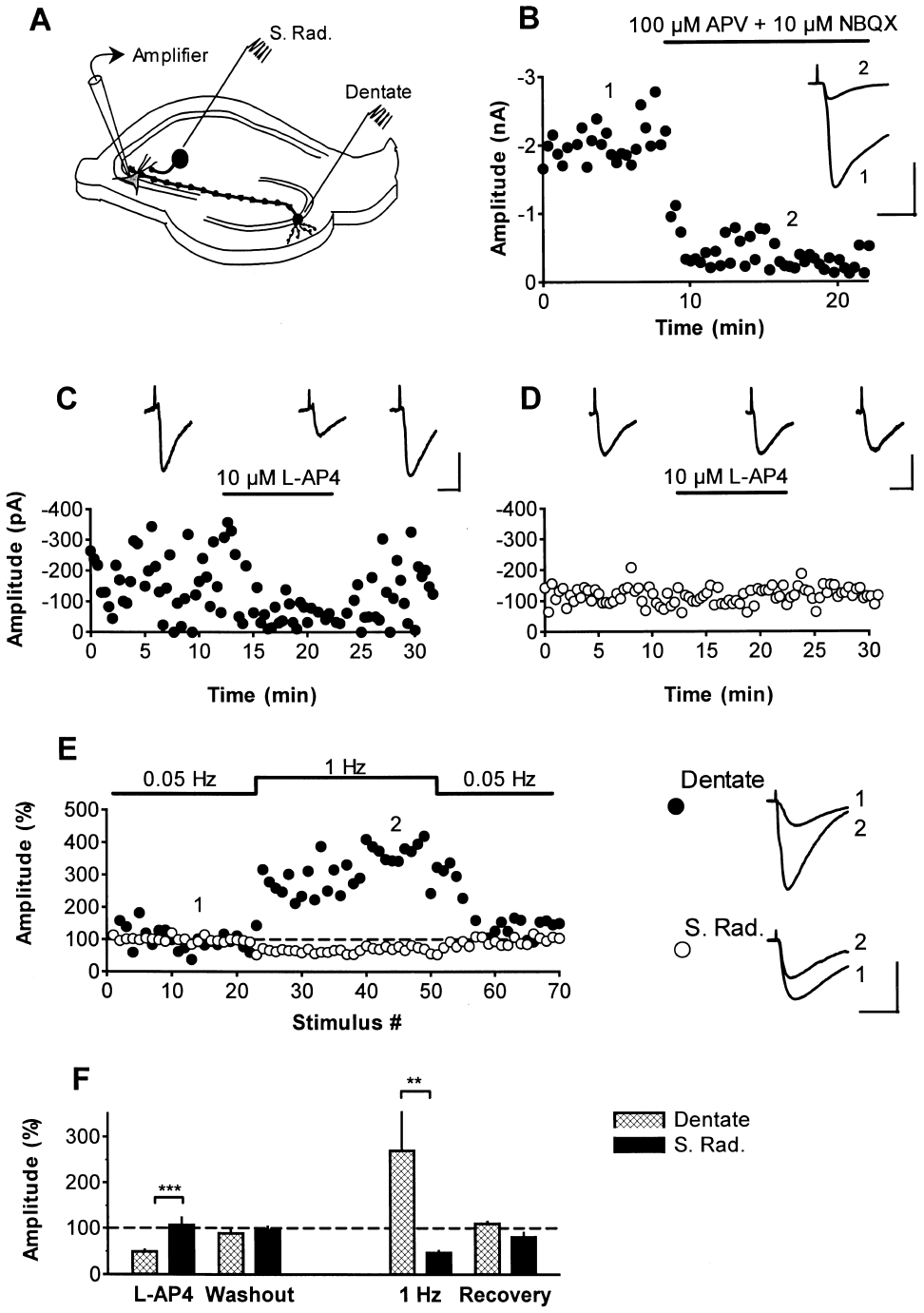

Figure 1. Nonglutamatergic PSCs Evoked by Stratum Granulosum Stimulation

(A) Position of stimulating electrodes: stratum granulosum of dentate gyrus and stratum radiatum (S. Rad.) of CA3.

(B) A residual dentate-evoked PSC remains following blockade of ionotropic glutamate receptors (results from one neuron).

(C and D) Effects of L-AP4 on PSCs. In (C), 10 μM L-AP4 depresses stratum granulosum–evoked PSCs; in (D), 10 μM L-AP4 has no effect on stratum radiatum–evoked PSCs.

(E) PSCs evoked by stratum granulosum (but not stratum radiatum) stimulation exhibit frequency-dependent facilitation. PSC amplitudes obtained in one neuron and normalized to the initial amplitude are plotted against stimulus number.

(F) Summary histogram showing reversible depression of dentate-evoked PSCs by L-AP4 and reversible facilitation in response to increasing the stimulation frequency from 0.05 Hz to 1 Hz. Stratum radiatum–evoked PSCs show no response to L-AP4 and are depressed by 1 Hz stimulation. Triple asterisks, $p < 10^{-6}$; double asterisks, $p < 0.01$.

(B–E) Insets show the averages of seven to ten successive PSCs recorded at the times indicated (the PSC illustrated in [B] contains polysynaptic components, which disappear upon blockade of ionotropic glutamate receptors). Calibration bars in (B), 1 nA, 50 ms; in (C) and (D), 100 pA, 50 ms; and in (E), 500 pA, 50 ms.

stratum radiatum. PSC amplitudes decreased to 51% ± 9% of control ($n = 6$; $p < 0.05$). The difference between dentate (stratum granulosum)- and CA3 (stratum radiatum)-evoked PSCs was significant at $p < 0.01$ (Mann–Whitney U test; Figure 1F).

Thus, dentate stimulus–evoked nonglutamatergic responses show both high sensitivity to L-AP4 and also pronounced frequency-dependent facilitation.

Residual Dentate Gyrus–Evoked PSCs Are Mediated by $GABA_A$ Receptors

We proceeded to investigate which postsynaptic receptors mediated the PSCs evoked by stimulation in stratum granulosum of the dentate gyrus in the presence of ionotropic glutamate receptor antagonists. We carried out the following experiments on PSCs recorded in the presence of NBQX (10 μM) and APV (100 μM) after routinely verifying that they were reversibly attenuated by application of L-AP4 (10 μM). With pipette solutions containing either CsCl ($n = 3$) or CsGluconate ($n = 3$), the PSCs reversed within 5mV of the predicted reversal potentials calculated for $GABA_A$ receptor–mediated responses (Figures 2A and 2B). The PSCs were abolished by the $GABA_A$ antagonists picrotoxin (100 μM; $n = 10$) or bicuculline (10 μM; $n = 6$; Figures 2C and 2E). Moreover, the benzodiazepine agonist zolpidem (ZPM; 200 nM) increased the decay time constant of the PSCs (Figures 2D and 2E) by 29% ± 2% ($n = 3$; t test, $p < 0.01$). Thus, the responses showed reversal potentials and pharmacological sensitivities typical of $GABA_A$ receptor–mediated IPSCs, with no evidence for a residual component mediated by other transmitter/receptor systems.

