Synaptic Transmission in Pair Recordings From CA3 Pyramidal Cells in Organotypic Culture

PAUL PAVLIDIS AND DANIEL V. MADISON
Department of Molecular and Cellular Physiology, Stanford University School of Medicine, Stanford, California, 94305-5345

Pavlidis, Paul and Daniel V. Madison. Synaptic transmission in pair recordings from CA3 pyramidal cells in organotypic culture. *J. Neurophysiol.* 81: 2787–2797, 1999. We performed simultaneous whole cell recordings from pairs of monosynaptically coupled hippocampal CA3 pyramidal neurons in organotypic slices. Stimulation of an action potential in a presynaptic cell resulted in an AMPA-receptor-mediated excitatory postsynaptic current (EPSC) in the postsynaptic cell that averaged ~34 pA. The average size of EPSCs varied in amplitude over a 20-fold range across different pairs. Both paired-pulse facilitation and depression were observed in the synaptic current in response to two presynaptic action potentials delivered 50 ms apart, but the average usually was dominated by depression. In addition, the amplitude of the second EPSC depended on the amplitude of the first EPSC, indicating competition between successive events for a common resource that is not restored within the 50-ms interpulse interval. Variation in the synaptic strength among pairs could arise from a variety of sources. Our data from anatomic reconstruction, $1/CV^2$ analysis, paired-pulse analysis, and manipulations of calcium/magnesium ratio suggest that differences in quantal size and release probability do not appear to vary sufficiently to fully account for the observed differences in amplitude. Thus it seems most likely that the variability in EPSC amplitude between pairs arises primarily from differences in the number of functional synapses. Injections of the calcium chelator bis-(*o*-aminophenoxy)-*N*,*N*,*N*′,*N*′-tetraacetic acid into the presynaptic neuron resulted in a rapid and nearly complete block of transmission, whereas injection of the slower-acting chelator EGTA resulted in a variable and partial block. In addition to demonstrating the feasibility of manipulating the intracellular presynaptic environment by injection into the presynaptic soma, these data, and the EGTA results in particular may suggest variability in the linkage between calcium entry sites an release sites in these synapses.

INTRODUCTION

The analysis of synaptic transmission in the mammalian CNS increasingly has turned to the use of techniques designed to monitor transmission between single pairs of neurons. These include minimal stimulation, where a small number (ideally only one) of presynaptic fibers are stimulated extracellularly, and paired recording, where intracellular recording from two synaptically coupled cells is performed.

Minimal stimulation, although relatively rapid and simple to perform, suffers from a number of drawbacks. The primary concern is that one rarely can be certain that a single cell is being reliably stimulated. Pair recordings, where simultaneous intracellular recordings are made from only two synaptically connected neurons, do not suffer from this problem and also permit direct electrophysiological characterization and pharmacological manipulation of the presynaptic cell (Miles and Poncer 1996). The major difficulty with pair recordings is that incidence of synaptic connection between any two cells is often low, and thus connected pairs can be difficult to obtain (Malinow 1991). One way around this is to use primary dissociated culture systems where connectivity is much higher (Bekkers and Stevens 1990). Of course, with dissociated cultured preparations come questions as to the identity of the recorded cells and whether the synaptic and connective properties are similar enough to those of mature synapses in brain for useful comparisons to be made.

The use of organotypic brain slice cultures partially has ameliorated such concerns because identification of cell types is much easier than in dissociated culture and the cells maintain a morphology and connectivity similar to that in native brain tissue (Gahwiler et al. 1997). Previous work on roller-tube cultures of hippocampal slices has supported the idea that organotypic cultures have properties closer to acute slices than dissociated cultures (Gahwiler et al. 1997). Another useful feature of organotypic cultures is that they express long-term potentiation (Stoppini et al. 1991) and other forms of synaptic plasticity including paired-pulse facilitation and depression (Debanne et al. 1996a) and thus are useful in studying these effects in pairs of neurons (Debanne et al. 1996b).

Here we describe some of the properties of synaptic transmission in pair recordings performed in synaptically connected CA3 pyramidal cells in organotypic slices maintained in interface-type culture (Stoppini et al. 1991). Overall we find that the properties of these recordings compare well with those performed in acute slices (Miles and Wong 1986) as well as roller cultures (Debanne et al. 1995). In addition, we demonstrate the feasibility of pharmacologically manipulating the presynaptic cell by including exogenous calcium buffers in the presynaptic recording electrode. Some of these results have been presented in abstract form (Pavlidis and Madison 1997).

