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CONDITIONS REQUIRED FOR POLYSYNAPTIC EXCITATION OF DENTATE GRANULE CELLS BY AREA CA3 PYRAMIDAL CELLS IN RAT HIPPOCAMPAL SLICES

H. E. SCHARFMAN

Neurology Research Center, Helen Hayes Hospital, West Haverstraw, NY 10993-1195, U.S.A. Department of Pharmacology and Neurology, Columbia University, College of Physicians and Surgeons, New York, NY 10032, U.S.A

Abstract

Under control conditions, stimulation of area CA3 pyramidal cells in slices can produce inhibitory postsynaptic potentials in granule cells by a polysynaptic pathway that is likely to involve hilar neurons [Müller W. and Misgeld U. (1990) *J. Neurophysiol.* **64**, 46–56; Müller W. and Misgeld U. (1991) *J. Neurophysiol.* **65**, 141–147; Scharfman H. E. (1993) *Neurosci. Lett.* **156**, 61–66; Scharfman H. E. (1994) *Neurosci. Lett.* **168**, 29–33]. When slices are disinhibited, excitatory postsynaptic potentials occur after the same stimulus [Scharfman H. E. (1994) *J. Neurosci.* **14**, 6041–6057]. The excitatory postsynaptic potentials are likely to be mediated by pyramidal cells that innervate hilar mossy cells, which in turn innervate granule cells. [Scharfman H. E. (1994) *J. Neurosci.* **14**, 6041–6057]. These pathways are potentially important, because they could provide positive or negative feedback from area CA3 to the dentate gyrus. However, it is not clear when the CA3-mossy cell-granule cell excitatory pathway operates, because to date it has only been described in detail when GABA_A receptors are blocked throughout the entire slice [Scharfman H. E. (1994) *J. Neurosci.* **14**, 6041–6057]. Furthermore, the monosynaptic excitatory synaptic connections between these cells have only been observed in the presence of bicuculline [Scharfman H. E. (1994) *J. Neurophysiol.* **72**, 2167–2180; Scharfman H. E. (1995) *J. Neurophysiol.* **74**, 179–194]. Yet *in vivo* data suggest that a CA3-mossy cell-granule cell excitatory pathway may be active under some physiological conditions, because granule cells discharge in association with sharp wave population bursts of CA3 [Ylinen A., *et al.* (1995) *Hippocampus* **5**, 78–90].

To address whether the CA3-mossy cell-granule cell pathway occurs without global disinhibition of the slice, and where in the network disinhibition may be required, the effects of area CA3 stimulation on granule cells was examined after focal application of the GABA_A receptor antagonist bicuculline to restricted areas of hippocampal slices. A micropipette containing 1 mM bicuculline was placed transiently either (i) in the area CA3 cell layer, (ii) the granule cell layer, (iii) the hilus, or (iv) more than one site in succession. If a small segment of the CA3 pyramidal cell layer or the hilus was disinhibited, or bicuculline was applied to both regions, area CA3 stimulation still evoked inhibitory postsynaptic potentials in granule cells. In fact, inhibitory postsynaptic potentials were enhanced under these conditions, probably because excitation of inhibitory cells was increased. When bicuculline was applied just to the area near an impaled granule cell, all inhibitory postsynaptic potentials evoked in that cell were blocked, but no underlying excitatory postsynaptic potential was uncovered. If bicuculline was applied focally to either area CA3 or the hilus and the impaled granule cell, CA3 stimulation subsequently evoked excitatory postsynaptic potentials in that granule cell, presumably because excitatory neurons innervating granule cells were disinhibited while the effects of inhibitory cells on granule cells were blocked. Excitatory postsynaptic potentials were produced without bicuculline application in three of seven cells, simply by stimulating the fimbria repetitively.

Thus, if bicuculline is applied to different sites in the slice, different effects occur on the inhibitory postsynaptic potentials of granule cells that are evoked by a fimbria stimulus. If bicuculline is applied to both the granule cell soma and either area CA3 or the hilus, inhibitory postsynaptic potentials are reduced, and reveal that excitatory postsynaptic potentials can be produced by the same stimulus. In addition, repetitive stimulation was sometimes able to change the fimbria-evoked inhibitory postsynaptic potential to an excitatory postsynaptic potential in granule cells. These results suggest that polysynaptic excitatory transmission from area CA3 to the dentate gyrus can occur when multiple, specific parts of the network are disinhibited or modified by prior stimulation. This implies that the excitatory polysynaptic pathway from area CA3 pyramidal cells to dentate granule cells does not require universal block of GABA_A receptors throughout the slice, and therefore it may operate under a variety of conditions that were not predicted by previous slice studies.

Keywords

disinhibition; GABA; hilus; interneuron; mossy cell; repetitive stimulation

According to some of the original conceptions of hippocampal signal processing, transmission from the dentate gyrus to the hippocampus proper was unidirectional, in that dentate granule cells were thought to excite CA3 pyramidal cells monosynaptically, but not vice versa. However, recent studies indicate that this perspective is incomplete, and pathways from CA3 to the dentate granule cells do exist. Specifically it has been shown that CA3 pyramidal cells innervate some hilar neurons,^{24,25,28,45} and many hilar neurons innervate granule cell.^{10,17,21,22,27,32,33,36,40,48,49,53} In fact, physiological studies in hippocampal slices have shown that stimulation of sites that selectively activate area CA3 pyramidal cells also evoke responses in granule cells.^{36,37,44} Such stimulation sites include the fimbria and stratum oriens/alveus of area CA3b. The pathways appear to be di- or trisynaptic because the latencies to onset of the granule cell responses are 8–10 ms.⁴⁴ Under control conditions in slices, only inhibitory postsynaptic potentials (IPSPs) are evoked from granule cells by fimbria stimulation. Therefore, the pathway from area CA3 to the dentate gyrus could provide negative feedback to dentate granule cells under control conditions.

