

Complex Synaptic Current Waveforms Evoked in Hippocampal Pyramidal Neurons by Extracellular Stimulation of Dentate Gyrus

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Xiang, Zixiu and Thomas H. Brown. Complex synaptic current waveforms evoked in hippocampal pyramidal neurons by extracellular stimulation of dentate gyrus. *J. Neurophysiol.* 79: 2475–2484, 1998. Excitatory postsynaptic currents (EPSCs) evoked in hippocampal CA3 pyramidal neurons by extracellular stimulation of the dentate gyrus typically exhibit complex waveforms. They commonly have inflections or notches on the rising phase; the decay phase may exhibit notches or other obvious departures from a simple monoexponential decline; they often display considerable variability in the latency from stimulation to the peak current; and the rise times tend to be long. One hypothesis is that these complex EPSC waveforms might result from excitation via other CA3 pyramidal cells that were recruited antidromically or trans-synaptically by the stimulus due to the complex anatomy of this region. An alternative hypothesis is that EPSC complexity does not emerge from the functional anatomy but rather reflects an unusual physiological property, intrinsic to excitation-secretion coupling in mossy-fiber (mf) synaptic terminals, that causes asynchronous quantal release. We evaluated certain predictions of our anatomic hypothesis by adding a pharmacological agent to the normal bathing medium that should suppress di- or polysynaptic responses. For this purpose we used baclofen (3 μ M), a selective agonist for the γ -aminobutyric acid B receptor. The idea was that baclofen should discriminate against polysynaptic versus monosynaptic inputs by hyperpolarizing the cells, bringing them further from spike threshold and possibly also through inhibitory presynaptic actions. Whole cell recordings were done from visually preselected CA3 pyramidal neurons and EPSCs were evoked by fine bipolar electrodes positioned into the granule cell layer of the dentate. To the extent that the EPSC complexity reflects di- or polysynaptic responses, we predicted baclofen to reduce the number of notches on the rising and decay phases, reduce the variance in latency to peak of the EPSCs, decrease the amplitudes and rise times of the individual and averaged EPSCs, and increase the apparent failures in evoked EPSCs. All of these predictions were confirmed, in support of the hypothesis that these complex EPSC waveforms commonly reflect di- or polysynaptic responses. We also documented a distinctly different, intermittent, form of EPSC complexity, which also is predicted and easily explained by our anatomic hypothesis. In particular, the results were in accord with the suggestion that stimulation of the dentate gyrus might antidromically stimulate axon collaterals of CA3 neurons that make recurrent synapses onto the recorded cell. We conclude that the overall pattern of results is consistent with expectations based on the functional anatomy. The explanation does not demand a special type of intrinsic asynchronous mechanism for excitation-secretion coupling in the mf synapses.

INTRODUCTION

Granule cells of the dentate gyrus send their mossy-fiber (mf) axons to hippocampal region CA3, where they form

large synapses onto the proximal dendrites of the pyramidal neurons (Amaral 1979; Blackstad and Kjaerheim 1961; Frotscher et al. 1981; Johnston and Brown 1983). The mf system long has been recognized as offering certain advantages for biophysical studies of synaptic microphysiology because of the large size of the pre- and postsynaptic structures and also because of the anatomic and electrotonic proximity of the input to the soma of the postsynaptic neuron (Barrionuevo et al. 1986; Bekkers 1994; Brown and Johnston 1983; Brown and Zador 1990; Brown et al. 1979, 1989; Chicurel and Harris 1992; Jaffe and Brown 1992, 1997; Jaffe and Johnston 1990; Johnston and Brown 1983, 1984; Jonas et al. 1993; Siegel et al. 1992; Spruston et al. 1993; Xiang et al. 1994; Yu and Brown 1994).

The functional anatomy of the dentate and CA3 region makes the mf synapses more difficult to examine in isolation than certain other hippocampal synapses (Amaral 1993; Claiborne et al. 1993). Each CA3 pyramidal neuron receives $\sim 15,000$ excitatory inputs, and only about 50 of these (or $\sim 0.33\%$) are from the granule cell mf axons (Amaral et al. 1990; Braitenberg and Schüz 1983; Claiborne et al. 1986). The vast majority of the synapses are associational inputs from other pyramidal neurons (Miles and Wong 1986). For this and other reasons, the likelihood of recording a pure mf input commonly is overestimated (Claiborne et al. 1993).

To eliminate or reduce contamination from di- or polysynaptic pathways, this and other laboratories developed procedures and criteria for isolating and identifying pure mf excitatory postsynaptic currents (EPSCs) (Brown and Johnston 1983; Claiborne et al. 1993; Williams and Johnston 1991). Our study of the quantal mechanism of mf long-term potentiation (LTP) expression (Xiang et al. 1994) was restricted to unitary EPSCs that had simple waveforms with a smooth, fast rising phase and a smooth, monoexponential decay. Unfortunately, it is much more common to observe complex EPSC waveforms. Such response complexity can be observed even with relatively low stimulus intensities. These complex EPSCs exhibit irregularities and/or apparent multiple components on the rising and decay phases, they often display considerable variability in the latency from stimulation to the peak current; the EPSC rise times (time to peak) tend to be long, and the decay phase often departs considerably from a simple mono-exponential relaxation (Claiborne et al. 1993; Johnston et al. 1992).

Our working hypothesis has been that these complex EPSCs emerge from the complex anatomy of this brain region (Claiborne et al. 1993; Xiang 1995; Xiang et al.

1994). For example, they could represent some combination of mono-, di-, and/or polysynaptic inputs activated orthodromically and/or antidromically. These possibilities will be elaborated in conjunction with the experimental observations. Barrionuevo and co-workers (Langdon et al. 1993) advanced an alternative hypothesis, according to which complex EPSCs do not reflect anatomic complexity, but rather they result, at least in large part, from unusual intrinsic properties of the mf axons and/or their presynaptic boutons.

Here we begin to test experimentally some predictions of our anatomic hypothesis. Specifically, we examined the effects of a "suppression medium" (cf. Langdon et al. 1993) that was designed to discriminate against di- or polysynaptic inputs versus monosynaptic inputs. For this purpose, we added baclofen, a selective agonist for the γ -aminobutyric acid-B (GABA_B) receptor, to the usual saline. In many central neurons, baclofen causes a resting hyperpolarization and a reduced input resistance by activating a potassium conductance (Crunelli et al. 1988; Doi et al. 1990; Newberry and Nicoll 1984) along with presynaptic actions that diminish transmitter release (Bowery et al. 1980; Dutar and Nicoll 1988; Lanthorn and Cotman 1981; Scanziani et al. 1992).