METHODS

Tissue culture

Interface cultures of hippocampal slices were prepared as described (Stoppini et al. 1991). We used 7- to 10-day-old Sprague-Dawley rats. Cultures were maintained at 37°C for 3 days and then kept at 34°C for the remaining culture period. Cultures were used after 7–14 days in culture. Healthy cultures selected for recording usually had a well-defined, raised border and a relatively clearly defined stratum pyramidale. Cultures with dark (apparently necrotic) material present in the CA3 region or a vacuolated (''cratered'') appearance or that had extensively flattened borders were rejected. On the basis of these

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criteria, approximately one-half to two-thirds of our cultures from any given preparation typically were judged to be sufficiently healthy for recording.

Electrophysiology

Individual slice cultures were transferred to a recording chamber perfused at 2–3 ml/min with artificial cerebrospinal fluid (ACSF) with the following composition (in mM): 119 NaCl, 2.5 KCl, 2.5 $CaCl_2$, 1.3 $MgSO_4$, 1 NaH_2PO_4, 26.2 $NaHCO_3$, and 11 glucose, pH 7.4, saturated with 95% O_2-5% CO_2. ACSF reagents were of molecular biology grade (Fluka). All experiments were performed at room temperature (21–23°C). 6-nitro-7-sulphamoylbenzo[*f*]quinoxaline-2,3-dione (NBQX), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), TTX, and picrotoxin were from RBI; all other reagents were from Sigma.

Whole cell recordings from CA3 pyramidal cells were made blindly (Blanton et al. 1989) or using an infrared-DIC microscope (Dodt and Zieglgansberger 1990). Recordings of excitatory postsynaptic current and potentials (EPSC and EPSPs) were made using an Axopatch 1C or Axoclamp 2A (Axon Instruments, Foster City, CA). Presynaptic current-clamp records were made with an Axoclamp 2A. Pre- and postsynaptic events were sampled at 10 kHz and low-pass filtered at 1–2 kHz. Series and input resistances of voltage-clamp recordings were monitored throughout experiments and did not vary by >20% over the course of the recording within experiments included in the data set.

In many experiments, we used the perforated-patch technique for the postsynaptic recording. Amphotericin (Fluka) was prepared at a concentration of 200–300 μg/ml by dilution of a 60 mg/ml DMSO stock (prepared at least weekly) into a solution of (in mM) 55 Cs methansulfonate, 75 Cs_2SO_4, 10 HEPES, and 8 $MgCl_2$ (pH 7.2 with CsOH). This solution was sonicated briefly to disperse the amphotericin and was usable for 1–2 h after preparation. The same solution without amphotericin was used to fill the tips of electrodes (2–5 MΩ), whereas the amphotericin solution was used for backfilling. Series resistances stabilized in 10–60 min between 15 and 40 MΩ. In experiments using broken patch whole cell mode, series resistance varied from 10 to 25 MΩ.

To establish a pair recording, a second whole cell recording was obtained in an adjacent area of the CA3 cell body layer (typically ~100–300 μm separation between cells in blind recordings, 10–100 μm in visualized recordings). The presynaptic electrode solution composition was (in mM) 120 K gluconate, 40 HEPES, 5 $MgCl_2$, 2 NaATP, and 0.3 NaGTP (pH 7.2 with KOH; in some experiments, we used $MeSO_4$ as the major cation). When EGTA or bis-(*o*-aminophenoxy)-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA) was included in the pipettes, the K gluconate concentration was lowered slightly so as to maintain osmolarity at 290 mOsm. This solution also was used for postsynaptic recordings in some experiments. Presynaptic cells were held in current clamp and induced to fire single action potentials by brief injection of depolarizing current (typically 20–50 pA for 20 ms). When a successful pair was obtained (i.e., a monosynaptic EPSC was evoked by a presynaptic action potential), the presynaptic cell was stimulated by current injection at 0.03–0.1 Hz throughout the experiment.

Data analysis

On- and off-line data analysis was performed using custom software developed in our laboratory in the Labview programming environment (National Instruments). Because the exact time of action potential occurrence during the depolarization of the presynaptic cell could vary slightly from trial to trial, analysis windows used for the postsynaptic EPSC were locked to the time of occurrence of the peak of the action potential. Sweeps in which no action potential occurred or in which the postsynaptic recording was distorted by spontaneous synaptic activity were excluded from analysis. In some experiments, polysynaptic events obscured the peak of the event in many sweeps, so in these cases the initial slope of the event was analyzed rather than the amplitude. Spontaneous synaptic events (mEPSCs) were detected automatically and measured as described (Ankri et al. 1994).