Neither (+)α-methyl-4-carboxyphenylglycine (MCPG; 500 μM; $n = 3$) nor methyllycaconitine (MLA; 200 nM; $n = 2$) significantly affected the current (Figure 2E; $p > 0.5$). These results indicate that the PSCs were neither directly mediated nor indirectly modulated by group I metabotropic glutamate receptors or nicotinic receptors. We also confirmed that CA3 stratum radiatum–evoked PSCs were GABAergic, as expected from the recruitment of local interneurons (data not shown).

Minimal Stimulation in Stratum Granulosum Evokes IPSCs in CA3 Pyramidal Neurons

Unitary granule cell–evoked responses can be elicited by minimal stimulation in stratum granulosum (Jonas et al., 1993). With this technique, we obtained all-or-none GABAergic responses in DL-APV (100 μM) and NBQX (10 μM) that appeared abruptly with a failure rate of 20%–30%, at stimulation intensities similar to those required for glutamatergic responses recorded in the same conditions ($n = 3$; Figure 3A). This failure rate is similar to that reported for the glutamatergic response (Jonas et al., 1993) and further supports the hypothesis that the GABAergic response is monosynaptic. L-AP4 (10 μM) depressed the minimal stimulation–evoked IPSCs (Figure 3A), further confirming a mossy fiber origin.

If mossy fibers can release both GABA and glutamate, it should be possible to record minimal stimulation–evoked PSCs mediated by both transmitters from the same presynaptic axon. We attempted to record such responses by using a CsGluconate recording pipette, while holding the postsynaptic neuron between the reversal potentials for glutamate and GABA. With this approach, however, we mainly found purely inward currents mediated by AMPA receptors (data not shown). This is consistent with a lower quantal content for the GABAergic component and prompts the hypothesis that most mossy fibers either do not release GABA or only release it with a low probability. We therefore took an alternative approach; by optimizing the stimulus to recruit a unitary GABAergic PSC with glutamate receptors blocked, can we subsequently record a glutamateric PSC, and, if so, does this have the same latency and abrupt threshold, implying the same presynaptic origin?

We applied minimal stimulation in stratum lucidum, with 5 mM kynurenic acid used to block ionotropic glutamate receptors. We then adjusted the position of the stimulating electrode to elicit a GABAergic response with an abrupt stimulus threshold and plateau consistent with single-fiber stimulation (Figure 3B). Once such a response was obtained, we washed out kynurenic acid but left NMDA receptors blocked with DL-APV (100 μM). We then held the cell at a membrane potential between the reversal potentials for $GABA_A$ and AMPA receptors. In each of four cells, the responses fluctuated between failures and inward (glutamatergic), outward (GABAergic), and biphasic (dual component glutamatergic and GABAergic) synaptic currents, with relative frequencies suggestive of independent release of the two transmitters (Figure 3C). Washing in bicuculline (10 μM) abolished the outward PSCs, confirming that they were GABAergic and leaving inward (glutamatergic) PSCs. These pure glutamatergic PSCs had the same latency and the same threshold as the GABAergic PSCs ($n = 4$), consistent with an identical presynaptic origin. These results imply that a single mossy fiber can release both GABA and glutamate, albeit from distinct populations of vesicles (although in common with all minimal stimulation experiments, we cannot exclude a coincidence where two fibers run close together with identical stimulus thresholds).

The 20%–80% rise time for the glutamatergic component of the unitary PSC (1.4 ± 0.2 ms; $n = 4$) was consistent with the rise times of mossy fiber–CA3 pyramidal cell PSCs obtained under similar conditions by others (Yeckel et al. 1999; Toth et al. 2000). The 20%–80% rise time of the GABAergic component recorded in the same cells was, however, always longer (3.7 ± 0.8 ms). The possible explanation for the relatively slow time course of the GABAergic component (which was also seen with the larger IPSCs recorded throughout this study) is considered in the Discussion.

Glutamate Application in Stratum Moleculare Evokes mGluR Agonist-Sensitive GABAergic IPSCs in CA3

Although the behavior of dentate-evoked IPSCs described above is compatible with GABA release from mossy fibers, the results do not exclude the possibility that they were generated by activation of interneurons that send axon collaterals into the dentate gyrus. Such interneurons would also have to show the otherwise unique frequency-dependent facilitation and metabo-