On the basis of this action of baclofen on intermediate cells in a di- or polysynaptic circuit, our hypothesis predicts that the suppression medium should reduce the number of notches on the rising and decay phases, diminish the variance in the latency to the peak of the EPSC, decrease the amplitude and rise time of the individual and averaged EPSCs, and increase the number of transmission failures in evoked EPSCs (expected in some cases where there is no monosynaptic input). All of these predictions were confirmed. Our hypothesis makes very different predictions for antidromically activated monosynaptic inputs. Here we also furnish experimental documentation in support of this form of synaptic input as well.

METHODS

Slice preparation and maintenance

Transverse hippocampal slices (350 μ m) from 14–18 day-old male Sprague Dawley rats were prepared using a vibratome in ice-cold oxygenated (95% O₂–5% CO₂) "dissection saline" (Aghajanian and Rasmussen 1989) [composition was as follows (in mM): 252 sucrose, 2 KCl, 1 CaCl₂, 3 MgSO₄, 1.25 NaH₂PO₄, 26 NaHCO₃, and 10 D-glucose]. Slices then were placed in oxygenated regular artificial cerebrospinal fluid (ACSF) at 5–10°C and allowed to recover for ≥ 1 h while the solution equilibrated with room temperature (26–29°C). The ACSF contained (in mM) 124 NaCl, 2 KCl, 1 CaCl₂, 3 MgSO₄, 1.25 NaH₂PO₄, 26 NaHCO₃, and 10 D-glucose. Individual slices then were transferred as needed to a submerged-type recording chamber and perfused with oxygenated ACSF at room temperature.

Whole cell recordings and electrical stimulation

Whole cell patch recordings were made from visually identified pyramidal neurons in region CA3 of the hippocampus (Xiang 1995). Except as indicated later, the whole cell patch pipette solution contained (in mM) 120 K-gluconate, 10 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, 10 ethylene glycol-bis(β -ami-

noethyl ether)-N,N,N',N'-tetraacetic acid, 20 KCl, 2 MgCl₂, and 2 Na₂ATP (adjusted to pH 7.3 and osmolarity 275–285 mOsm). In acceptable recordings, the electrode resistances were 2–3 M Ω in the bath; the access resistance was 6–10 M Ω in the whole cell mode, and it changed <15% during the experiment. The cell input resistance was ≥ 0.25 G Ω . Electrical signals were amplified using an Axopatch 200A (Axon Instruments) in current- or voltage-clamp modes. Signals were filtered at 1–2 kHz, digitized at 22 kHz (Neuro-corder), stored on video tape, and analyzed off-line using software written for IGOR PRO.

In most experiments, we blocked GABA_A currents by addition of picrotoxin (10 μ M) and bicuculline (10 μ M) to the perfusion medium (Xiang et al. 1994). In other experiments, picrotoxin (10 μ M) was directly added to the pipette solution (Nelson et al. 1994). Under the latter conditions, we did not see any evidence of evoked inhibitory postsynaptic currents (IPSCs), but these may have been absent even without this procedure (cf. Langdon et al. 1993). The data obtained under these conditions were pooled because we found no differences in the results.

EPSCs were recorded at a holding potential of –80 mV and elicited every 5 s by extracellular stimulation (biphasic, 50 or 100 μ s/phase) of the stratum granulosum of the dentate gyrus using a bipolar stimulation electrode (2 25- μ m insulated platinum wires glued together with the centers ~ 30 μ m apart). The amplitude of the first (cathodal) phase of the biphasic pulse was 250–500 μ A, and the amplitude of the second (anodal) phase was smaller (100–300 μ A). The purpose of the second phase was to reduce the stimulus artifact. While adjusting the current, the polarity of the two stimulating electrodes was switched back and forth to determine which was preferable. The stimulation currents were larger than we previously used in a study designed to evoke pure unitary mf EPSCs (Xiang et al. 1994). That study used 60–200 μ A (typically ~ 100 μ A) for the first (cathodal) phase (see Fig. 1 of Xiang et al. 1994), proportionately smaller currents for the second (anodal) phase, and extensive "hunting" was required to find suitable recording and stimulation sites using these smaller stimulation currents.

In the first group of experiments, we established the dose-response relationship for the effect of baclofen on the passive membrane properties of these cells. Once a stable recording was maintained for ~ 5 min in control medium, baclofen was added to the bath in various concentrations (1, 3, 5, and 10 μ M), and we measured the resulting effect on the resting potential and input resistance. After perfusing for 7–15 min with the baclofen-containing solution, we then returned to the control medium to verify that any baclofen-produced effects were reversible.

In subsequent experiments on complex EPSCs, we restricted the analysis of the baclofen "suppression effect" to a single concentration (3 μ M) of baclofen. The purpose of these experiments was to use a dose of baclofen and a time window such that we could expect a suppression effect on polysynaptic circuits—given our particular recording and perfusion conditions, general procedures, and age, species, and strain of animal. In addition to postsynaptic actions, which were measured, one could anticipate, based on the literature (Blaxter and Carlen 1985; Bowery et al. 1980; Dutar and Nicoll 1988; Lanthorn and Cotman 1981; Scanziani et al. 1992) presynaptic actions that also could discriminate against polysynaptic circuits.

Video microscopy

Stimulation and whole cell recording electrodes were positioned under direct visual control using a fixed-stage, upright microscope (Zeiss Axioskop) equipped with a water immersion objective ($\times 40$, 0.75 NA, 2 mm working distance), infrared filtered light, differential interference contrast (DIC) optics, a video camera and a video enhancement device (Brown and Jaffe

1994; Edwards et al. 1989; Jaffe and Brown 1992, 1994, 1997; Keenan et al. 1988; MacVicar 1984; Stuart et al. 1993; Xiang 1995). This enabled recordings from visually preselected CA3 neurons that had an appearance that we have come to associate with cells displaying the characteristics of healthy CA3 pyramidal neurons (Xiang 1995).