The IPSPs are probably mediated by dentate inhibitory (GABAergic) neurons, because inhibitory neurons in the hilus and granule cell layers receive CA3 input and terminate on granule cells.^{17,21,25,27,28,32,33,39,48,49} This CA3-inhibitory neuron-granule cell pathway would be a candidate disynaptic circuit underlying the IPSPs. The IPSPs could also be due to CA3 pyramidal cell activation of hilar mossy cells which in turn innervate inhibitory neurons that produce granule cell IPSPs. This CA3-mossy cell-inhibitory neuron-granule cell circuit would be trisynaptic and explain the IPSPs with the longest latencies. These hypothetical circuits were suggested by experiments showing that pyramidal cells innervate mossy cells,⁴⁵ mossy cells innervate some dentate “fast-spiking” cells (putative inhibitory neurons⁴⁸), and fast-spiking cells in the hilus and granule cell layer produce monosynaptic IPSPs in granule cells.⁴⁹ They are also supported by *in vivo* studies showing that granule cells hyperpolarize during sharp wave population bursts in area CA3.⁶¹

In contrast to control conditions, in which granule cell IPSPs are produced by CA3 stimulation, CA3 stimulation produces excitatory postsynaptic potentials (EPSPs) in granule cells when slices are perfused with the GABA_A receptor antagonist bicuculline.⁴⁴ In such experiments, the same stimulus that evokes an IPSP prior to bicuculline application evokes EPSPs and granule cell discharges after bicuculline is added to the buffer perfusing the slice. The EPSPs occur after a variety of synchronous discharges in area CA3, whether they are stimulus-evoked population bursts, afterdischarges, or spontaneous population spikes.⁴⁴ The

latencies to onset of the EPSPs, like the IPSPs, are long (8–10 ms), so the excitatory pathway is likely to be polysynaptic. The most likely pathway underlying the EPSP is CA3-mossy cell-granule cell.⁴⁴

Thus, transmission from area CA3 to the dentate gyrus under control conditions could limit excitation of granule cells, but after disinhibition, the pathway could produce positive feedback. The consequences of positive feedback could be epileptogenic, since positive feedback to granule cells could lead to more excitation of CA3 pyramidal cells, and reverberatory activity among granule cells and pyramidal cells. Therefore, it is important to determine the conditions that control the net effects of these pathways on granule cells. What must change in order for area CA3 activity to trigger excitation rather than inhibition of granule cells? Does the entire slice require disinhibition? If so, it would be unlikely for EPSPs to occur in granule cells under any but the most severe pathological conditions. However, if only some of the cells or some of the synapses involved in the pathway need to be disinhibited, the excitatory pathway might operate under more common conditions.

To address this issue, focal bicuculline application was used to disinhibit small parts of the hippocampal network selectively. The sites that were chosen were those that were likely to be involved in the pathway(s) from CA3 to the dentate gyrus. First, disinhibition of part of the CA3 cell layer was tested. This would address whether enhancing excitation of area CA3 neurons alone could change the net effect of the pathway(s) on granule cells from inhibitory to excitatory. Second, disinhibition of part of the hilus was tested, to address whether evoking large excitatory responses in these cells could enhance excitation of granule cells. The logic behind this part of the study was based on the premise that glutamatergic hilar cells (mossy cells) produced the EPSPs in granule cells. If the mossy cells were selectively disinhibited, then CA3 activation of mossy cells might be facilitated, and the net effect of CA3 activation on granule cells might become excitatory rather than inhibitory. Conversely, if inhibitory hilar cells are involved in the pathways from CA3 to granule cells, then disinhibition of the hilus might facilitate activation of inhibitory cells and the net effect of CA3 stimulation on granule cells might remain inhibitory. Third, blocking GABA_A receptors on the granule cells was tested to determine if the release of GABA onto granule cells was a critical factor.

Finally, experiments were also performed without bicuculline. In this case, inhibition was reduced by repetitive stimulation. This approach was suggested by previous studies in other areas of the hippocampus, which showed that IPSPs are reduced subsequent to increased frequency of afferent stimulation.^{6,8,31,52,58} Increasing stimulus frequency would allow one to test the hypothesis that GABA receptor blockade was not necessarily a requirement, but decreases in inhibitory function by other mechanisms might be sufficient. If true, then a variety of mechanisms that decrease inhibitory function might allow CA3 stimulation to evoke EPSPs in granule cells rather than IPSPs.

EXPERIMENTAL PROCEDURES

Preparation and maintenance of slices

Animal care followed the regulations of the National Institutes of Health and the New York State Department of Health. Adult Sprague-Dawley rats (150–300 g; Charles River) were anesthetized with ether and decapitated. The brain was removed and the hippocampus was placed in ice cold (approximately 4°C), oxygenated (95%O₂, 5%CO₂) artificial cerebrospinal fluid (ACSF, in mM: 126.0 NaCl, 5.0 KCl, 2.0 MgSO₄, 2.0 CaCl₂ 1.25 NaH₂PO₄, 26.0 NaHCO₃, and 10.0 D-glucose; pH 7.4). A Vibratome (Vibroslice, Campden Instruments) was used to cut 400-μm-thick slices in the horizontal plane while the hippocampus was immersed in ice cold ACSF. Slices were placed immediately in a

recording chamber (Fine Science Tools) that was modified so that only the uppermost surfaces of the slices were exposed to warm, humidified air. Slices were maintained at 33–34°C. Recordings were made between one and seven hours after the end of the slicing procedure.

Recording and stimulation of afferent pathways

Extracellular recordings were made with 2–5 megohm glass (0.75mm inner diameter, 1.0 mm outer diameter borosilicate glass with a capillary fibre, A &M Systems) and pipettes were filled with ACSF or 1 mM bicuculline methiodide (the latter was diluted in ACSF, as described below). Intracellular electrodes were made from the same glass but were 60–90 megohms and filled with 1 M potassium acetate. Recordings used a two channel intracellular amplifier with a bridge circuit (Axoclamp 2A, Axon Instruments) and bridge balance was monitored during intracellular recordings. Signals were displayed on an oscilloscope (Model 410, Nicolet Instruments) and recorded on tape (Neurocorder Model DR-484, Neurodata Instruments) for analysis offline. Taped data were reproduced with an X–Y plotter (Model HC100, Tektronix Instruments).

The stimulating electrode was monopolar and made from Teflon-coated stainless steel wire (75 μ m diameter). The electrode was placed on the surface of the slice in the outer molecular layer to stimulate perforant path axons, or in the ventral fimbria. Stimuli were composed of monopolar current pulses (25–100 μ A, 10–200 μ s duration). For any stimulus site, current was set at a fixed level and stimulus duration was altered to vary stimulus intensity.