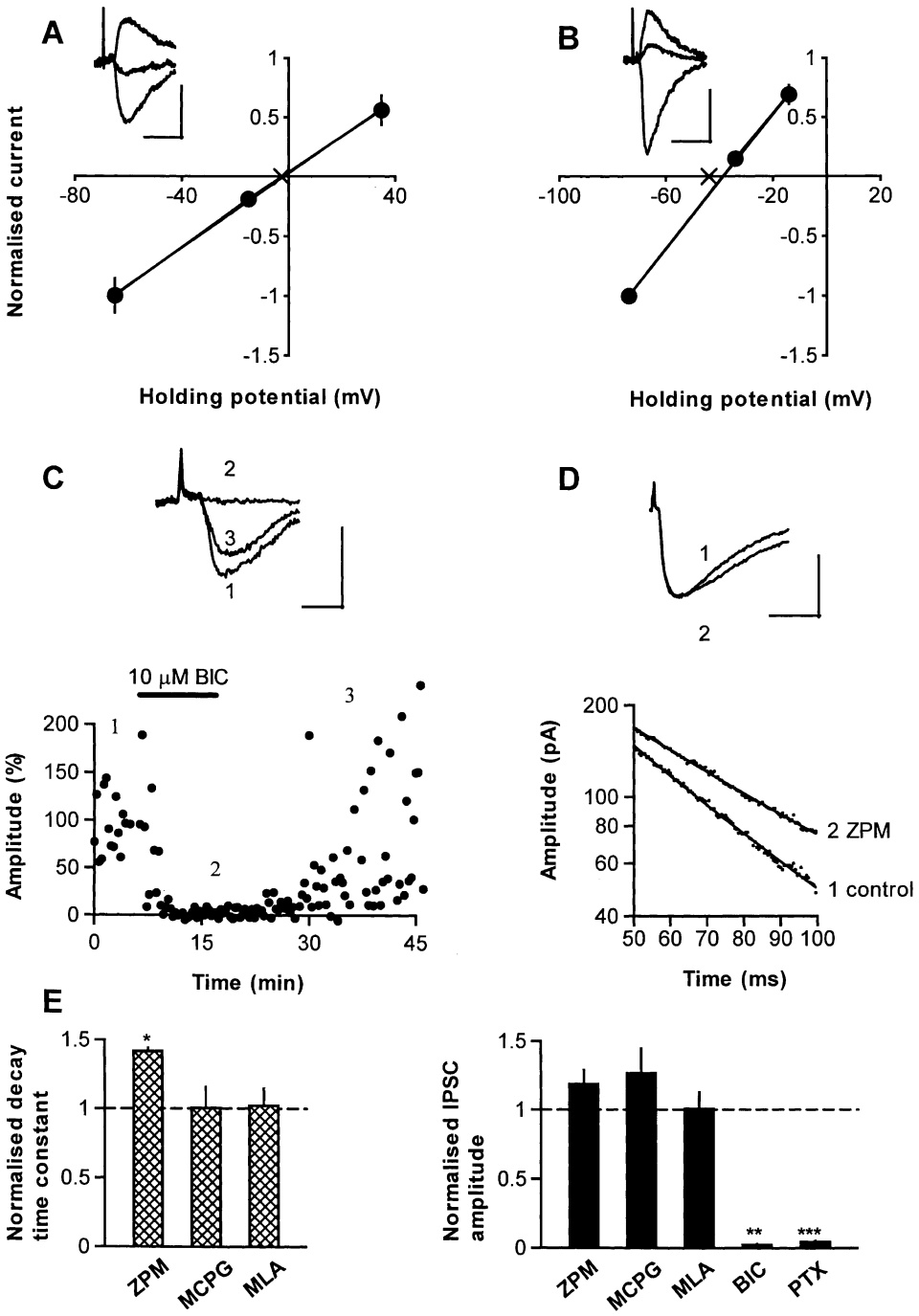

Figure 2. Dentate-Evoked PSCs Recorded in NBQX and APV Are Mediated by $GABA_A$ Receptors

All PSCs showed a reversible >40% reduction by 10 μM L-AP4.

(A and B) The reversal potential is determined by the intracellular Cl^- concentration. (A) I-V relation for stratum granulosum–evoked PSC recorded in one neuron with a CsCl-based pipette solution. In (B), replacing CsCl with Cs gluconate in another neuron shifts the reversal potential. The calculated reversal potentials for $GABA_A$ receptors ([A], –2.7mV; [B], –43.8mV) are marked with a cross. Each point represents the mean (± SEM) amplitudes of three PSCs. Insets, average traces corresponding to each point.

(C) Bicuculline methiodide (10 μM) reversibly depresses the stratum granulosum–evoked GABAergic IPSC.

(D) The benzodiazepine agonist zolpidem (ZPM; 200 nM) increases the decay time constant of dentate-evoked PSCs (data from one neuron). The averaged decay phases of the PSCs are shown together with monoexponential fits. Inset, average traces.

(E) Pharmacological sensitivity of dentate-evoked PSCs recorded in NBQX and APV (data from six cells). ZPM significantly prolonged the decay time constant ($n = 3$; control $\tau = 40 \pm 9$ ms). MCPG (500 μM; $n = 3$) and MLA (200 nM; $n = 2$) were without effect. Bicuculline (BIC; 10 μM; $n = 6$) and picrotoxin (PTX; 100 μM; $n = 10$) both abolished the responses.

(A–E) Asterisk, $p < 0.01$; double asterisks, $p < 10^{-7}$; triple asterisks, $p < 10^{-12}$. Calibration bars in (A), (B), and (D), 100 pA, 50 ms; in (C), 50 pA, 20 ms.

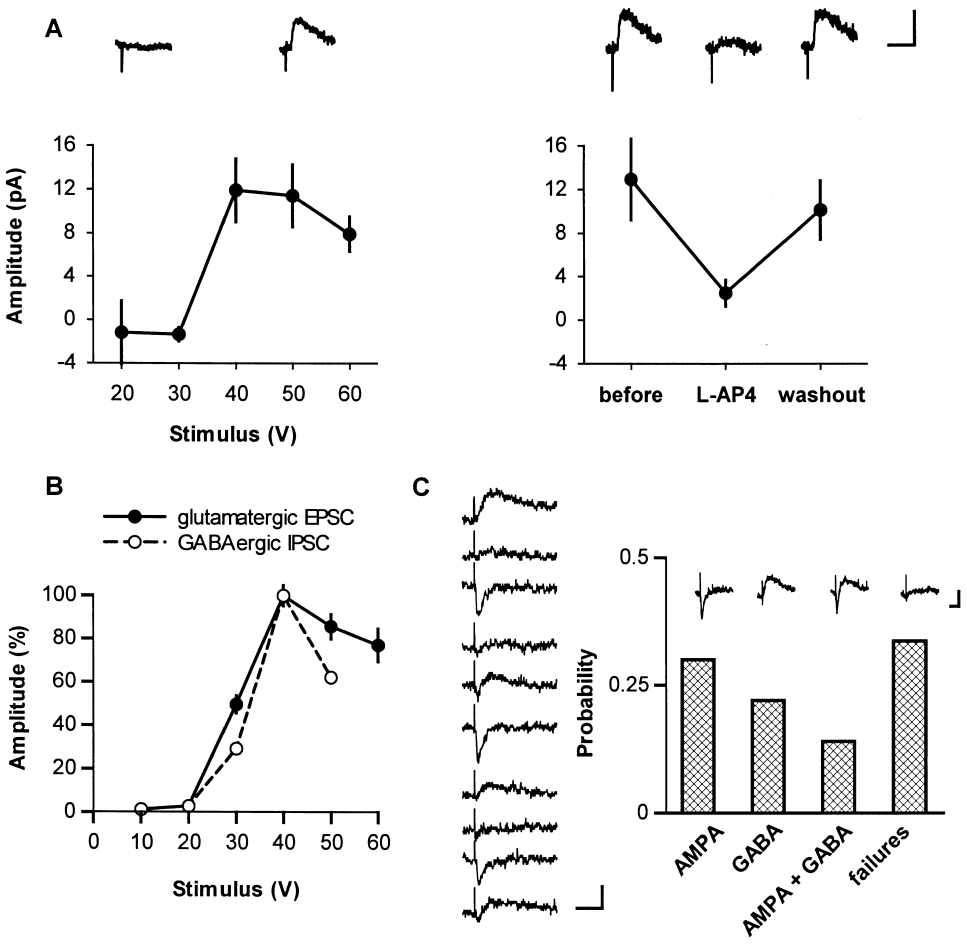

Figure 3. Minimal Stimulation Evokes GABAergic PSCs in CA3 Pyramidal Neurons that Respond to L-AP4 and Have the Same Threshold as Glutamatergic PSCs