Statistical analysis

Several measures were used to quantify the effect of baclofen on the EPSC waveforms: the number of notches or inflections on the rising phase, the number of notches on the decay phase, the latency from stimulation to the peak of the EPSC, the variance in the latency to peak, the peak time (total time from response onset to peak), the peak amplitude, and the rate of response failures. A notch was considered to occur on the rising phase if a subsequent peak was greater than a previous one and if the latency between them was <5 ms. Otherwise, the notch was considered part of the decay phase. Notches on single sweeps were identified visually and manually marked using the data analysis package (written using IGOR PRO, mentioned above). The analysis software facilitated identification of notches by allowing visual inspection at a variety of time bases (sweep speeds). The peak time was defined as the time between response onset and the largest peak during the rising phase, as defined above. Results are stated as means \pm SE unless indicated otherwise. The statistical significance of the effects of the baclofen "suppression medium" were determined with a two-tailed Student's *t*-test for paired comparisons ($\alpha = 0.05$).

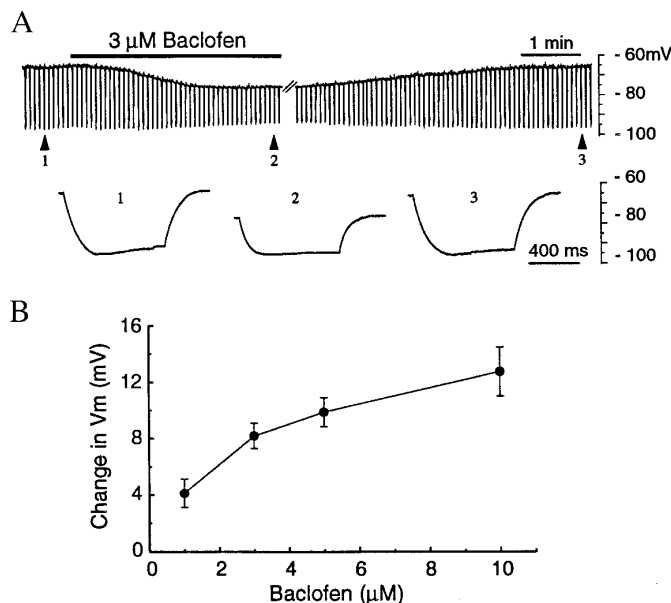


FIG. 1. Effects of baclofen on resting membrane properties of CA3 pyramidal neurons. *A*, top: record resting potential and voltage responses to hyperpolarizing current steps (0.2 Hz, 100 pA, 800 ms) in control medium, during wash-in of baclofen, and after wash-out of baclofen. Break in the record indicates an 8-min gap. Bottom: voltage responses to the current steps taken from the indicated portion of the top trace and illustrated with an expanded time base. Baclofen-produced hyperpolarization was accompanied by a reduction in the input resistance. Bath contained picrotoxin (10 μ M) and bicuculline (10 μ M). *B*: hyperpolarization of the resting potential as a function of the baclofen concentration (1, 3, 5, or 10 μ M) in the perfusion medium. Each point is the average of 4–6 experiments, and the vertical error bars indicate \pm SE. In 6 neurons, the bath contained picrotoxin (10 μ M) and bicuculline (10 μ M). In the other 13 neurons, these GABA antagonists were omitted from the bath and picrotoxin (10 μ M) was added to the pipette. Results for 3 μ M baclofen under these 2 conditions were within 5% of each other.

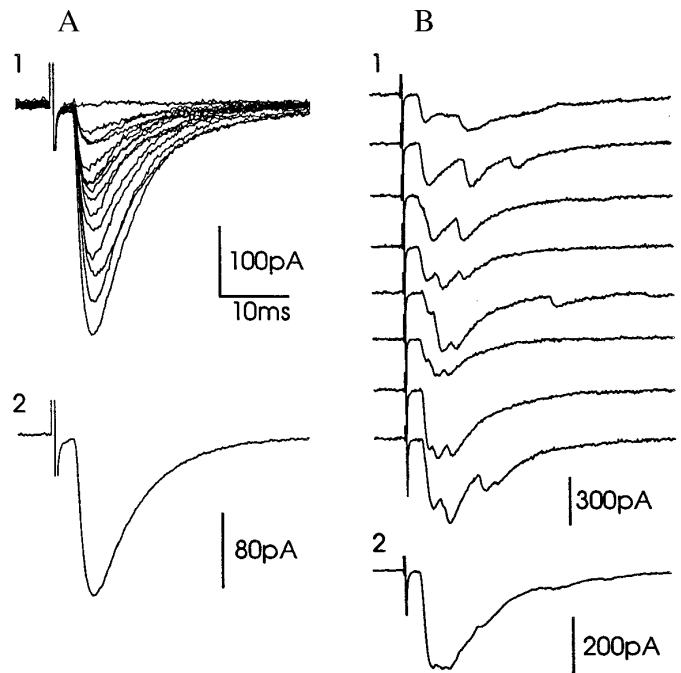


FIG. 2. Simple and complex excitatory postsynaptic currents (EPSCs) in evoked in CA3 pyramidal neurons. *A*: examples of simple EPSCs that satisfied our criteria for a mossy-fiber (mf) input. *A1*: 15 EPSCs are superimposed, illustrating marked fluctuations in the amplitude but little change in the waveform or latency to peak. *A2*: average of the 15 EPSCs, illustrating that the average waveform closely resembles the individual waveforms. *B*: examples of complex EPSCs recorded in another CA3 pyramidal neuron. *B1*: 8 EPSCs showing extreme variability and complexity. They are not shown superimposed because the variability makes the composite difficult to interpret. Note the notches and inflections on the rising phase, notches on the decay phase, and variability in the latency to peak. *B2*: average of 15 of these EPSCs that shows little similarity to the individual EPSCs. Note the apparent complexity of the average in the vicinity of the peak and during the decay. In these recordings, artificial cerebrospinal fluid contained 5 mM Ca^{2+} , 5 mM Mg^{2+} , 50 μ M APV, 10 μ M picrotoxin and 10 μ M bicuculline.

Unless stated otherwise, analysis is based on 20 successive EPSC waveforms per cell.

RESULTS

Data on the effects of the baclofen "suppression medium" were taken from 32 neurons that satisfied our selection criteria as described in METHODS. The resting potentials of these cells (not corrected for junctional potentials) ranged from -60 to -71 mV, with an average value of -65 ± 0.6 (SE) mV. The input resistances ranged from 0.25 to 0.46 G Ω , with an average value of 0.29 ± 0.01 G Ω .