Histology

In some experiments, neurobiotin (Vector Laboratories) was included in the recording electrodes (0.5%) to allow anatomic reconstruction of the cells. Cells were filled for 15–60 min before the electrodes were withdrawn gently after which the culture was usually left in the recording chamber for an additional 15–60 min. A sketch of the location of the cells within the slice was made to allow later identification of the pre- and postsynaptic cells. The cultures were fixed overnight at 4°C in 1% glutaraldehyde/1% paraformaldehyde in phosphate-buffered saline (PBS). The cultures then were washed in PBS, teased away from the support membrane, then permeabilized by freeze-thaw on dry ice or liquid nitrogen and stained using the ABC Elite Kit (Vector Labs) with nickel enhancement. Stained cultures were whole-mounted in Permount. Selected well-filled pairs were traced using a Neurolucida system (Microbrightfield). Tracing was carried out with ×63 and ×100 oil-immersion objectives (Zeiss), and identification of potential contact sites was performed at ×100 and with the condenser diaphragm fully open to give the narrowest plane of focus. The photographs taken to illustrate the contacts in Fig. 4 were not taken on the same microscope used for tracing [a ×63 water-immersion objective was used (1.2 NA), and images were captured digitally using the transmitted light detector of a BioRad confocal microscope]. Images were adjusted for contrast and composed using Corel PhotoPaint.

RESULTS

Recording from monosynaptically coupled pairs in CA3

We established simultaneous whole cell recordings from pairs of CA3 pyramidal cells (Fig. 1*A*). Each pair was tested for connection by stimulating one cell, designated as presynaptic, to fire an action potential by passing a depolarizing current pulse via the recording electrode. The postsynaptic current trace then was examined for the presence of synaptic currents occurring at short (typically <3 ms), constant latencies after the peak of the action potential. Although it was not unusual for the first potential presynaptic cell tested to be coupled synaptically to the postsynaptic cell, in most experiments several potential presynaptic cells were tested before a connection was obtained. Overall, approximately one-third of potential presynaptic CA3 cells were found to be monosynaptically coupled to the postsynaptic CA3 cell. The success rate was higher when using visualized recordings from cells that were <100 μm apart, although there was a great deal of variability in the success rate even when cells were directly adjacent to each other. Data from >150 pairs are presented in this paper.

When the presynaptic cell was a pyramidal neuron (almost all of our recordings), synaptic responses were blocked completely by bath application of AMPA receptor antagonists (NBQX or CNQX, 10 μM) at a holding potential of −65 mV (Fig. 1*B*). Long depolarizing pulses delivered to the presynaptic cell resulted in a train of action potentials, and postsynaptic responses during the train showed rapidly developing depression of transmission that was often apparent after the first action potential. This depression was characterized both by smaller EPSCs as well as failures of transmission (Fig. 1*C*).