(A) Stimulation (50 μs pulse width) via a monopolar glass electrode (1 MΩ) positioned in stratum granulosum (Jonas et al., 1993) in the presence of NBQX (10 μM) and APV (100 μM) elicits a response in a CA3 pyramidal cell with an abrupt stimulus threshold (left). The plot shows mean ± SEM current amplitude (including failures) averaged at each stimulation voltage. The failure rate decreased from 100% at 20V–30V to 27% at 40V–60V. The response was reversibly depressed by 10 min of L-AP4 (10 μM) application (right). Traces are averages of ten successive PSCs.
(B) Minimal stimulation via a monopolar glass electrode (3 MΩ; 25 μs pulse width) in stratum lucidum elicits unitary GABAergic responses in 5 mM kynurenic acid that have the same threshold as unitary glutamatergic responses in 10 μM bicuculline and 100 μM DL-APV.
(C) In the same cell, in the presence of DL-APV (100 μM) at a holding potential of −40mV, stimulation at 40V elicits failures and inward (glutamatergic), outward (GABAergic), and biphasic (dual component) PSCs. Single traces (left) are shown together with a histogram showing the relative frequency of each type of response, classified by eye (right, traces show averages of ten responses of each type).
(A–C) Calibration bars in (A), 10 pA, 50 ms; in (B) and (C), 5 pA, 50 ms.

tropic receptor agonist sensitivity of mossy fibers. We addressed this possibility by activating presynaptic neurons via ionotropic receptors that are expressed on dendrites and cell bodies but are generally agreed to be absent from axons. We pressure-applied glutamate via a pipette positioned in stratum moleculare, close to a site where electrical stimulation evoked mossy fiber fEPSPs. NBQX (10 μM) was present, but APV was omitted from the perfusion solution in order to allow excitation of neurons via dendritic NMDA receptors (Weisskopf and Nicoll, 1995).

We first performed control experiments to verify that this method could recruit granule cells without activating hilar interneurons. Glutamate was applied in various sites in stratum moleculare while recording from a granule cell (Figure 4). When applied in the dendritic region of the recorded granule cell, a depolarization lasting 5–10 s was recorded (Figure 4A). This was unaffected by L-AP4. Displacement of the glutamate application pipette by >150 μm abolished the depolarization (Figure 4B). Similarly, no depolarization was seen when recording from hilar interneurons in three experiments. Conversely, if the glutamate application pipette was moved into the hilar region, the interneuron could be depolarized ($n = 3$).

These control experiments imply that glutamate application in stratum moleculare is sufficiently selective to prevent activation of hilar interneurons, many of which send axons through the granule cell layer. Can chemical activation of granule cells elicit GABAergic IPSCs in CA3 pyramidal neurons?

We positioned the glutamate application pipette in

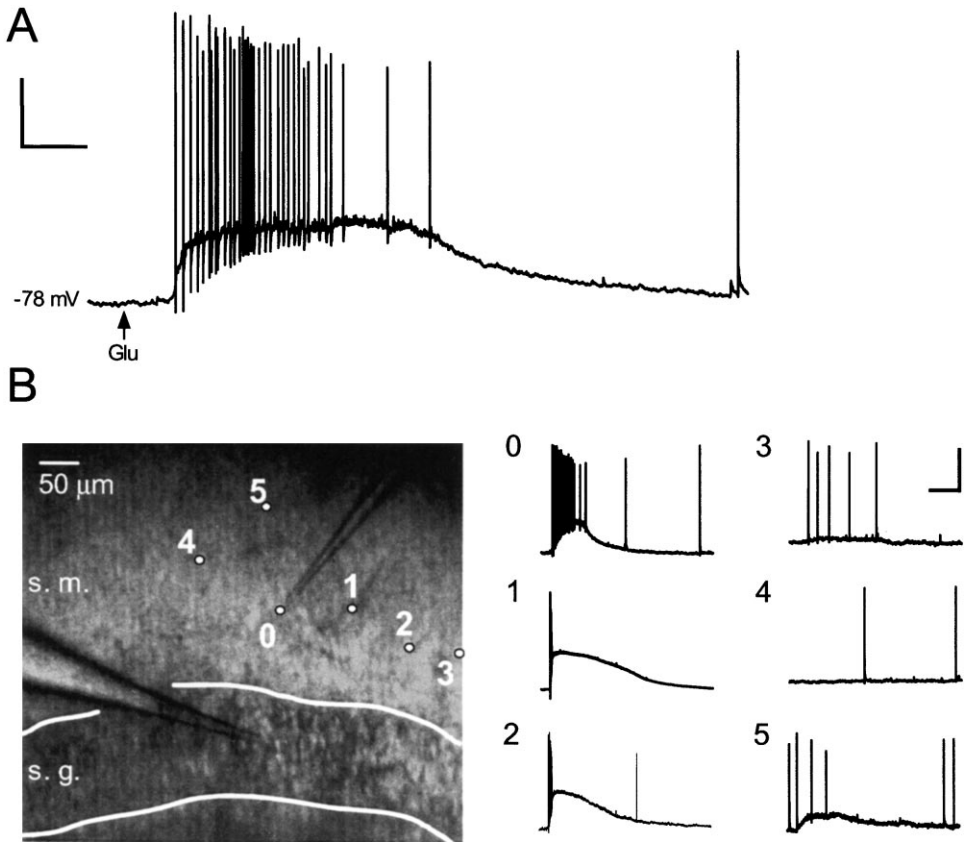

Figure 4. Puff Application of Glutamate Evokes Trains of Action Potentials in Dentate Granule Cells Recorded in Current Clamp

(A) Example of an action potential train following glutamate application (10 μM NBQX). The amplitude of the depolarization and number of action potentials elicited by glutamate application were not significantly affected by L-AP4 (10 μM) but were abolished by APV (100 μM) ($n = 3$). L-AP4 similarly had no effect on current–voltage relationships obtained from intrasomatic current injection ($n = 3$).