Baclofen-produced resting hyperpolarization

The time course of the effect of baclofen on the resting potential and input resistance was recorded continuously in 19 cells. As illustrated in Fig. 1A, bath application of 3 μ M baclofen had a pronounced effect on the cell, causing a hyperpolarization (from -66 to -76 mV) accompanied by a decrease in the input resistance (from 0.28 to 0.21 G Ω). The input resistance was measured as the ratio of the peak hyperpolarizing voltage response to a 100-pA inward current step (800 ms). The current steps were delivered at 0.2 Hz.

The effect of baclofen on the input resistance and resting membrane potential was fully reversible (Fig. 1A).

For the suppression medium, we wanted the lowest concentration of baclofen that would produce a sufficiently large effect to reduce the likelihood of evoking di- or polysynaptic responses in the cell under observation. Various bath concentrations of baclofen (1, 3, 5, and 10 μM) were applied to slices to obtain the dose-response relationship of the hyperpolarizing effect of baclofen. Within this concentration range, baclofen hyperpolarized the membrane potential in a dose-dependent manner (Fig. 1B, total $n = 19$ cells from 19 slices). At 1 μM , baclofen caused a mean hyperpolarization of 4.1 mV, and at 10 μM , it caused a mean hyperpolarization 12.8 mV. We decided to use 3 μM in baclofen in the subsequent experiments on EPSC complexity because it reliably produced a sizable hyperpolarization (8.2 ± 0.9 mV; $n = 5$), and there was the additional possibility of a presynaptic action that could further discriminate against polysynaptic circuits (Blaxter and Carlen 1985; Bowery et al. 1980; Dutar and Nicoll 1988; Lanthorn and Cotman 1981; Scanziani et al. 1992). As will be seen, this concentration proved to be adequate.

Simple and complex synaptic current waveforms

Before proceeding to discuss the effects of our suppression medium on complex EPSCs, we first should illustrate the

much less commonly observed simple EPSCs that we ordinarily study (Xiang et al. 1994). A typical example of a simple EPSC that satisfies our criteria for a mf input (Claiborne et al. 1993) is shown in Fig. 2A. Fifteen superimposed individual EPSCs are shown on the *top* (Fig. 2A1), and their average is shown on the *bottom* (Fig. 2A2). Note that the waveforms have a smooth rise and decay without any obvious notches, the rise time is short, and there is little variation in latency to peak (Fig. 2A1). Although the peak amplitude fluctuated considerably from trial to trial, as expected of a quantal process, the overall shape of the EPSCs remained relatively constant. Thus the shape of the average of the EPSCs (Fig. 2A2) is similar to that of the individual EPSCs (Fig. 2A1).

These simple EPSCs, which have a better chance of representing the mf synaptic input, are difficult to obtain, as would be expected from the functional anatomy (Claiborne et al. 1993). To increase the probability of doing so, we use very fine stimulating electrodes, low stimulation currents, and engage in extensive ‘‘hunting’’ for a suitable stimulation site, an appropriate stimulation intensity, and desirable recording site. Much more commonly we see complex EPSCs, which are easy to obtain without any special procedures. They are in fact by far the most usual response.

In the past, we never have examined systematically or reported on these complex EPSCs, an example of which is

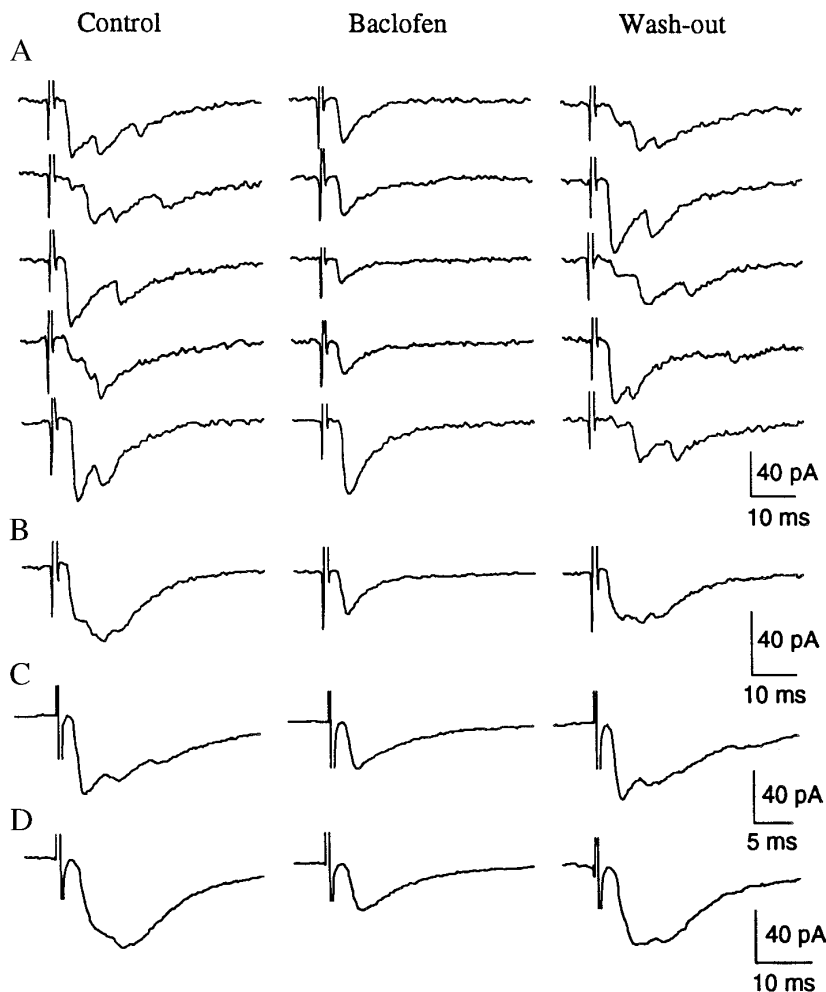


FIG. 3. Suppression of evoked EPSC waveform complexity by baclofen. A: examples of single traces of EPSCs in the control medium (left), during bath application of 3 μM baclofen (middle), and after wash-out (right). Note that the responses became simpler in the baclofen suppression medium and that this effect was reversible. B: averaged waveforms of 20 evoked EPSCs excluding response failures (from the same cell illustrated in A). In the control medium and after wash-out, note the obvious complexity in the vicinity of the peak and the long latency to the peak. C and D: 2 other examples of the effect of baclofen on average of 20 EPSC waveforms (excluding response failures; see Fig. 4). In A, B, and D, γ -aminobutyric acid (GABA) antagonists were omitted from the bath and picrotoxin (10 μM) was added to the pipette; in C, the bath contained picrotoxin (10 μM) and bicuculline (10 μM).

shown in Fig. 2*B*. Note that the latency to peak in these eight evoked EPSCs is quite variable; there are obvious notches on the rising and/or falling phases of many of the waveforms; and the averaged EPSC waveform (Fig. 2*B2*, based on 15 successive responses) does not closely resemble the individual EPSC waveforms (Fig. 2*B1*). Typically the rise time of the averaged EPSC is long and/or there is obvious complexity in the vicinity of the peak (Figs. 2*B2* and 3, *B* and *D*). In previous studies, if any of these features were present, the data were discarded because we assumed that the synaptic response was not a pure mf EPSC and might not even contain a mf component to the EPSC (Barrioneuvo et al. 1986; Brown and Johnston 1983; Claiborne et al. 1993; Xiang 1994).