Bicuculline application

Bicuculline methiodide (Sigma Chemical Co.) was stored in concentrated aliquots (10 mM in 0.9% NaCl) at –20°C. On the day of use, one aliquot was brought to room temperature and diluted in ACSF so the final concentration was 1 mM. Low resistance glass microelectrodes (2–5 megohm) were filled with the 1 mM bicuculline solution and were used to apply bicuculline as well as record extracellularly from the site of bicuculline application.^{52,57} Bicuculline-containing electrodes were placed on the surface of the slice at the desired location and remained there until recordings from the electrode indicated a loss of inhibition had occurred. At that point the electrode was removed from the slice.

Loss of inhibition was defined, for the purposes of this study, by changes in evoked responses recorded extracellularly. Responses of granule cells were evoked by molecular layer stimulation. Loss of inhibition was defined by blockade of paired-pulse inhibition (Fig. 1). Paired-pulse inhibition is the decrease in the response to the second stimulus of a pair of identical stimuli (interstimulus interval, 20 ms; Fig. 1). In the case of responses recorded in the dentate granule cell layer, each response consists of a population spike superimposed on a large positive potential; paired-pulse inhibition involves a reduction in the population spike and sometimes the positivity as well. In this study, paired-pulse inhibition was said to be “blocked” when the ratio of (population spike amplitude evoked by the first stimulus)/(population spike amplitude evoked by the second stimulus) changed from > 1 to < 1 . Two population spikes after any single stimulus was additional evidence of disinhibition.

To assess inhibition in area CA3, the response to fimbria stimulation was monitored. Loss of inhibition was not defined by changes in paired-pulse inhibition, because in control conditions paired-pulse inhibition is weak in area CA3.⁵⁵ Instead, multiple population spikes were used as an indication of reduced inhibition (Fig. 1). Ordinarily two population spikes were evoked by a single fimbria stimulus in area CA3 (one antidromic, one orthodromic); disinhibition was defined by the development of more than two population spikes (usually more than five) in response to a single stimulus.

For bicuculline application to the hilus, extracellular recording from the bicuculline-containing pipette was not used to gauge disinhibition of nearby cells, because field potentials recorded from the hilus do not necessarily reflect hilar cell activity. Therefore, the bicuculline electrode was placed in the hilus for approximately the same time as was required to produce disinhibition of granule cells or area CA3 pyramidal cells (approximately 60s). Intracellular recordings from hilar cells next to the bicuculline electrode verified that they were disinhibited at this time, because multiple discharges were evoked in response to a molecular layer or fimbria stimulus, as has been previously shown.^{34,43,47}

Spread of bicuculline to areas around the bicuculline-containing electrode was monitored by placing an ACSF-containing recording electrode at several sites near the bicuculline-containing electrode. For bicuculline application to either the granule cell layer or the pyramidal cell layer, the ACSF-containing electrode was positioned in adjacent portions of the cell layer. For bicuculline application in the hilus, the ACSF-containing electrode was placed at the nearest site in the granule cell layer or pyramidal cell layer. Evoked potentials recorded by the ACSF electrode did not demonstrate a loss of inhibition if the ACSF electrode was over 200 μm from the bicuculline electrode, and the bicuculline electrode remained in position for a short time (typically 30–90 s). If the ACSF electrode was moved to a site less than 200 μm from the bicuculline electrode, disinhibited responses could be recorded from the ACSF-containing electrode. After the bicuculline electrode was removed from the slice, evoked responses did not become progressively more disinhibited, but slowly returned to control conditions. Therefore, spread of bicuculline over a 30–90 s period appeared to be limited to a circular area with a radius of 200 μm , centred around the bicuculline electrode. Distances were measured by an ocular micrometer and were accurate within 10 μm . Leakage of vehicle (ACSF) did not produce a detectable loss of inhibition.

RESULTS

Focal application of bicuculline disinhibited small areas of the slice

This study was based on recordings from 35 slices from 23 rats. In each slice, bicuculline was applied focally by placing an extracellular recording electrode filled with 1 mM bicuculline on the slice surface. Approximately 30–90 s after placement of the bicuculline electrode in the granule cell layer, the response to a molecular layer stimulus recorded by the bicuculline electrode showed evidence of disinhibition (Fig. 1). Specifically, multiple population spikes could be evoked and paired-pulse inhibition was blocked (Fig. 1A–B). Simultaneous recording from a second recording electrode containing ACSF showed that cells over 200 μm away were not detectably disinhibited, indicating that the effects of bicuculline were restricted to a small part of the dentate gyrus (Fig. 1A–B). Local disinhibition could also occur in part of the CA3 cell layer if the bicuculline electrode was placed there (Fig. 1C–D). If the bicuculline electrode was left on the slice surface for over 90 s, the effects of bicuculline were not as restricted, and disinhibited responses to stimulation could be recorded over 200 μm from the bicuculline electrode (Fig. 1C–D).

Bicuculline application to area CA3 or the hilus increased fimbria-evoked inhibitory postsynaptic potentials of granule cells

Stimulation of the fimbria in control conditions evoked a small IPSP in granule cells (Fig. 2). To test whether disinhibition of area CA3 pyramidal cells or hilar cells could modify these IPSPs, a granule cell was impaled and stimulation of the fimbria was tested before and after bicuculline was focally applied to one site in the CA3 cell layer or the hilus. Application of bicuculline to area CA3c ($n = 4$) or area CA3b ($n = 5$) or the hilus ($n = 5$) increased the IPSP evoked by fimbria stimulation (Fig. 2). The hilar electrode containing

bicuculline was placed either near the crest, centre of the lower blade, or near the upper blade. In each case the bicuculline electrode was located over 100 μm from the granule cell layer and over 300 μm from the impaled granule cell. The mean amplitude of an IPSP (evoked by a maximal stimulus) was 2.1 ± 0.5 mV in control, 7.3 ± 0.4 mV when area CA3 was disinhibited, and 6.7 ± 0.4 mV when the hilus was disinhibited. The effect required up to 30 min to reverse, and reversed in every case tested ($n = 3$; Fig. 2). The recovery of disinhibition in area CA3 always had a similar timecourse as the recovery of the IPSP amplitude (Fig. 2), supporting the premise that area CA3 activity might have been involved in the changes in the IPSP. If bicuculline was applied at two sites (two sites in area CA3, two sites in the hilus, or one site in the hilus and one site in area CA3) an increased IPSP also occurred ($n = 4$; data not shown).