(B) Glutamate application elicits a depolarization and action potentials in a restricted area of stratum moleculare. (Left) DIC image showing the positions (numbered 0–5) where glutamate was applied during recording from one granule cell (s. g., stratum granulosum; s. m., stratum moleculare. (Right) Traces obtained in response to glutamate application at the corresponding positions. When a hilar interneuron was subsequently recorded, glutamate application in stratum moleculare elicited no response; action potentials could be elicited when the glutamate application pipette was placed in the hilus, within 50 μm of its soma ($n = 3$). Calibration: 20mV, 1s.

stratum moleculare, close to the site where electrical stimulation evoked a mossy fiber field EPSP, and recorded from a CA3 pyramidal neuron after adding NBQX (10 μM) to the perfusion solution. The pyramidal neuron was held hyperpolarized (−60mV) to prevent NMDA receptor opening. Glutamate application resulted in a burst of PSCs (Figure 5A). The train lasted a similar duration to the depolarization seen with granule cell recordings (Figure 4). Perfusion of L-AP4 (10 μM) reversibly reduced the frequency of glutamate-evoked PSCs to 23% ± 12% of control ($n = 6$; $p < 0.001$; Figures 5B and 5D). Their mean amplitude was simultaneously reduced by 17% ± 4% ($p < 0.05$; Figure 5C). In the same neurons, PSCs evoked by electrical stimulation in dentate stratum granulosum were reversibly depressed by L-AP4 (Figure 5E). Both glutamate-evoked and stimulus-evoked PSCs were abolished by picrotoxin, indicating that they were mediated by $GABA_A$ receptors (Figure 5D; stimulus-evoked IPSCs not shown). The background frequency of spontaneous IPSCs occurring in between successive glutamate applications was much less sensitive to L-AP4 (reduced to 85% ± 2% of control; $p <$ 0.05). The lesser sensitivity of spontaneous IPSCs to L-AP4 is compatible with the possibility that most of these arise from interneurons.

Glutamate application to the dendritic region of dentate granule cells thus elicits GABAergic PSCs in CA3 pyramidal neurons with a high sensitivity to L-AP4. Note that AMPA and kainate receptors were blocked in these experiments. This allowed the GABAergic signal to be detected without extensive contamination by glutamatergic mossy fiber PSCs. Moreover, blockade of AMPA and kainate receptors minimized or prevented the recruitment of interneurons. The IPSCs seen in CA3 pyramidal neurons are thus highly likely to be monosynaptic and to arise from neurons with dendrites in stratum moleculare, namely, granule cells.

Because the terminals of hilar interneurons may have presynaptic metabotropic receptors (Poncer et al., 1995), we repeated the experiments by applying glutamate in the dentate hilus in order to deliberately recruit hilar interneurons but not mossy fibers passing through this region. Although glutamate application again evoked trains of PSCs in CA3 pyramidal neurons, they were only

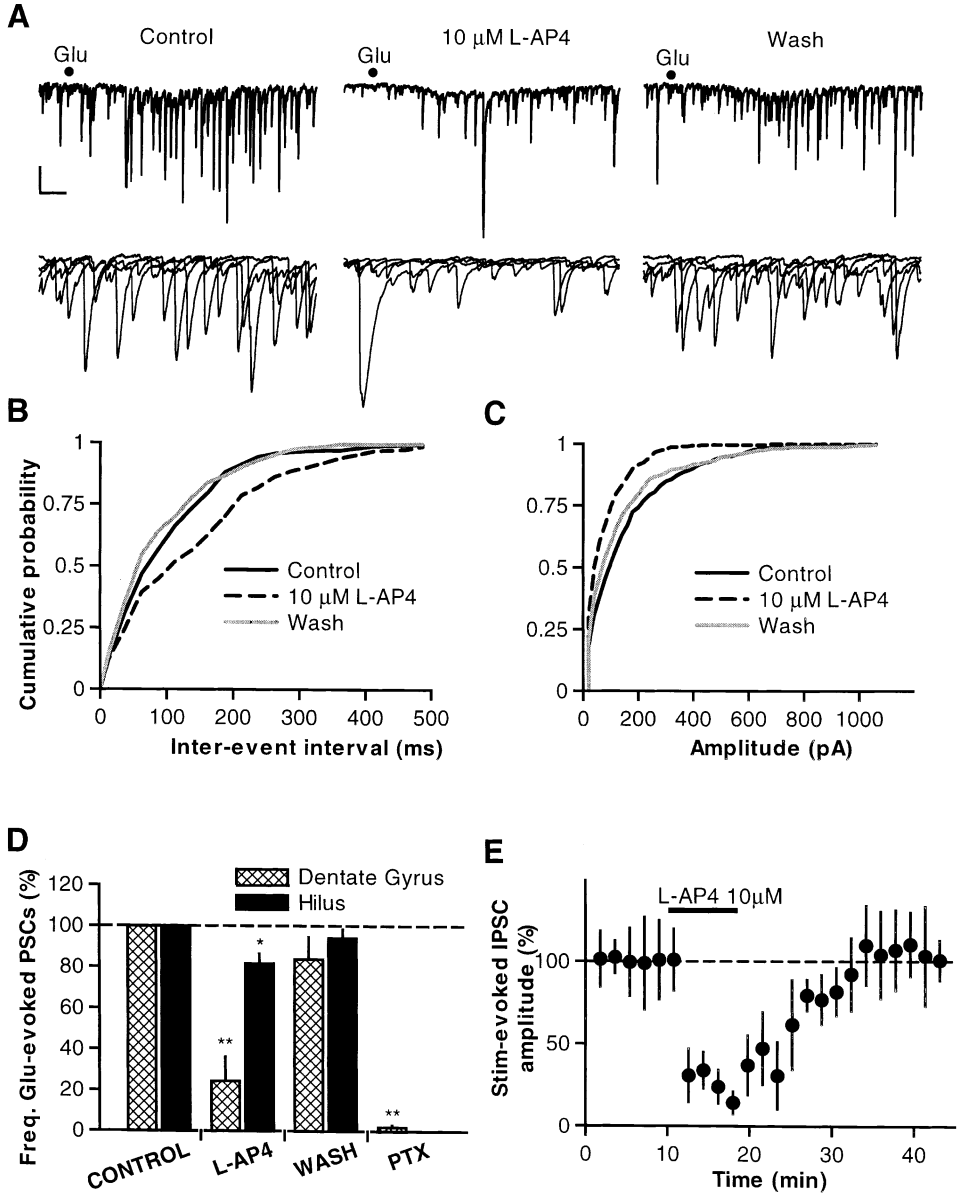

Figure 5. GABAergic PSCs Evoked by Pressure Application of Glutamate in the Dentate Gyrus Are Sensitive to L-AP4

(A–C) The effect of L-AP4 (10 μM) on the frequency and amplitude of PSCs elicited in one CA3 pyramidal neuron by pressure application of glutamate in stratum moleculare. In (A), the upper traces show glutamate-evoked PSCs before, during, and after L-AP4. (Lower traces) Four consecutive 1 s epochs superimposed. Calibration: 100 pA, 1 s (upper traces), 100 ms (lower traces). Activation of group III mGluRs by L-AP4 causes a reversible rightward shift of the cumulative distribution of interevent intervals (B) and a leftward shift of the cumulative distribution of PSC amplitudes (C).