Complex waveform suppression by baclofen

In the remaining experiments, where no effort was made to elicit pure mf EPSCs that satisfied our criteria (Claiborne et al. 1993), only complex EPSC waveforms were obtained. An example of a complex EPSC is shown in Fig. 3*A* (left). Note the multiple notches on the rising and/or decay phases, the variable latency of the peak response, the fact that the average of 20 waveforms (Fig. 3*B*, left) does not resemble the individual waveforms (Fig. 3*A*, left), and the long latency to peak and long time to peak of the averaged EPSC (Fig. 3*B*, left; see also Figs. 3*D* and 4, top). The individual EPSC waveforms evidence enormous variability from trial to trial (Fig. 3*A*, left; also see Figs. 2*B1* and 4, top).

After exposure to 3 μ M baclofen, the complex waveforms were dramatically simplified (Fig. 3*B*, middle). Many of the obvious notches or inflections clearly were suppressed, leaving a simpler response with relatively smoother rising and decay phases. The averaged waveform (Fig. 3*B*, middle) resembled more closely the individual traces (Fig. 3*A*, middle). This suppression effect was fully reversible, as judged both by the individual waveforms (Fig. 3*A*, left and right) and the average (Fig. 3*B*, left and right). Also illustrated is the effect of the suppression medium on averaged EPSCs in two additional cells (Fig. 3, *C* and *D*). In both cases, baclofen produced a dramatic change in the shape of the waveform and the effect was reversible.

If the evoked responses in a cell entirely reflect polysynaptic inputs, then one might expect baclofen sometimes to block synaptic transmission completely. An example of this is shown in Fig. 4, where individual EPSCs are on the left and averages are on the right. Notice that in the control bathing medium, the individual EPSCs had relatively long and highly variable latencies to peak (Fig. 4*A1*), consistent with the possibility that there was no monosynaptic input to this cell. Bath application of baclofen completely abolished the response, as illustrated in the individual EPSCs (Fig. 4*B1*) and the average of 20 consecutive EPSCs (Fig. 4*B2*). After baclofen wash-out, a response with a long and variable latency reappeared (Fig. 4*C1*). In control medium and after baclofen washout, the averaged EPSCs exhibit a long latency to peak and slow rise time (Fig. 4, *A2* and *C2*). This cell was otherwise similar to the larger set, with a resting potential of -61 mV and an input resistance of 0.32 G Ω . Such a complete block by baclofen was not observed in cells the response of which appeared to include a monosynaptic input (Fig. 5, discussed in the following text).

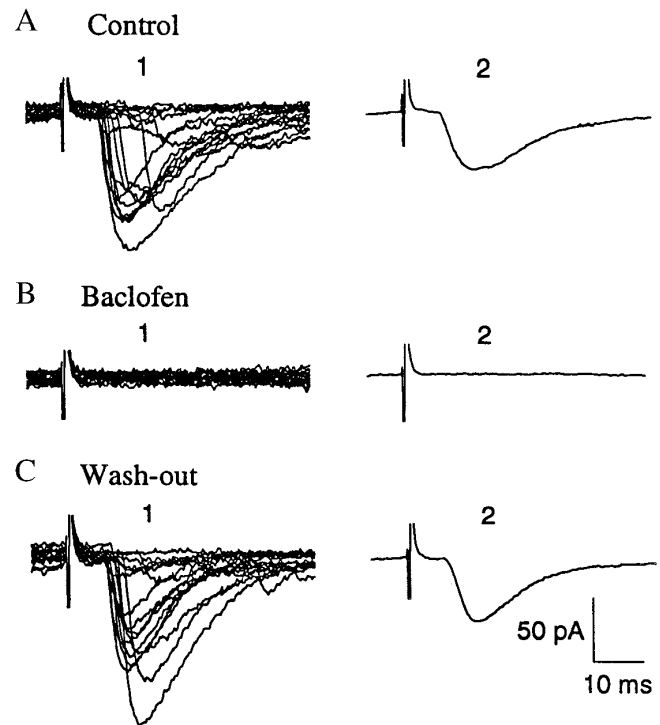


FIG. 4. Example of the abolition of responses in the baclofen suppression medium. *A*: evoked EPSCs in control medium. *A1*: 16 superimposed traces of complex EPSCs in control medium, showing with long and variable latencies to peak. *A2*: average of these EPSCs (including response failures). Note that the average has a long rise time and a hint of complexity in the vicinity of the peak. *B*: abolition of EPSCs in the baclofen suppression medium. *B1*: 16 superimposed traces in the baclofen suppression medium, showing consistent response failures. *B2*: average of these response failures, showing complete block of evoked synaptic current in the presence of 3 μ M baclofen. *C*: evoked EPSCs after wash-out of baclofen. *C1*: 16 superimposed traces of complex EPSCs after washout of baclofen, again showing long and variable latencies to peak. Effect of baclofen was fully reversible. *C2*: average of these EPSCs (including response failures). Bath contained picrotoxin (10 μ M) and bicuculline (10 μ M).

Quantification of baclofen suppression effect

Qualitative impressions of baclofen-associated changes in waveform complexity were reinforced by seven different quantitative measures that revealed statistically significant effects in 10 cells (Table 1). Superscripts in Table 1 indicate specific statistical comparisons among conditions. Data for each of these additional 10 cells were taken from 20 successive evoked EPSCs (200 total EPSCs). For the 10 cells in Table 1, the mean input resistance and resting potential were 0.28 G Ω and -64 mV, respectively, similar to the total set of 32 cells. Unless otherwise stated, measurements were made on individual sweeps and the results then were averaged.