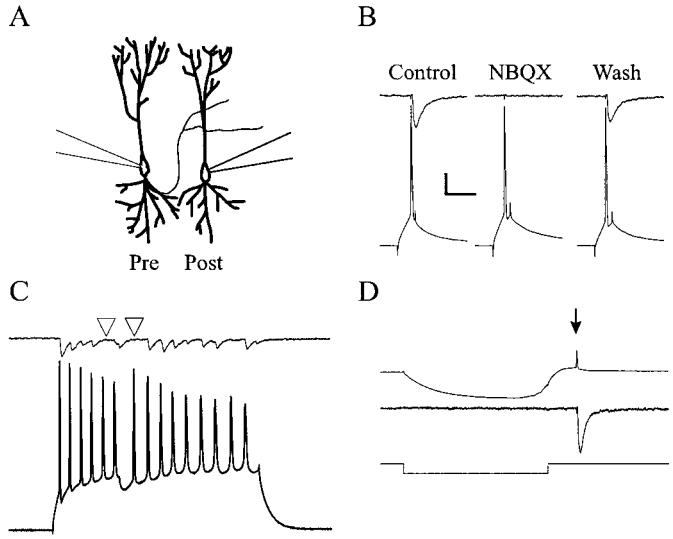

FIG. 1. *A*: schematic of the recording configuration for recording from 2 CA3 pyramidal cells. In some experiments, perforated-patch recording was used for the postsynaptic cell. *B*: recordings from a synaptically connected pair of CA3 neurons. Postsynaptic responses (*top*) after depolarization-evoked action potentials in the presynaptic cell (*bottom*) are blocked by the AMPA receptor antagonist 6-nitro-7-sulphamoylbenzo[*f*]quinoxaline-2,3-dione (NBQX, 10 μM). *C*: response of pair to a long depolarization of the presynaptic cell. Postsynaptic cell (*top*) responds to a train of action potentials in the presynaptic cell (*bottom*) with a train of excitatory postsynaptic currents (EPSCs) that decline in amplitude and give way to failures of transmission (▽). *D*: pairs are not electrotonically coupled. Large hyperpolarization of the presynaptic cell (*top*) does not result in current in the postsynaptic cell (*middle*). On return to rest, the presynaptic cell fired an anode-break action potential, resulting in a monosynaptic synaptic current in the postsynaptic cell (↓). *Bottom*: command potential. Scale: 30 ms/15 mv/75 pA for *B*, 50 ms/10 mV/25 pA for *C*, 50 ms/100 mV/50 pA/1 nA for *D*.

We saw no evidence of electrotonic coupling in our pairs, as was reported in acute slices (MacVicar and Dudek 1981). Action potentials generated by one cell never were observed to produce nonsynaptic currents or depolarizations in the other cell. We also tested this explicitly in five pairs by delivering large hyperpolarizing pulses to the presynaptic cell. This never resulted in any current passing to the postsynaptic cell (Fig. 1*D*).

Some pairs exhibited polysynaptic connections with no apparent monosynaptic connection. These generally had longer (>5 ms) and more variable latencies to the first synaptic potential. Such pairs were rejected from analysis. Typically, these polysynaptic synaptic potentials were apparently inhibitory because they had reversal potential of around −55 mV. Such polysynaptic inhibitory currents were also common in pair recordings where there was also a monosynaptic excitatory component; being present in about half of experiments (Fig. 2*A*). As expected these polysynaptic potentials were blocked by CNQX because blocking glutamatergic synapses removes excitatory links to intervening inhibitory neurons (not shown).

Polysynaptic inhibitory events were probably mediated by $GABA_A$ receptors, but we could not pharmacologically block GABAergic inhibition in our experiments due to the disruptive hyperactivity this produced. These polysynaptic inhibitory events always occurred later than monosynaptic excitatory synaptic potentials and generally did not prevent measurement of the early excitatory event. Thus pairs having a monosynaptic response were included in the data set whether or not polysynaptic events were also observed. However, in some experiments the presence of polysynaptic inhibitory events made the analysis of paired-pulse responses difficult or impossible because polysynaptic activity induced by the first action potential affected measurement of monosynaptic responses induced by the second action potential. Such pairs were excluded from this analysis. Pairs exhibiting polysynaptic excitatory connections were much less common and also were excluded from analysis.

In a few experiments, monosynaptic inhibitory events were observed. This can be attributed to the presynaptic cell being an inhibitory interneuron rather than a pyramidal cell. Eight such recordings were obtained in the course of our studies. The identity of the presynaptic cell as an inhibitory cell always was corroborated by differences in the electrical properties of the cells as compared with pyramidal cells. Specifically, putative interneurons had shorter action potentials [64.6 ± 10.1 (SD) mV vs. 85.4 ± 4.8 mV for pyramidal cells, $P < 0.0001$], briefer action potentials (2.4 ± 0.47 ms vs. 3.6 ± 0.4 ms, $P < 0.001$), a larger fast afterhyperpolarization (10.8 ± 2.5 mV vs. 5.8 ± 1.7 mV, $P < 0.001$), and fired at a higher rate without accommodation in response to long depolarizations (Fig. 2*B*). Such pairs were not studied in detail and are not included in the analyses described in the following text. In many experiments, the postsynaptic cell was cesium-loaded in perforated-patch