(D) Summary of L-AP4 action on the frequency of glutamate-evoked PSCs and inhibition by picrotoxin (PTX). Glutamate was applied five times under each condition, and the background rates of PSCs were subtracted to give the measured frequencies. Glutamate-evoked PSCs in the dentate were reduced by L-AP4 and completely abolished by PTX (double asterisks: $p < 0.001$, Student's paired t test, $n = 6$). PSCs evoked by glutamate application to the hilus were less sensitive to L-AP4 (asterisk: $p < 0.05$, $n = 6$).

(E) Stratum granulosum stimulus-evoked PSC amplitudes (mean ± SEM), recorded in the same cells as shown in (B).

minimally depressed by 10 μM L-AP4 (82% ± 6% of control; $n = 7$; Figure 5D).

To verify that the above results were not a peculiarity of guinea pigs, we examined stratum granulosum–evoked PSCs in hippocampal slices from rats. In this species, L-AP4 cannot be used to identify mossy fibers (Lanthorn et al., 1984), so we instead applied the group II agonist (2S,2′R,3′R)-2-(2′,3′-dicarboxycyclopropyl)-glycine (DCG-IV) (Kamiya et al., 1996). We tested the effect of applying 1 μM DCG-IV on the residual PSCs recorded in the presence of NBQX (10 μM). The mean PSC amplitude was reduced to 38% ± 8% of control ($p < 0.02$; $n = 3$; washout = 90% ± 5%) and was subsequently completely abolished by picrotoxin (100 μM). In the same experiments, pressure application of glutamate via a pipette positioned in stratum moleculare

close to the stimulating electrode evoked bursts of PSCs as in the guinea pig experiments. Their frequency was reduced by DCG-IV (to 16% ± 10% of control; $p < 0.001$), and they were abolished by picrotoxin. Thus, GABAergic PSCs can be elicited both in guinea pigs and in rats by stimulating dentate granule cells, and these PSCs show a similar sensitivity to metabotropic glutamate receptor agonists as do conventional mossy fiber signals.

Dentate-Evoked IPSCs Show NMDA Receptor–Independent LTP

An unusual feature of mossy fiber synapses is that they exhibit NMDA receptor–independent LTP, which is expressed presynaptically (Harris and Cotman, 1986). We therefore examined the effect of tetanization on the GABAergic IPSCs evoked by electric stimulation in stratum granulosum of the dentate gyrus in the presence of NBQX (10 μM) and APV (100 μM). Two high-frequency tetani (100 Hz; 1 s, 10 s interval) were followed by a very pronounced initial potentiation, which gradually decayed to reach a plateau of 188% ± 35% after 10–20 min ($n = 9$; $p < 0.05$; Figures 6A and 6B). This plateau was accompanied by an increase in the statistic $1/CV^2$ (LTP/baseline ratio of $1/CV^2 = 3.7 \pm 1.3$; Wilcoxon signed rank, $p < 0.05$; Figure 6C), consistent with a presynaptic site of expression, as has been reported for mossy fiber LTP of glutamatergic signals.

Because tetanic stimulation has been reported to cause a slow-onset potentiation of GABAergic IPSCs in CA1 pyramidal neurons (Shew et al., 2000), we examined the effect of delivering tetani via a CA3 stratum radiatum stimulating electrode. The IPSCs evoked via this electrode showed no potentiation, either early or late (Figures 6A and 6B; $n = 4$; $p = 0.4$).

Mossy fiber LTP is mimicked and partially occluded by application of the adenylate cyclase activator forskolin (Weisskopf et al., 1994). We therefore tested the effect of forskolin application (50 μM 10–20 min) on GABAergic IPSCs evoked by stratum granulosum stimulation. This treatment led to an increase in IPSC amplitude to 228% ± 61% ($n = 7$). When the IPSCs reached a plateau, two 100 Hz tetani were applied. In contrast to the effect in naive slices, this stimulus produced no further potentiation (Figure 6D). Thus, GABAergic IPSCs elicited by stratum granulosum stimulation shows the same frequency- and forskolin-evoked plasticity as mossy fiber EPSPs.

Discussion

GABAergic PSCs elicited by dentate gyrus stimulation showed the same pharmacological sensitivity and use-dependent plasticity normally associated with glutamatergic mossy fiber signals: reduction by metabotropic glutamate receptor agonists (Yamamoto et al., 1983; Kamiya et al., 1996), frequency-dependent facilitation (Regehr et al., 1994; Salin et al., 1996), and NMDA receptor–independent LTP (Nicoll and Malenka, 1995). Indeed, these properties are widely used as defining criteria for mossy fiber synaptic responses (Scanziani et al., 1997; Maccaferri et al., 1998). The present results show that these phenomena apply not only to glutamatergic mossy fiber transmission but also to GABAergic signaling from the dentate gyrus to CA3. In contrast, IPSCs elicited by CA3 stratum radiatum stimulation were unaffected by L-AP4, were depressed by increasing the stimulus frequency, and showed no LTP following tetanic stimulation. The results are consistent with the release of GABA from mossy fibers, as suggested by the immunohistochemical evidence for the presence of GABA, GAD65, and GAD67 in this pathway. Alternatively, the responses originate from a population of unidentified interneurons that project from stratum moleculare and show all the physiological and pharmacological features of granule cells.

Although the GABAergic signals elicited from stratum granulosum stimulation satisfied all the criteria used to identify glutamatergic mossy fibers, some reports have described quantitatively larger effects of metabotropic glutamate agonists or stimulation frequency increments on field EPSPs recorded in stratum lucidum (e.g., Regehr et al., 1994). This discrepancy may reflect the fact that the GABAergic IPSCs recorded here were inevitably contaminated by stimulation of some interneuron axon collaterals, some of which synapse on the postsynaptic CA3 cell. This would have the effect of diluting the mossy fiber "signature" of the responses elicited by granule cell stimulation. In contrast, contamination of mossy fiber stimulation would not have this effect on field EPSPs, because only synapses in stratum lucidum contribute to the local current sink that generates the response.