The mean number of notches on the rise of each response was reduced from the control value by 74% during baclofen application, an effect that was statistically significant (Table 1). The mean number of notches in the decay phase also was found to decrease significantly during baclofen application and by about the same amount. The mean latency from stimulation to the peak of the EPSC decreased significantly as did the variance in the latency of the peak. In the suppression medium, rise times were significantly shorter, and the

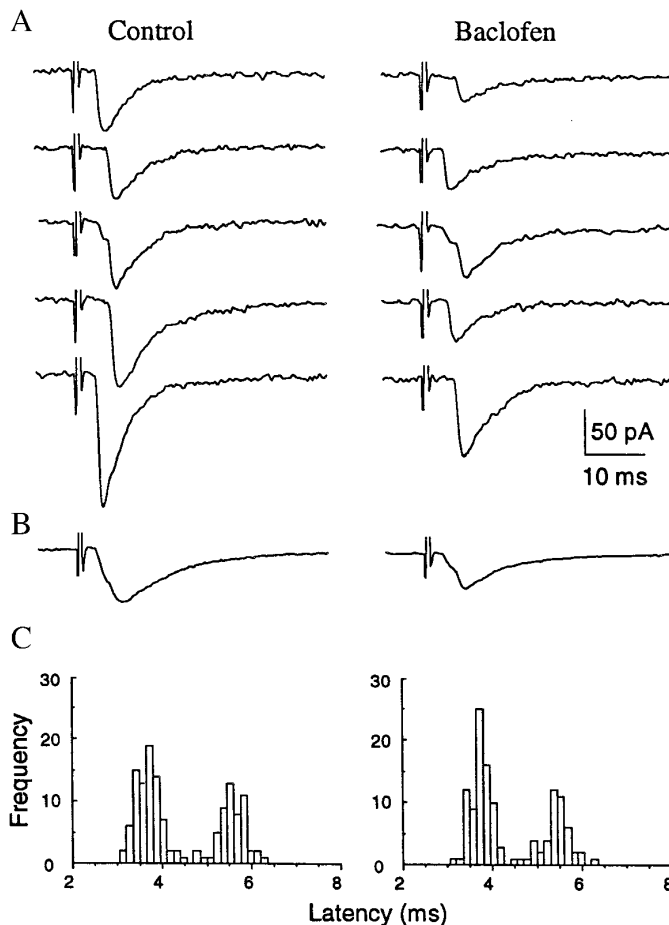


FIG. 5. Effects of the suppression medium on intermittently complex EPSCs. *A*: in the control medium (left) the individual EPSCs are relatively simple on most trials, showing a smooth rising and decay phase. In some trials, however, there was a clear inflection and/or notch on the rising phase (left, 3rd trace from top). This complexity was not blocked by 3 μ M baclofen (right, 3rd trace from top). Both in the control medium and in the suppression medium there was considerable variability in the onset latency and the latency to peak. *B*: average of 20 EPSCs in the control medium (left) and in the suppression medium (right). Baclofen did not eliminate fluctuations in the latency to peak or the inflections in the rising phase. Note that the inflection on the rising phase remained evident in the averaged EPSC in both the control medium and in baclofen. *C*: frequency histograms of EPSC onset latencies in the control medium (left) and in the suppression medium (right). The distribution is clearly bimodal, indicating fluctuations around 2 distinct latencies. The general shape of the frequency distribution was unaffected the suppression medium. GABA antagonists were omitted from the bath and picrotoxin (10 μ M) was added to the pipette.

peak amplitudes were significantly smaller (excluding response failures). Baclofen also caused a 60% increase in the failure rate, which was statistically significant. In 5 of these 10 cells, the same measures also were made after wash-out of baclofen (Table 1). As indicated, the mean values of our seven response measures after washout were not significantly different from the corresponding values in the control period, indicating that the effect was reversible.

Resistance to baclofen suppression effect

The responses in two additional cells were interesting because their evoked EPSCs evidenced only *intermittent* complexity. Using our normal classification scheme, these EPSCs could not be categorized as being typical of either simple or complex responses. In both cells, the EPSC waveforms were simple on most trials, consisting of smooth and monophonic rising and decay phases, as shown for one of these cells in Fig. 5*A* (left). However the onset latencies fluctuated from trial to trial (Fig. 5*A*, left), and in some trials, there was a clear notch on the rising phase (Fig. 5*A*, left, 3rd trace from top). This notch remained evident at the same latency in the averaged EPSCs (Fig. 5*B*, left). In these two cells, bath application of baclofen reduced the response amplitudes slightly but it did not suppress the latency fluctuations (Fig. 5*A*, right) or the notch on the rising phase, which remained evident both in the individual EPSCs (Fig. 5*A*, right) and in the averaged EPSCs (Fig. 5*B*, right). In contrast to what we saw before, the averaged EPSC does appear to be a simple composite of the individual EPSCs. In these two cells, the mean resting potential and input resistance were -65 mV and 0.32 G Ω , respectively, similar to the larger group.

Closer inspection of individual EPSCs revealed that the response latency fluctuated around two values, as illustrated in the frequency histograms of onset latencies (Fig. 5*C*), a phenomenon that was never seen in the typical complex EPSCs. Each latency measurement was to the onset of synaptic current, regardless of whether there was a notch on the rising phase. In control medium, measurements of 136 EPSCs revealed a bimodal distribution of latencies (Fig. 5*C*, left). In the suppression medium, analysis of 124 EPSCs revealed a similar bimodal distribution (Fig. 5*C*, right).

For the example illustrated in Fig. 5, we divided the data at 4.6 ms into two distributions and analyzed the early and late subsets separately. In the control medium, the mean latency was 3.70 ± 0.03 ms for early subset and 5.57 ± 0.04 ms for late subset. Similarly, in baclofen, the mean