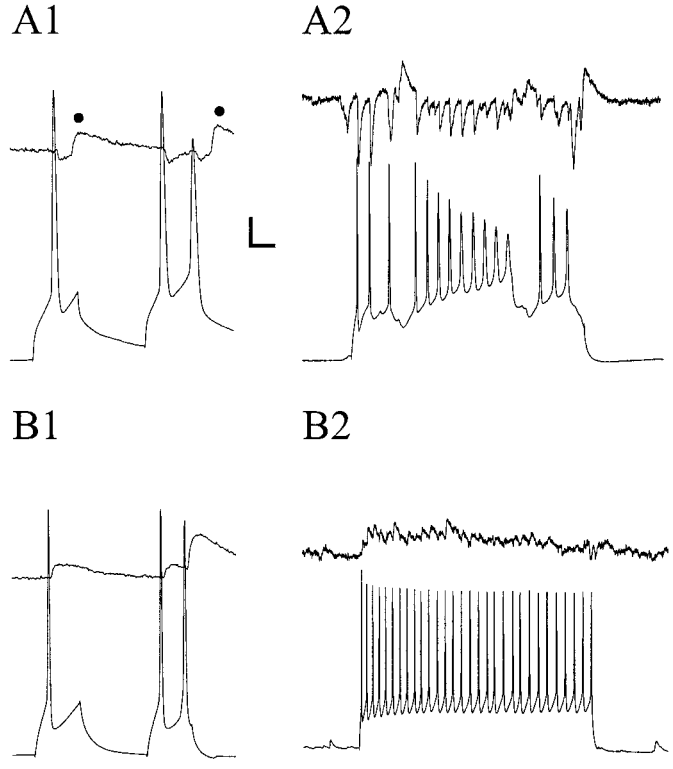

FIG. 2. Synaptic inhibition in pairs. *Top*: current records from the postsynaptic cell showing synaptic responses after depolarization-induced action potentials in the presynaptic cell (*bottom*). *A*: both cells are pyramidal cells, but after some of the action potentials in the presynaptic cell (evoked by 2 20-s current injections delivered 50 ms apart), a biphasic response is observed. Outward currents (bulleted in *A1*) are polysynaptic inhibitory events. *A2*: response to a long depolarization of the presynaptic cell. *B*: presynaptic cell is an interneuron as evidenced by the difference in action potential characteristics and firing pattern (*B2*). Action potentials give rise to monosynaptic inhibitory currents in the postsynaptic cell (*B1*). Scale bar: 10 ms/50 pA/10 mV for *A1* and *B1*, 50 ms/25 pA/12 mV for *A2* and *B2*.

mode, which precluded electrophysiological confirmation of the identity of the postsynaptic cell as a pyramidal cell based on its electrical properties. However, based on the observed frequency of presynaptic interneuron recordings, from the same population of neurons, we estimate that the accidental inclusion of a postsynaptic interneuron would have occurred only a few times in the course of our many experiments. This would be minimized further by our visualized recording technique, which permits more accurate identification of cell types. In addition, analysis of neurobiotin-labeled pairs confirmed the identity of both cells as pyramidal cells in all cases tested (see following text).

Properties of pair responses

One of the striking properties of excitatory synaptic transmission between single pairs of connected neurons was the great variability in size of the synaptic potential observed from pair to pair. In some synaptically coupled pairs of pyramidal cells, average responses were extremely small, <10 pA, and failures of transmission often were observed. In others, the responses were as large as 200 pA with few if any failures. In a representative subset of 136 pairs, the average response was 34 pA; the median response was 21 pA (Fig. 3*A*). We also examined transmission in a few pairs while holding the postsynaptic cell in current clamp to determine size of the depolarization generated by these events. The range of average EPSP amplitudes induced by a presynaptic action potential in eight experiments varied from ~200 to 1,000 μV, and the average size was 450 μV.