Bicuculline application to the granule cell layer, next to an impaled granule cell, blocked inhibitory postsynaptic potentials evoked by fimbria stimulation in that cell

In other slices, bicuculline was applied next to an impaled granule cell ($n = 4$). Within 90 s after the bicuculline electrode was placed on the slice, the response to a molecular layer stimulus, recorded by the bicuculline electrode, consisted of multiple population spikes (Fig. 3). At this time, the IPSP evoked in the impaled granule cell in response to a fimbria stimulus was blocked completely (Fig. 3). Even if the bicuculline pipette was left next to the impaled granule cell for several minutes, so more bicuculline leaked and a larger area was disinhibited, no EPSP could be evoked in the impaled granule cell in response to a fimbria stimulus (Fig. 3). Even if the fimbria stimulus was increased to a maximal intensity (100 μA , 200 μs) EPSPs were still not evoked. These data suggest that blockade of GABA_A receptors on granule cells alone is insufficient to change fimbria-evoked IPSPs into EPSPs.

Bicuculline application to the granule cell layer, and area CA3 or the hilus, caused fimbria stimulation to evoke excitatory postsynaptic potentials in granule cells

Bicuculline was applied to two sites in the slice in five other experiments. One site was next to an impaled granule cell and the other site was either in the area CA3 pyramidal cell layer (area CA3b, $n = 2$; area CA3c, $n = 1$) or the hilus ($n = 2$). As described above, only IPSPs were evoked by fimbria stimulation before bicuculline was applied. Following application of bicuculline to both sites, stimulation of the fimbria evoked EPSPs in granule cells (Fig. 4). The EPSP reverted to an IPSP after the bicuculline electrode was removed from the slice. The recovery of the IPSP and reversal of disinhibition recorded in the cell layers had a similar time course, suggesting that the changes might be related.

Figure 4 shows data from a representative experiment. In this case, bicuculline was first placed transiently in area CA3b and after 20 s multiple population spikes were recorded in that location in response to a fimbria stimulus. The fimbria-evoked IPSP of the simultaneously-recorded granule cell increased. The bicuculline electrode was then removed. At that time, an ACSF-containing recording electrode positioned in area CA3c did not record a disinhibited response to the same stimulus, indicating that the bicuculline had not spread far from area CA3b.

Subsequently, the bicuculline electrode was moved adjacent to the impaled granule cell. A molecular layer stimulus initially produced a response consisting of a large positivity and small superimposed population spike, which is a typical extracellular response to such stimulation. Paired-pulse inhibition was demonstrated by the responses to two identical stimuli that were triggered 20 ms apart (Fig. 4B4). After 25 s, the bicuculline electrode recorded a loss of paired-pulse inhibition. At this time the simultaneously-recorded granule cell's IPSP was blocked. After another 45 s, the loss of paired-pulse inhibition was more pronounced and an EPSP was produced in the granule cell in response to the same fimbria

stimulus. When the ACSF electrode was subsequently moved to a site in the granule cell layer approximately 200 μm away from the bicuculline electrode, there was no evidence of disinhibition at that location (Fig. 4B5). Thus, disinhibition of a subpopulation of CA3 pyramidal cells, paired with disinhibition of part of the granule cell layer near the impaled cell, was sufficient to change the IPSP evoked by fimbria stimulation to an EPSP. Similar experiments showed that hilar disinhibition coupled with disinhibition of an impaled granule cell also changed fimbria-evoked IPSPs to EPSPs (data not shown).

The effects of repetitive stimulation on fimbria-evoked inhibitory postsynaptic potentials

The effects of repetitive stimulation were tested by monitoring the fimbria-evoked IPSP of a granule cell as stimulation frequency was increased from 0.1 Hz to 1 Hz. In all seven cells tested, the IPSP was reduced during such stimulation, after as few as five stimuli (Fig. 5). Similar effects occurred when stimulus frequency was 3 Hz, 10 Hz, or 100 Hz (Fig. 5). In each slice, the four different frequencies were tested in succession with 5–10 s between high frequency trains; each high frequency stimulus train lasted 1 s.

After high frequency stimulation was terminated, responses were retested at slower stimulus frequencies (0.05–0.1 Hz). In three of the seven cells, the same fimbria stimulus that evoked an IPSP before high frequency stimulation evoked an EPSP immediately after high frequency stimulation (Fig. 5). EPSPs were able to trigger a single action potential (Fig. 5). These EPSPs began at a long latency after the stimulus, similar to the previously-evoked IPSPs (Fig. 5). In two cells EPSPs persisted long after the stimulus trains were over (at least 15 min). In the slices containing those two cells, five of five granule cells recorded later also produced EPSPs in response to fimbria stimulation (data not shown). These data suggest fimbria-evoked EPSPs can be produced in granule cells without the use of bicuculline and some pathways exhibit long-term potentiation.

DISCUSSION

Summary

The results show that disinhibition of small areas of the dentate granule cell layer, hilus, or the area CA3 pyramidal cell layer can have a predictable effect on the IPSPs evoked in granule cells by stimulation of the fimbria. If bicuculline is applied to part of the area CA3 cell layer or the hilus, the IPSPs increase. If bicuculline is applied to the granule cell layer next to the impaled granule cell, IPSPs are blocked. However, if bicuculline is applied to both the granule cell and part of area CA3 or the hilus, fimbria stimulation can evoke EPSPs in granule cells. EPSPs can also occur without bicuculline application, after repetitive stimulation of the fimbria. These findings are important because they demonstrate that only small parts of the network must be disinhibited for the CA3—dentate excitatory pathway to operate. In addition they show which parts of the network are key: the area around the granule cell somata, and either the hilus or part of the CA3 cell layer.