TABLE 1. Effect of baclofen on seven measurements of EPSC waveforms

EPSC Waveform Measurements	Control Saline*	Baclofen Suppression*	Baclofen Washout†
Rise phase notches	$0.68 \pm 0.09^{\ddagger}\S$	$0.18 \pm 0.06^{\ddagger}$	$0.61 \pm 0.12\S$
Decay phase notches	$0.58 \pm 0.05^{\ddagger}\S$	$0.15 \pm 0.03^{\ddagger}$	$0.44 \pm 0.12\S$
Peak time, ms	$5.59 \pm 0.51^{\ddagger}\S$	$3.56 \pm 0.46^{\ddagger}$	$5.82 \pm 0.89\S$
Latency to peak, ms	$10.53 \pm 0.79^{\ddagger}\S$	$8.86 \pm 0.77^{\ddagger}$	$10.86 \pm 1.02\S$
Latency variance, ms ²	$8.49 \pm 2.29^{\ddagger}\S$	$2.74 \pm 0.54^{\ddagger}$	$15.31 \pm 8.82\S$
Peak amplitude, pA	$73.1 \pm 17.0^{\ddagger}\S$	$37.9 \pm 7.6^{\ddagger}$	$75.4 \pm 27.9\S$
Failure rate, %	$7.7 \pm 3.1^{\ddagger}\S$	$35.2 \pm 10.8^{\ddagger}$	$9.4 \pm 5.3\S$

EPSC, excitatory postsynaptic current. * $n = 10$; † $n = 5$; ‡ $P < 0.05$ for paired t -test on 10 cells; each entry is the mean \pm SE of 200 EPSCs (10 cells \times 20 EPSCs per cell in each condition); § $P > 0.05$ for paired t -test on 5 of the 10 cells that also were held throughout the washout period; each entry is the mean \pm SE of 100 EPSCs (5 cells \times 20 EPSCs per cell in each condition).

latency was 3.75 ± 0.03 ms for early subset and 5.44 ± 0.05 ms for late subset. This pattern of results differs qualitatively from that described above and quantified in Table 1 for the majority of the cells, and it suggests a fundamentally different mechanism, considered later.

DISCUSSION

Simple and complex synaptic current waveforms

When appropriate procedures are used, focal extracellular stimulation of the stratum granulosum of the dentate gyrus sometimes produces simple EPSCs (Fig. 1A) with smooth and short rising phases, smooth and monoexponential decay phases, and little variance in the latency to onset or peak (Claiborne et al. 1993; Xiang et al. 1994). More commonly, however, complex EPSCs are evoked by extracellular stimulation of dentate (Fig. 1B, 2–4) (Claiborne et al. 1993). These can have some combination of the following features: obvious notches and inflections or just inflections on the rising phase, notches on the decay phase, considerable variance in the latency to peak, and slow rise times.

We previously hypothesized that complex EPSCs emerge from the complexity of anatomy of the CA3 region and dentate gyrus (Claiborne et al. 1993). Since formulating that hypothesis, evidence regarding the anatomic complexity in this brain region has continued to mount (Amaral 1993; Scharfman 1993–1996). We proposed that in some cases complex responses represent di- or polysynaptic inputs. Here we used a suppression medium to test this suggestion. The suppression medium, which contained 3 μ M baclofen, was shown to hyperpolarize CA3 cells and reduce their input resistance (Fig. 1). In many cells, it is known to have presynaptic inhibitory effects as well (Bowery et al. 1980; Dutar and Nicoll 1988; Lanthorn and Cotman 1981; Scanziani et al. 1992). These actions of baclofen, we reasoned, should reduce the likelihood of activating polysynaptic inputs to these cells and thereby reduce the complexity of the evoked EPSCs.

The prediction was confirmed qualitatively simply by observing obvious changes in the evoked EPSCs (Figs. 2–4) and quantitatively based on several different measures of the EPSC waveforms (Table 1), all of which were shown to undergo large and statistically significant changes in the expected direction. In particular, the suppression medium significantly reduced the number of notches or inflections on the rising phase and the number of notches on the decay phase of the EPSC, shortened the EPSC rise time and latency to peak, diminished the variance in the latency to peak, reduced the peak amplitude, and increased the response failure rate.

All of these actions are consistent with the idea that the demonstrated postsynaptic effect of baclofen on the resting membrane properties of these cells (Fig. 1), and the probable presynaptic effect on transmitter release (Bowery et al. 1980; Dutar and Nicoll 1988; Lanthorn and Cotman 1981; Scanziani et al. 1992) should diminish the likelihood that extracellular stimulation would activate polysynaptic inputs to any given CA3 pyramidal neuron. In cases where there are only polysynaptic inputs to a neuron, one might expect baclofen sometimes to abolish throughput altogether, and indeed we have shown this to occur (Fig. 4).

Intermittently complex synaptic current waveforms

Claiborne et al. (1993) also suggested a second respect in which irregular waveforms could result from the anatomic complexity; namely that some of the evoked responses could result from antidromic activation of an axon collateral in the dentate. In particular, they noted that some CA3 neurons send an axon collateral to the dentate, this collateral could be antidromically stimulated, thereby antidromically spiking the CA3 soma, and resulting in an orthodromic associational response in another CA3 neuron (Claiborne et al. 1993).

Supporting of this possibility, Claiborne et al. (1993) pointed to anatomic evidence that CA3 collaterals can project into the hilus (Ishizuka et al. 1990) and the inner third of the molecular layer (Li et al. 1992; more fully reported in Li et al. 1994) and electrophysiological evidence that they can be antidromically fired by a stimulating electrode in the dentate (Misgeld et al. 1979). The result could be a “monosynaptic input” that was *not* from a mf synapse. They also called attention (Fig. 2 of Claiborne et al. 1993) to the possibility of antidromic stimulation of other cell types, including mossy cells and granule cells, as well as the possibility of monosynaptic inputs from the perforant pathway. Along a similar vein, Langdon et al. (1993) considered, but then discounted, the possibility of antidromic stimulation of axon collaterals of the granule cells in the dentate gyrus.

For any of these cases of antidromic stimulation, one could imagine (depending on the conduction time associated with the axon collateral) compound monosynaptic synaptic responses occurring asynchronously and thus possibly resembling in certain respects the complex responses that result from compound di- or polysynaptic inputs. However, in the case of antidromic stimulation of a monosynaptic input, we would expect the suppression medium to have a very different effect. The two cells in which we observed intermittent EPSC complexity (Fig. 5) would be consistent with the possibility of two monosynaptic inputs, at least one of which resulted from antidromic activation of an axon collateral, consistent with the original suggestion of Claiborne et al. (1993).

In these two cases, many individual EPSCs did in fact have a simple waveform. However there was considerable variability in the onset latency from trial to trial, and on some occasions, there was an obvious notch or inflection on the rising phase of the EPSC (Fig. 5A). This inflection was preserved in the averaged EPSC (Fig. 5B, *left*), indicating that its time of occurrence was not highly variable. The baclofen suppression medium had no effect on these phenomena (Fig. 5, A and B, *right*). These two cases also differed from the others in that the response latencies revealed a distinctly bimodal frequency distribution both in the control medium (Fig. 5C, *left*) and in baclofen (Fig. 5C, *right*). This pattern suggests that two monosynaptic inputs, with distinctly different latencies, were being activated probabilistically in a manner that was unaffected by baclofen (Fig. 5C). Under these circumstances, baclofen clearly does not block synaptic transmission (unlike the results in Fig. 4).