The present results can be explained by action potential–dependent release of GABA from mossy fiber terminals, which activates postsynaptic $GABA_A$ receptors on CA3 pyramidal neurons. Alternatively, a previously unknown population of abundant GABAergic neurons projects from the dentate gyrus to CA3, with terminals that have presynaptic pharmacological and physiological properties identical to those of mossy fibers. We cannot exclude this possibility, but, if such neurons exist, they have yet to be identified. Interneurons have been described that have dendrites projecting through stratum granulosum, but they are not known to project to CA3a and CA3b (Sik et al., 1997; Lubke et al., 1998). They are unlikely to be the recently described "mossy fiber–associated" interneurons (Spruston et al., 1997; Vida and Frotscher, 2000), as these should not be recruited by glutamate application in stratum moleculare. Moreover, the GABAergic PSCs generated by these interneurons do not show pronounced frequency-dependent facilitation.

The results are highly unlikely to arise from the transsynaptic recruitment of interneurons by mossy fibers. First, in the presence of ionotropic glutamate receptor blockers, it is unclear how they could be depolarized to firing threshold. Second, there was no evidence for an increase in PSC latency upon addition of NBQX and APV. Third, tetanic stimulation elicited NMDA receptor–independent LTP of the GABAergic signals, in contrast to mossy fiber to interneuron synapses (Maccaferri et al., 1998). Finally, L-AP4-sensitive GABAergic PSCs could be elicitied with a low failure rate by minimal stimulation in stratum granulosum (Jonas et al., 1993).

If the present results do reflect GABA release from mossy fiber terminals, an important question is whether

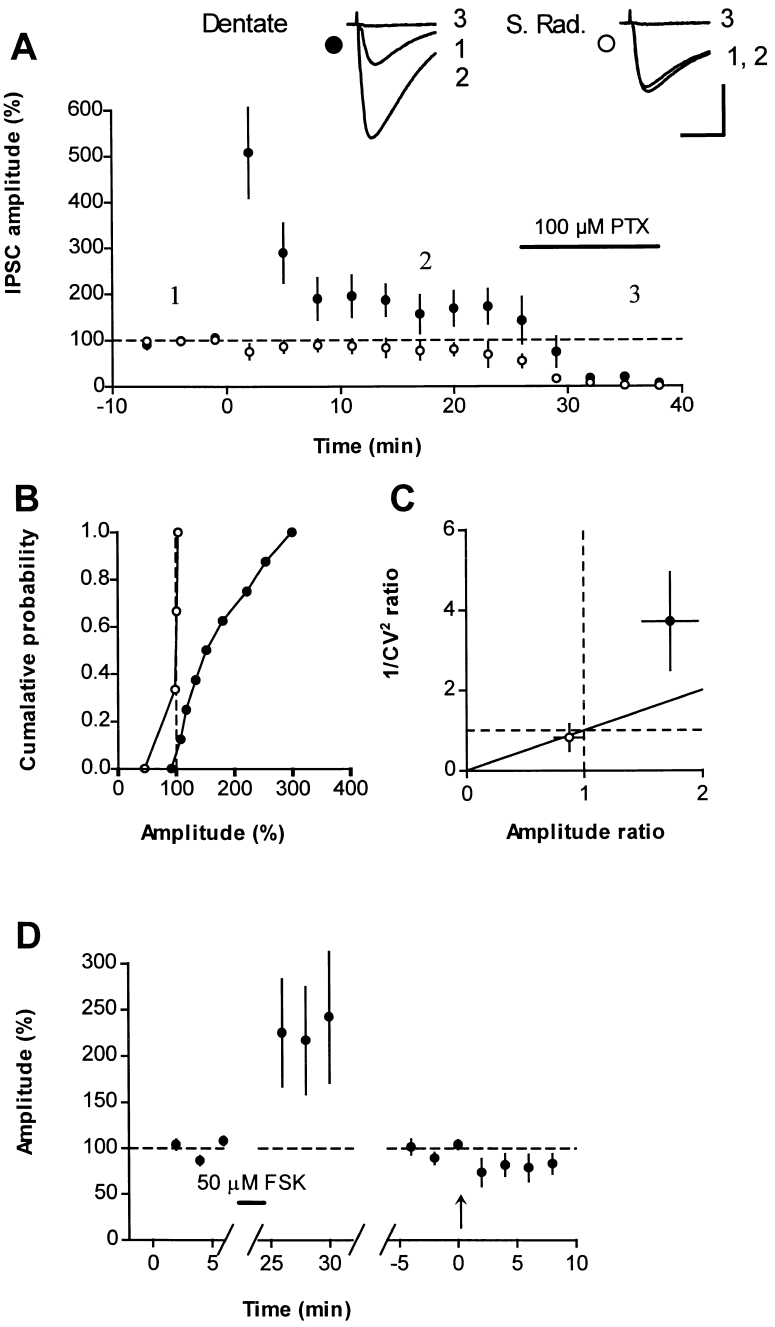

Figure 6. GABAergic PSCs Evoked by Stimulation of Granule Cells Show NMDA Receptor–Independent LTP

(A) Mean PSC amplitudes evoked by dentate ($n = 9$; closed circles) or stratum radiatum ($n = 4$; open circles) stimulation. Both pathways were tetanized at time 0 (2×100 Hz, 1 s, 10 s interval), resulting in LTP of dentate-evoked PSCs but not stratum radiatum–evoked PSCs. PSCs evoked by both stimuli were subsequently abolished by picrotoxin (100 μM). Sample traces were averaged from seven to ten responses recorded in one cell at the time points indicated. Calibration bar in (A): 400 pA, 50 ms.

(B) Cumulative probability histogram showing the change in PSC amplitude at 10–20 min (closed circles, dentate evoked; open circles, stratum radiatum evoked; Kolmogorov–Smirnov test, $p < 0.01$).

(C) Evidence for presynaptic locus of LTP. The mean fractional change in $1/CV^2$ is plotted against the mean fractional change in PSC amplitude for dentate-evoked (filled circle) or stratum radiatum–evoked (open circle) signals.

(D) LTP is occluded by prior application of forskolin (FSK). Application of 50 μM forskolin for 10–20 min followed by washout results in a potentiation of the PSC to 288% ± 61%. The PSCs were then renormalized. Tetanization (2×100 Hz, 1 s, 10 s interval) at time indicated by arrow results in no further potentiation.

both glutamate and GABA are released from the same terminals and, if so, whether they are packaged in the same vesicles, as has been argued for glycine and GABA at some spinal interneuron synapses (Jonas et al., 1998). Our results argue against corelease from the same vesicles. First, the emergence of transmission failures after blocking glutamate receptors suggests either that there are mossy fiber synapses that lack postsynaptic $GABA_A$ receptors or that many glutamate release events are not accompanied by GABA release. Second, with minimal stimulation, we observed independent GABAergic and glutamatergic PSCs as well as biphasic responses, all with the same threshold, consistent with single-fiber stimulation. If, as our results suggest, GABA release only occurs from a minority of mossy fibers, it will be important to determine if these fibers differ with respect to maturation, because GABAergic transmission may play an important role in the formation of glutamatergic synapses (Ben-Ari et al., 1997).