Pathways responsible for granule cell responses to fimbria stimulation

The results are most easily explained by the circuit shown in Fig. 6A. Other pathways may contribute to the responses of granule cells to fimbria stimulation, but the pathways shown in Fig. 6 are the simplest that are consistent with the available data from a number of different studies (see below). This circuit suggests that the effects of fimbria stimulation on granule cells are mediated by area CA3 pyramidal cells that excite dentate non-granule cells, which in turn innervate the granule cells. The non-granule cells innervated by area CA3 pyramidal cells are excitatory (the mossy cells^{48,54}) and inhibitory (including various GABAergic neurons in different areas of the dentate gyrus^{2,17,21,22,27,29,32,33,39,50,53}), so the effects on granule cells can be excitatory or inhibitory. In addition, it is assumed that

inhibitory inputs from a variety of sources, both intrinsic and extrinsic to the hippocampus, innervate both the mossy cells and the inhibitory cells involved in these pathways.^{11,19,21,22,39,49,50,55}

Previous studies support this scheme. It has been demonstrated that the fimbria contains axons of area CA3 pyramidal cells and fimbria stimulation produces antidromic and orthodromic action potentials in these cells.^{5,26,35} Area CA3 pyramidal cells project to the hilus,^{24,28} and innervate mossy cells,⁴⁵ and probably inhibitory cells⁴⁷ such as the basket cells of the granule cell layer.²⁵ There is considerable evidence that mossy cells are excitatory^{48,54} and many other hilar neurons are inhibitory.^{21,34,36,37,49,50} Mossy cells and inhibitory hilar neurons, as well as other inhibitory dentate neurons, innervate granule cells.^{9,22,36,39,41,49,53} There also is evidence that mossy cells innervate inhibitory cells in the ipsilateral and contralateral dentate gyrus.^{13,48,51}

The latencies of responses to fimbria stimulation and their pharmacology further support the circuit diagram shown in Fig. 6. Of all the cells shown, fimbria stimulation produces the shortest latency responses in pyramidal cells (antidromic or 1–2 ms), followed by dentate non-granule cells,⁴² and the longest latency responses are recorded from granule cells (8–10 ms to onset of EPSP or IPSP⁴⁴). This suggests that the order of activation is pyramidal cells, then dentate non-granule cells, and finally granule cells. The glutamate receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) blocks EPSPs and IPSPs in pyramidal cells, hilar cells, and granule cells occurring in response to fimbria stimulation, but cholinergic antagonists do not.⁴³ Because pyramidal cells use glutamate as a neurotransmitter,¹⁵ those data support pyramidal cell involvement in the granule cell responses rather than septohippocampal cholinergic pathways. Antidromic action potentials are not evoked in hilar mossy cells by fimbria stimulation, probably because their axons are cut in stratum oriens/alveus of area CA3b/c, before they reach the fimbria (unpublished observations based on intracellularly-labeled mossy cells from slices); thus, taken together with the long latencies of granule cell responses to fimbria stimulation, it is unlikely that hypothetical antidromic activation of mossy cells alone can explain responses of granule cells. In summary, these results support the premise that the pathway initially involves excitation of pyramidal cells and do not necessarily involve other pathways that travel in the fimbria.

Another possibility raised by the data and consistent with the circuit depicted in Fig. 6 is that mossy cell-mediated excitation of granule cells is relatively weak. This possibility must be tested with further experiments, but is a reasonable hypothesis at this time because it is supported by previous studies and can explain the effects of bicuculline observed in the present study (see below and Fig. 6). Previous studies showed that mossy cells may excite granule cells weakly relative to inhibitory cells because they do not evoke discharge in granule cells unless the granule cell is depolarized; in contrast, mossy cells evoke discharge in inhibitory cells without depolarization of the inhibitory cell.⁴⁸ Studies *in vivo* have shown that the major effect of hilar stimulation on the contralateral dentate gyrus is inhibition of granule cells and excitation of putative inhibitory interneurons;^{13,18} a few studies have shown that such stimulation elicits weak excitatory responses.^{16,56} Presumably these effects were mediated by mossy cells, because these cells contribute the most to the hilar commissural projection.^{2,40} The *in vivo* data suggest that the predominant effect of mossy cells is inhibitory to granule cells, probably by exciting inhibitory neurons that innervate granule cells. Finally, lesions of mossy cells produce hyperexcitability of granule cells, which has been interpreted as evidence that their predominant input is to inhibitory cells.⁵¹

Effects of bicuculline

Figure 6B–E illustrates how bicuculline could have altered the responses of granule cells to fimbria stimulation. In the case of bicuculline application to the CA3 cell layer (Fig. 6B),

larger IPSPs were produced in granule cells. This can be explained by presuming that bicuculline blocked inhibitory inputs to CA3 cells, which allowed the fimbria stimulus to activate CA3 cells more strongly. Such strong excitation of CA3 neurons would lead to stronger excitation of their dentate target cells, and an enhanced response would result in granule cells.

Bicuculline application to the hilus also increased IPSPs in granule cells, and the possible alterations in the circuit responsible for this effect are shown in Fig. 6C. Bicuculline probably reduced inhibitory inputs to hilar neurons, so any subsequent excitatory input from area CA3 would be more effective in discharging them. This would be expected to increase the effects of dentate non-granule cells on their targets, i.e. produce a larger IPSP in granule cells.

Figure 6D suggests how bicuculline application to granule cells led to blockade of their IPSPs, but did not reveal an EPSP. By blocking the effects of inhibitory inputs to granule cells, the only remaining input that would have had an effect would have been the mossy cell input. However, if this is a weak input, no response might be detected, and in fact that was the result that was obtained. Another reason why an EPSP was not detected could have been that the site of synaptic input responsible for the EPSP was electrically remote from the soma, such as on a spine head on a distal dendrite. However, the inputs of mossy cells are on proximal dendrites,^{10,40,53} so this argument is unconvincing.

Finally, Fig. 6E presents an explanation for the effect of bicuculline application to both the hilus and granule cell somata. These applications changed the response of granule cells to fimbria stimulation from IPSPs to EPSPs. There were probably two reasons for this effect: bicuculline application to the granule cell blocked inhibitory inputs to granule cells, and bicuculline application to the hilus enhanced excitation of mossy cells. Enhancing the excitation of mossy cells would be expected to make their normally weak input to granule cells much stronger. Simultaneously blocking inhibition of granule cells would enable one to detect mossy cell-mediated EPSPs. Bicuculline application to the granule cell and area CA3 had a similar effect as bicuculline application to the granule cell and the hilus, presumably for similar reasons: input from mossy cells to granule cells was enhanced and inhibitory inputs to granule cells were reduced. (Note that the granule cell/CA3 applications are not shown in Fig. 6.)