The simplest explanation is that the stimulating electrode intermittently fired or caused release from two monosynaptic inputs to CA3 and that these two inputs differed in latency. It is possible that one monosynaptic does in fact represent

the mf synapse, but this need not be the case. The results can be explained if one assumes probabilistic activation of any two inputs to CA3 that have different latencies—by virtue of intrinsic conduction velocity and/or the actual distance traveled (see later).

Viability of antidromic stimulation

On the basis of inferences about conduction velocities and results of microdissections, Barrionuevo and coworkers (Langdon et al. 1993) concluded that antidromic stimulation of granule cells makes only a small contribution to the extent of desynchronization of inputs and is therefore is not a likely candidate for most of the observed response complexity. As already indicated, we had previously suggested (Claiborne et al. 1993) an alternative possibility involving antidromic stimulation of axon collaterals of CA3 neurons. On the basis of the present data (Fig. 5) and the work of others (Ishizuka et al. 1990; Li et al. 1994; Misgeld et al. 1979), we suspect that extracellular stimulation in the dentate may in fact commonly activate some inputs to CA3 via antidromic stimulation. Indeed our computer simulations (Y.-W. Lam and T. H. Brown, unpublished data) suggest that the added conduction time could be considerable. At least qualitatively, it is easy to see how probabilistic activation of two monosynaptic inputs that differ in latency could in principle produce the kind of results shown in Fig. 5. In light of the known anatomy of this brain region (Amaral 1993; Claiborne et al. 1993), it is simple to account for the results presented here, without having to resort to the unorthodox neurophysiological mechanisms discussed later.

Intrinsically asynchronous excitation-secretion coupling

Barrionuevo and coworkers (Langdon et al. 1993) raised a radical alternative to our hypothesis that EPSC complexity reflects anatomic complexity. According to their interesting hypothesis, the mf axons and/or their synaptic boutons themselves have the intrinsic physiological property of causing asynchronous activation of neurotransmitter release. The mechanism for this asynchronous activation was suggested to involve an impedance mismatch, between the mf axon and its synaptic bouton, that causes a large (1–3 ms) delay in the invasion of the mf bouton by a nerve impulse. Our computer simulations have failed to find support for this possibility (Y.-W. Lam and T. H. Brown, unpublished results) and, in any case, it is not obvious how this mechanism could account for the range of data reported here.

In examining the consequence of suppression media on complex EPSCs in six CA3 pyramidal cells, Barrionuevo and coworkers (Langdon et al. 1993) saw little effect. The complex EPSCs were evoked by “bulk” extracellular stimulation of the dentate and the suppression medium contained either a low $[Ca^{2+}]/[Mg^{2+}]$ ratio ($n = 3$) or glutamate receptor antagonists ($n = 3$). Although they do not quantify the neurophysiological effects of the suppression medium on individual EPSCs, they noted that in five of six cells, the suppression medium in general “had little or no effect on the shape” of the averaged waveforms. Negative results, based on qualitative impressions of a few averaged compound EPSCs, are not compelling.

By contrast, we furnished compelling positive evidence that a suppression medium in fact can alter seven different quantitative measures of individual complex EPSCs. Our suppression medium also affects the averaged EPSC waveforms, and the effects reported here are qualitatively obvious and quantitatively the effects are statistically significant (Figs. 2B, 3, and 4; Table 1, $P < 0.05$).

Neurophysiological implications and conclusions

These findings have important implications for studies of LTP in the mf synapses because they suggest that discrepant results in the literature may reflect that fact that different populations of synapses are being studied. In our experience, mf EPSCs have simple waveforms, fast rise times, short onset latencies, and relatively invariant latencies to peak (Fig. 2A1) (Barrionuevo et al. 1986; Brown and Johnston 1983; Claiborne et al. 1993; Xiang 1995; Xiang et al. 1994). In spite of considerable variance in the amplitudes of mf EPSCs, the waveform shapes remain relatively invariant. Thus the average waveform (Fig. 2A2) is similar to the individual waveforms (Fig. 2A1). These features of the simple EPSCs are dramatically different from the complex EPSCs that we illustrated and described here (Figs. 2B, 3, and 4) and the waveforms of which are significantly altered by the suppression medium (Table 1).

In considering these differences between the two types of evoked EPSC, and the relative ease of evoking them, it is important to keep the functional anatomy in mind (Amaral 1993; Braitenberg and Schüz 1983; Claiborne et al. 1993). Recall that the mf inputs constitute only 0.33% of the total number of excitatory synapses into CA3 pyramidal neurons (50 of 15,000). The total number of *intact* mf inputs to a given CA3 neuron that could be activated by a stimulating electrode in a typical thin brain slice is presumably much more than 50 and might well be zero, especially if the slice is not taken at the optimal angle (Claiborne et al. 1993). The somata of the few granule cells that have intact connections to any particular CA3 neuron presumably are distributed throughout the extent of the stratum granulosum within the plane of the slice.

Consistent with this functional anatomy, we find that special procedures and extensive hunting are required to evoke simple EPSCs that satisfy our criteria for mf synapses, whereas stimulating almost anywhere in the dentate with a large electrode and enough current will invariably generate complex EPSCs. The intermittent complexity, which we observe less often, could arise from two monosynaptic inputs with different latencies—one or both of which could be activated antidromically. Langdon et al. (1993) failed to observe this intermittent complexity with a bimodal frequency distribution of latencies. Although the reason is unclear, one could hypothesize that the failure resulted from the fact that their compound EPSCs reflected stochastic activation of a sufficiently large number of synaptic inputs that the latency variance associated with the numerous different inputs obscured the appearance of distinct modes in the overall distribution.

In conclusion, given what we now know, the most parsimonious and natural explanation is that the complexity of excitatory postsynaptic potentials evoked in CA3 by extra-

cellular stimulation of the dentate emerges naturally from the known anatomy of this region and not, as proposed by Langdon et al. (1993), from unorthodox mechanisms of excitation-secretion coupling in the mf boutons or unusual action potential propagation delays due to impedance mismatching between the axon and synapse.

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