There have been extensive studies of the kinetics of glutamatergic mossy fiber transmission (see Williams and Johnston, 1991; Jonas et al., 1993) demonstrating a fast rise time for AMPA-mediated responses at this synapse. It has, however, also been argued that the criteria used in these studies were designed to select for fast rise times and that the full distribution of unitary mossy fiber EPSC kinetic parameters were not accurately described (Henze et al., 2000; Toth et al., 2000).

The rise times for unitary mossy fiber glutamatergic responses that we observed using uncompensated series resistance were consistent with those seen in other studies in similar conditions (Yeckel et al. 1999; Henze et al., 2000; Toth et al. 2000). The GABAergic PSC, however, had a relatively slow rise time, consistent with the hypothesis that the $GABA_A$ receptors are exposed to a relatively low concentration of GABA following presynaptic release (Maconochie et al., 1994). This may reflect dilution of vesicular GABA into the large mossy fiber cleft, with $GABA_A$ receptors positioned relatively far from the release site.

What functional roles could be served by GABA release from mossy fibers? One possibility is that the postsynaptic $GABA_A$ receptor–mediated signals are of little quantitative importance and that the major target of GABA is actually presynaptic $GABA_B$ receptors. This could explain why heterosynaptic depression among mossy fibers is sensitive to $GABA_B$ antagonists (Min et al., 1998; Vogt and Nicoll, 1999). If so, GABA-mediated autoreceptors at mossy fiber terminals could represent a further level of modulation of transmission, in addition to that mediated by zinc, metabotropic glutamate, and dynorphin receptors.

Interestingly, the expression of GAD67 and GAD65 as well as GABA-like immunoreactivity has been reported to increase in granule cells following prolonged stimulation (Sloviter et al., 1996; Makiura et al., 1999) or kainate injection (Schwarzer and Sperk, 1995). Kainate-induced seizures are also followed by a selective increase in expression of γ_3 subunits of $GABA_A$ receptors restricted to stratum lucidum (Schwarzer et al., 1997). These observations suggest that the balance of glutamatergic and GABAergic signaling at mossy fiber synapses is under dynamic control. Independent regulation of the two signals could provide an additional degree of freedom in the transmission of information from the dentate gyrus to the hippocampus.

One possible evolutionary advantage of upregulating GABAergic transmission from mossy fibers would be to act as a brake that reduces excitatory flow through the hippocampus following seizures. Such a phenomenon could have antiepileptogenic effects. However, because interneurons have been shown to represent numerically a larger target than CA3 pyramidal neurons (Acsady et al., 1998), it will be important to determine whether mossy fiber signaling to interneurons also shows a GABAergic component. Since these terminals also show GABA-like immunoreactivity (Sandler and Smith, 1991), an enhancement of GABA release following seizures could actually have a net disinhibitory effect on the hippocampus and could therefore contribute to epileptogenesis. Indeed, upregulation of GABA release at mossy fiber–interneuron synapses could contribute to the functional disconnection of inhibition that may underlie the development of hyperexcitability following prolonged seizures (Sloviter, 1991).

In conclusion, we have demonstrated that the unique use-dependent and pharmacological plasticity of mossy fiber synapses is shared by a monosynaptic GABAergic projection from dentate gyrus to CA3 pyramidal neurons. These results provide a compelling candidate synaptic signal mediated by the release of GABA from mossy fiber terminals.

Experimental Procedures

Slice Preparation and Electrophysiology

Hippocampal slices (450 μm thick) were obtained from guinea pigs (3–5 weeks) or rats (9–21 days) and were maintained at room temperature in artificial cerebrospinal fluid (ACSF) containing (in mM) NaCl, 119; KCl, 2.5; $MgCl_2$, 4; $CaCl_2$, 4; $NaHCO_3$, 26.2; NaH_2PO_4, 1; and glucose, 11; gassed with 95% O2/5% CO_2. We placed bipolar stainless-steel stimulating electrodes in stratum granulosum of the dentate gyrus and in stratum radiatum of CA3b, as shown in Figure 1A (20 μs; 20V–80V). Field EPSPs were recorded via a glass pipette containing ACSF. If a mossy fiber response was not identified, the slice was discarded. Whole-cell voltage-clamp recordings were obtained from CA3 pyramidal neurons in CA3a or CA3b. Pipettes (3–5 MΩ) contained (in mM) CsCl, 135; HEPES, 10; NaCl, 8; EGTA, 2; $MgCl_2$, 0.2; MgATP, 2; GTP, 0.3; and QX314 Br, 5 ([pH 7.2] osmolarity corrected to 295 mOsm). In some experiments, CsGluconate was substituted for CsCl. Granule cell recordings were obtained using KCl-containing pipettes. The stimulus response latencies for field EPSPs and PSCs were consistent with several studies of mossy fiber transmission in guinea pig hippocampal slices that have used a similar positioning of stimulating (stratum granulosum) and recording (CA3a or CA3b) electrodes (Weisskopf et al., 1994; Salin et al., 1996; Vogt and Nicoll, 1999). Series resistances were ≤20 MΩ and were not routinely compensated. $1/CV^2$ was calculated as (mean PSC)^2/(Var_{PSC} − Var_{noise}).

Minimal stimulation was delivered via a monopolar glass electrode (1 MΩ resistance; 50 μs pulse width) filled with artificial CSF, advanced into stratum granulosum as described by Jonas et al. (1993). The site for stimulation was chosen on the basis of prior identification of field EPSPs with stimulation via a bipolar electrode as described above. To search for dual component GABAergic-glutamatergic PSCs, a monopolar glass electrode (3 MΩ resistance; 25 μs pulse width) filled with artificial CSF was advanced into stratum lucidum and repositioned until the stimulus threshold for the GABAergic component was minimized.

Drugs were obtained from Tocris Cookson or Sigma.

Glutamate Application

Glutamate was applied via a pipette containing 50–100 mM Na glutamate (pH 7.2) connected to a Picospritzer (General Valve Corp; 0.5–1.0 bar; pulse duration 20–100 ms). The pipette was positioned under infrared differential interference contrast (DIC) imaging (Olympus BX50WI) in stratum moleculare, close to a stimulating electrode that evoked a mossy fiber fEPSP. Glutamate was applied every 36 s. Presynaptic neurons were activated via NMDA receptors, because GABAergic PSCs evoked by pressure application were abolished when APV (100 μM) was added to the bath. Glutamate-evoked PSCs were identified offline by detecting events with an onset >5 pA/ms and peak amplitude >20 pA.

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