Repetitive stimulation

Repetitive stimulation of the fimbria reduced fimbria-evoked IPSPs at several stimulus frequencies from 1 to 100 Hz. The depression of IPSPs was rapid and sustained throughout the period of repetitive stimulation. This result is consistent with the reports of others who showed that repetitive stimulation can reduce IPSPs.^{6,8,31,58} Immediately after the train, stimulation produced an EPSP rather than an IPSP in granule cells of some slices. One possible explanation for this effect was that repetitive stimulation produced a depression of inhibitory inputs coupled with potentiation of excitatory inputs to granule cells. That is, inhibitory neuron inputs to granule cells were depressed and mossy cell inputs to granule cells were potentiated.

In some cases the effects of the repetitive train was long lasting, because a stimulus still could produce an EPSP several minutes after the train was over. This result suggests that there is plasticity in the pathways that underlie the EPSP. Long-term depression of inhibitory inputs could have been induced, long-term potentiation of excitatory inputs, or both. For example, pyramidal cell inputs to mossy cells might have been potentiated, but pyramidal cell inputs to inhibitory cells might not have been. The result would be a potentially stronger excitatory input to granule cells. These possibilities for plasticity are

consistent with the examples of potentiation that have already been identified in dentate and CA3 neurons. For example, previous studies have identified long-term potentiation of granule cell to CA3 transmission^{1,60} and mossy cell to granule cell transmission^{23,56} (but see Ref. 7).

Significance

The results illustrate how certain polysynaptic pathways from area CA3 to the dentate gyrus can be expressed. Under most conditions, inhibition is favoured. This suggests that under control conditions feedback from area CA3 to the dentate gyrus is inhibitory, and would be predicted to limit excitation in the dentate gyrus. This polysynaptic feedback may contribute to the high inhibitory tone in granule cells that has been reported previously.^{30,38} It is consistent with recent findings *in vivo* which show that mossy cells depolarize and granule cells hyperpolarize during theta activity.^{53,61}

However, the results also show that EPSPs can be produced in granule cells by fimbria stimulation if disinhibition of granule cells is coupled to enhanced activity of some area CA3 or hilar neurons. This is important because it suggests that the excitatory pathway might function *in vivo*, specifically when there is disinhibition of small populations of granule cells and either hilar neurons or CA3 neurons. Indeed, it has been shown *in vivo* that repetitive discharges occur in granule cells during sharp wave bursts of CA3,⁶¹ and sharp waves may be due to transient disinhibition.¹² Thus, the CA3–dentate excitatory pathway is unlikely to necessitate global disinhibition, as a previous slice study indicated.⁴⁴

It will be important to further determine exactly how the CA3–dentate excitatory pathway functions in the intact animal. The slice may underestimate the role of mossy cells, or the role of inhibitory neurons, because the axons of these cells are cut in a slice.^{4,9,62} Anesthesia involved in *in vivo* studies may also be misleading in some ways. One of the complexities that needs to be clarified is whether mossy cells innervate granule cells and inhibitory cells in similar ways and to a similar extent throughout their axonal projection. Another issue that is important in relating the results of *in vitro* experiments to the *in vivo* situation is what factors *in vivo* would be able to disinhibit granule cells and enhance input to mossy cells. There are several possibilities. For example, there is inhibitory input to inhibitory neurons that could be suppressed or activated. An example would be the septoGABAergic input which selectively targets inhibitory cells in the dentate gyrus.²⁰ If this input were activated, one would expect depression of inhibitory neurons in the dentate gyrus, decreased IPSPs in granule cells, and facilitation of the mossy cell-granule cell EPSP might result. Another example involves enkephalin, which is known to be released onto inhibitory cells preferentially and to disinhibit granule cells.^{14,59} Several factors could enhance excitation of mossy cells. For example, their excitatory inputs from area CA3 could be increased, during sharp wave bursts in area CA3¹² or long-term potentiation of area CA3. Increased entorhinal input to the hippocampus would produce enhanced excitation of mossy cells, because these cells are directly and indirectly excited by the perforant path.^{2,41} Brainstem inputs to the hilar region are diverse,^{2,3} and may provide other possibilities for increasing excitation of mossy cells, either by potentiating their excitatory inputs or suppressing their inhibitory inputs.

Thus, there are many factors present *in vivo* that could function analogous to focal bicuculline application used in this study. It is likely that situations might arise *in vivo* where the EPSPs mediated by the CA3-mossy cell-granule cell pathway could become significant, and negative feedback to the dentate could become positive feedback. This would have substantial implications for signal processing in the hippocampus. In addition, the presence of a “switch” from negative to positive feedback has potential importance to epilepsy,

because it suggests a mechanism that could contribute to the generation of epileptiform activity in the hippocampus.

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Abbreviations

ACSF	artificial cerebrospinal fluid
EPSP	excitatory postsynaptic potential
IPSP	inhibitory postsynaptic potential

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Fig. 1. Effects of focal bicuculline application on extracellularly-recorded responses recorded in the granule cell layer and area CA3. (A) A diagram shows where recording and stimulating electrodes were placed to produce the responses in part B. A monopolar stimulating electrode was placed in the outer molecular layer just below the subiculum (STIM). A recording electrode containing bicuculline (BIC; 1 mM) was placed in the granule cell layer of the upper blade, and another recording electrode containing ACSF was placed approximately 200 μm away in the granule cell layer. (B) Changes in evoked responses following focal bicuculline application to the granule cell layer. *Left:* The responses to two stimuli of the molecular layer are shown 5 s after the bicuculline electrode was placed on the slice in the location shown in part A. There was paired-pulse inhibition recorded by both the ACSF electrode (top; ACSF) and the bicuculline electrode (bottom; BIC). *Right:* Responses to the same stimuli are shown 45s after the bicuculline electrode was placed on the slice. The responses recorded by the ACSF electrode were similar to those evoked at 5 s, but the responses recorded by the bicuculline electrode were disinhibited. For this figure and others, small dots mark the stimulus artifacts. (C) A diagram shows where recording and stimulating electrodes were placed to produce the responses shown in part D. The stimulating electrode was located in the ventral fimbria and the ACSF and bicuculline electrodes were placed in area CA3b, approximately 200 μm apart. (D) Changes in evoked responses following focal bicuculline application to area CA3b. *Left:* The responses to a single fimbria stimulus are shown 2 s after the bicuculline electrode was placed on the slice. The top trace was recorded by the ACSF recording electrode and the bottom trace was recorded by the bicuculline electrode. A large antidromic and small orthodromic population spike were recorded at both sites. *Centre:* Approximately 40 s after the bicuculline electrode had touched the slice, the response to the same fimbria stimulus as used on the left produced a similar effect at the ACSF recording electrode but the response at the bicuculline electrode included four population spikes. *Right:* Approximately 3 min after the bicuculline electrode touched the slice, the responses recorded at both electrodes had changed, showing a burst of numerous population spikes after a single stimulus. Voltage calibration for all traces in part D, 5 mV. Time calibration for traces recorded at 2 s and 40 s, 10 ms; calibration for traces recorded at 3 min is 50 ms.

Fig. 2.

Focal application of bicuculline to area CA3 produces an increase in fimbria-evoked IPSPs of granule cells. (A) A diagram of the experimental arrangement includes a stimulating electrode placed in the ventral fimbria, a bicuculline-containing extracellular electrode placed in area CA3c, and an intracellular electrode that was used to record from a granule cell in the upper blade. (B) Simultaneous recordings of fimbria-evoked responses in a granule cell (1) and area CA3c extracellularly (2) at several times after the bicuculline electrode was placed in area CA3c. Three seconds after the bicuculline electrode touched the slice, a small IPSP with a long latency was recorded in the granule cell, and a series of antidromic and orthodromic population spikes were recorded in area CA3c. Approximately 40 s after the bicuculline electrode had touched the slice, the IPSP of the granule cell increased in amplitude and the response of area CA3c to the same stimulus included numerous population spikes. At this point the bicuculline electrode was raised above the slice. After 14 min it was lowered to the slice surface and the response to a fimbria stimulus evoked at that time was similar to the response recorded at the start of the experiment. The IPSP of the granule cell had also returned to a small amplitude, similar to the start of the experiment. The membrane potential of the granule cell (-62 mV) was depolarized relative to its resting potential (-79 mV) by injecting current intracellularly.

Fig. 3.

Focal application of bicuculline to the granule cell layer disinhibits granule cells and blocks fimbria-evoked IPSPs. (A) A diagram of the recording and stimulating electrodes used to produce responses shown in part B. Two stimulating electrodes were used, one placed in the outer molecular layer near the subiculum and the other in the ventral fimbria. Two extracellular recording electrodes were used (one containing bicuculline, one containing ACSF) and one intracellular electrode was used to record from a granule cell (GC). (B) Responses recorded from granule cells following application of bicuculline to the impaled cells. 1. Responses to fimbria stimulation recorded from a granule cell 5 s (left) and 3 min (centre) after a bicuculline electrode was placed near the impaled cell. The trace on the far right was recorded 25 min after the bicuculline electrode was removed from the slice. Prior to bicuculline application, and at the 5 s timepoint, the response to a fimbria stimulus was a small IPSP. Thirty seconds after the bicuculline electrode was placed in the slice, the IPSP was blocked (not shown). The bicuculline electrode remained in the slice for 3 min, at which time the IPSP was still blocked (centre trace). The effects on the IPSP were reversible (right). Membrane potential, -60 mV. Resting potential, -77 mV. Note that the extracellular response recorded from the granule cell layer to fimbria stimulation in control conditions is a small negativity (usually 1 mV amplitude) that corresponds to depolarizing IPSPs ranging from 2–7 mV in granule cells.⁴⁶ 2. Responses to a molecular layer stimulus recorded extracellularly by a bicuculline electrode placed near the impaled granule cell. The extracellular responses were recorded simultaneously to the intracellular responses shown in part B1. Note that the extracellular recording showed a typical response 5 s after the bicuculline electrode was placed in the slice (left), multiple population spikes 3 min later (centre), and almost complete recovery after removal of the bicuculline electrode (right). 3. Responses to molecular layer stimulation, recorded with an ACSF-containing electrode, 3 min after the bicuculline electrode was placed on the slice. This response was recorded

immediately after the bicuculline pipette was removed from the slice. The ACSF electrode was approximately 200 μm from the site of the bicuculline electrode. Note that the ACSF electrode recorded a response composed of multiple population spikes, indicating that the granule cell layer was extensively disinhibited.

Fig. 4.

Effects of bicuculline application to the pyramidal cell layer and granule cell layer. (A) A diagram illustrates the two stimulation sites in the outer molecular layer and fimbria, as well as the sites where recording electrodes were placed. One intracellular electrode was positioned in a granule cell for the entire experiment (1). An electrode containing bicuculline was placed first in the pyramidal cell layer of CA3b (2) and then next to the granule cell (4). An ACSF-filled electrode recorded extracellular responses in area CA3c after bicuculline was applied to CA3b (3) and then was moved to the granule cell layer where it recorded responses after bicuculline was applied to the granule cell (5). (B) Recordings from the sites shown in part A, at several times after the bicuculline-containing electrode was placed in area CA3b (approximately 3 s, 20 s, 45 s, 1.5 min and 30 min). 1. These responses were recorded from the granule cell intracellularly. "3 s": Three seconds after the bicuculline electrode was placed in area CA3b, a small IPSP was recorded in response to a fimbria stimulus. This response was similar to the IPSP evoked before the bicuculline electrode touched the slice. "20 s": Twenty seconds after the bicuculline was placed in area CA3b, the same fimbria stimulus evoked a larger IPSP. Then the bicuculline pipette was repositioned near the granule cell. "45 s": After 25 s of bicuculline leak in the granule cell layer, the IPSP was blocked. "1.5 min": After another 45 s of bicuculline leak, an EPSP was produced by the same stimulus. "30 min": After the bicuculline pipette was removed from the slice, the response reverted to the original small IPSP evoked at the start of the experiment. This response was recorded a total of 30 min after the bicuculline electrode was placed in CA3b. 2. Recordings from area CA3b with the bicuculline-containing electrode evoked 3 s and 20 s after the electrode was placed in area CA3b. Note that 20 s after it was placed in area CA3b, it recorded an enhanced response (burst of multiple population spikes), suggesting that there was disinhibition of the cells around the recording electrode. After the bicuculline electrode was removed from the slice the response reversed ("30 min"). 3. Recordings from area CA3c with the ACSF electrode. Immediately after the burst of population spikes was recorded in CA3b, the bicuculline pipette was removed and a pipette containing ACSF recorded a typical response to the same fimbria stimulus in area CA3c, indicating no detectable disinhibition in that area of the slice. This

indicated that the bicuculline leaked onto area CA3b had not spread to CA3c. 4. Recordings using the bicuculline electrode after it was placed in the granule cell layer, adjacent to the impaled granule cell. “20 s”: Immediately after placement in the cell layer, paired-pulse inhibition was recorded. “45 s”: After 25 s of bicuculline leak, the electrode recorded a disinhibited response without paired-pulse inhibition. “1.5 min”: Approximately 45 s later, multiple population spikes were recorded after a single stimulus. Note that at this time an EPSP was recorded simultaneously in the granule cell. “30 min”: After the bicuculline pipette was removed there was reversal of the disinhibition of the population spike. 5. A recording using the ACSF electrode after it was placed in the granule cell layer. This recording was made shortly after the bicuculline pipette was removed from the granule cell layer (“1.5 min”). It shows that there was a typical response in an area of the granule cell layer approximately 200 μm from the impaled granule cell, suggesting that bicuculline’s effects were restricted.

Fig. 5.

Changes in fimbria-evoked responses of granule cells after repetitive fimbria stimulation. (A) Successive responses of a granule cell to fimbria stimulation during a depolarizing current pulse are shown. The first response is on the left, second in the centre, and third at right. The stimuli were triggered at 1 Hz. Note that the IPSP decreased in amplitude from left to right. The first stimulus was followed by an action potential. The depolarizing current pulse commands are shown below each trace. Membrane potential = -68 mV. (B) Response of a different granule cell to 10 Hz stimulation of the fimbria. Membrane potential was -76 mV, so the polarity of the IPSP was depolarizing. Note that the IPSP decreased in amplitude with successive stimuli. (C) Response of the same granule cell as in part B to fimbria stimulation, triggered during responses to intracellular current injection, before (left) and after (right) 10 Hz stimulation. Note that the IPSP was evoked prior to the 10 Hz train, but an EPSP was triggered after the train. When the cell was depolarized by intracellular current injection, the EPSP triggered an action potential. Successive responses triggered during depolarizing current injection and hyperpolarizing current injection are superimposed. The commands for intracellular current injection are shown below the responses of the cell. Membrane potential = -76 mV. (D) Responses of a different granule cell to fimbria stimulation triggered at 100 Hz. The onset of the train is shown on the left and the end of the train is at right. Note that the response was initially depolarizing but then decreased so that no detectable response occurred at the end of the train, which was one second in duration. Membrane potential was -81 mV, so the response to the first stimuli were depolarizing IPSPs.

Fig. 6.

Interpretation of fimbria-evoked responses of granule cells based on a simplified circuit diagram. (A) In control conditions, fimbria stimulation evoked small IPSPs in granule cells. This result can be explained by the circuit diagram that is illustrated (for additional discussion see text). Fimbria stimulation excites pyramidal cells (triangle cell) that innervate dentate neurons (excitatory mossy cells represented by the square cell, and inhibitory neurons represented by the filled circle) that in turn innervate granule cells (open circle). Mossy cells innervate both inhibitory cells and granule cells. Thus, fimbria stimulation leads to a combination of EPSPs and IPSPs in granule cells and the net effect is a small IPSP. Note that the inhibitory neurons are represented by one cell in one location but could be in many areas of the dentate gyrus. Note also that there are multiple sources of inhibitory input to all cells, as indicated by the parenthetical minus signs near the cell bodies. (B) When bicuculline is selectively applied to area CA3, IPSPs in granule cells increased. The modified circuit illustrated suggests an explanation for this result. Bicuculline application to CA3 would be expected to block the effects of GABAergic inputs to the CA3 cells. This would be likely to increase the excitation of CA3 cells in response to a fimbria stimulus. If fimbria stimulation produced increased excitation of CA3 cells, one would expect dentate cells innervated by CA3 to be excited more by a fimbria stimulus also. These dentate cells should then produce larger effects in their target cells, the granule cells. Since the response of granule cells to a fimbria stimulus under control conditions was a small IPSP, a larger IPSP would be expected after bicuculline application. (C) Bicuculline application to the hilus also led to an enhanced IPSP in granule cells. This result can be explained by assuming that bicuculline blocked inhibitory inputs to the hilar neurons that normally innervate granule cells. By removing this inhibition, there would be enhanced excitation of hilar neurons in response to their excitatory inputs, such as those from CA3 pyramidal cells. Thus, under these conditions, fimbria stimulation would excite CA3 pyramidal cells and then pyramidal cells would excite hilar neurons more strongly. The enhanced activation of hilar neurons would lead to greater effects on their targets, the granule cells. (D) When bicuculline was selectively applied to granule cells, there was blockade of fimbria-evoked responses. The control response of a granule cell to fimbria stimulation, an IPSP, was blocked because bicuculline was applied to the site where GABA is likely to be released, the cell layer. The diagram shows that an excitatory cell, the mossy cell, innervates the granule cell, so one might expect an EPSP to be produced by fimbria stimulation under these conditions. However, blockade of the IPSP did not reveal an underlying EPSP, probably because the mossy cell input is weak (represented as a dashed line from the mossy cell to the granule cell; see text for further discussion). (E) When bicuculline was selectively applied to both granule cells and the hilus, fimbria stimulation evoked EPSPs in granule cells. The

explanation for this result stems from part D, where it was hypothesized that the mossy cell input to granule cells was normally too weak to produce a detectable EPSP in granule cells, even when inhibitory inputs to granule cells were blocked. One prediction arising from this hypothesis is that enhancing excitation of mossy cells would lead to a detectable EPSP in granule cells. This result was obtained by using bicuculline application to the hilus to enhance mossy cell excitation in response to a fimbria stimulus. Thus, disinhibiting granule cells and disinhibiting hilar neurons allows a response to a fimbria stimulus to change from an IPSP to an EPSP.