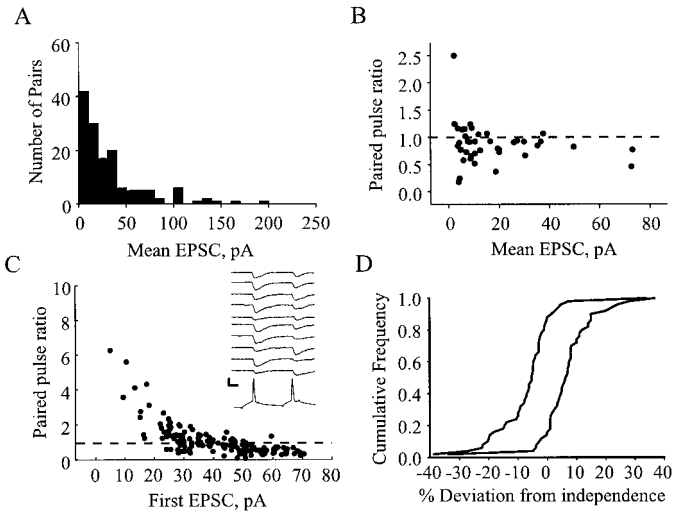

FIG. 3. Properties of pair responses. *A*: histogram of the average EPSC amplitude observed in a total of 134 pairs. *B*: average paired-pulse ratio in each of 42 pairs (each point represents 1 pair recording) plotted against the average EPSC amplitude in each pair. *C*: variability in responses in 1 pair. ●, ratio resulting from 1 paired-pulse trial with a 50-ms interspike interval. There is a wide variability in the 1st EPSC (abscissa; sweeps *inset*). Paired-pulse ratio varied widely as well, giving rise to both depression and facilitation (ordinate: - - -, paired-pulse ratio = 1). Scale bar for *inset*: 60 pA, 25 mV, 10 ms. *D*: occurrence of depression and facilitation is nonrandom and depends on the amplitude of the first EPSC. In this graph, the results of 50 experiments are summarized by plotting the cumulative frequency of the deviation of EPSC2 from the mean EPSC2 when EPSC1 was smaller than average (*left-hand curve*) or larger than average (*right-hand curve*). Distributions are significantly different from that predicted for 2 independent responses ($P < 0.01$). See text for details.

Paired-pulse characteristics of pair responses

We have examined the postsynaptic responses in pairs of pyramidal cells in response to two presynaptic action potentials, delivered 50 ms apart. In general the synaptic response to the second presynaptic action potential (EPSC2) could be larger or smaller than the response to the first (EPSC1), and this varied from trial to trial in a given pair (Fig. 3*C*). When the average paired-pulse ratio (EPSC2/EPSC1; PPR) was calculated for each pair, most pairs were found to exhibit paired-pulse depression (PPR < 1; the average EPSC2 was smaller than the average EPSC1, within a given pair recording). The average PPR varied greatly from pair to pair, from high levels of facilitation (2.5-fold) to depression (0.2-fold). The average PPR across 42 pairs was 0.88 ± 0.35 (Fig. 3*B*). However, there was no significant relationship between the average EPSC size in a pair and the average PPR ($P > 0.1$; Fig. 3*B*). As expected for a presynaptic effect, the inverse of the coefficient of variation squared ($1/CV^2$) for the second pulse compared with the first pulse was correlated with the degree of depression or facilitation observed (correlation coefficient 0.79; $P < 0.001$; measured for 25 experiments; not shown). Specifically, in pairs showing strong depression, the ratio of CV^2(pulse 1)/CV^2(pulse 2) was relatively low.

When the trial-to-trial variability in PPR is examined within a single pair, there is a strong relationship between the amplitude of the first EPSC and the PPR for that trial. For trials with a small EPSC1, the PPR is typically larger, and PPR is smaller for trials with large first EPSCs (Fig. 3*C*). To analyze these data, the trials within each pair were ranked by EPSC1 amplitude and then divided into two groups, those with EPSC1s larger than average for that pair and those with EPSC1s smaller than that average. The mean EPSC2 was taken for each group and compared the mean EPSC2 for the experiment. If EPSC2 was independent of EPSC1 and could vary over the same range of amplitudes, the mean EPSC2 should be the same whether or not EPSC1 was large or small. In contrast, there was often a significant deviation from this expectation. Data from 50 pairs are plotted in Fig. 3*D* to represent the relationship across all of those paired recordings. The left-hand curve displays the cumulative probability of the deviation from the mean of EPSC2 where the EPSC1 was larger than average. The right-hand curve is the analogous data for trials when the EPSC1 was smaller than average. As can be seen, there is a tendency for trials with large EPSC1s to have small EPSC2s and vice versa (Fig. 3*D*). On average, across 50 experiments, EPSC2 was 6.8 ± 10% smaller than the mean EPSC2 when EPSC1 was larger than the mean EPSC1 and 6.8 ± 10% larger than the mean EPSC2 when EPSC1 was smaller than the mean EPSC1 ($P < 0.01$, Kolmogorov-Smirnov test).

Estimating the number of active synapses in a pair

We observed a great deal of variability in the size of responses from pair to pair, with a range of ~20-fold between the ''weakest'' pair and the ''strongest'' pair. Such variability could arise from a number of sources: variation in the number of active synapses between different pairs, variation in the probability of release between pairs, variation in quantal size between pairs, and variation in the dendritic location of synapses. Each of these could, in theory, account for the variation

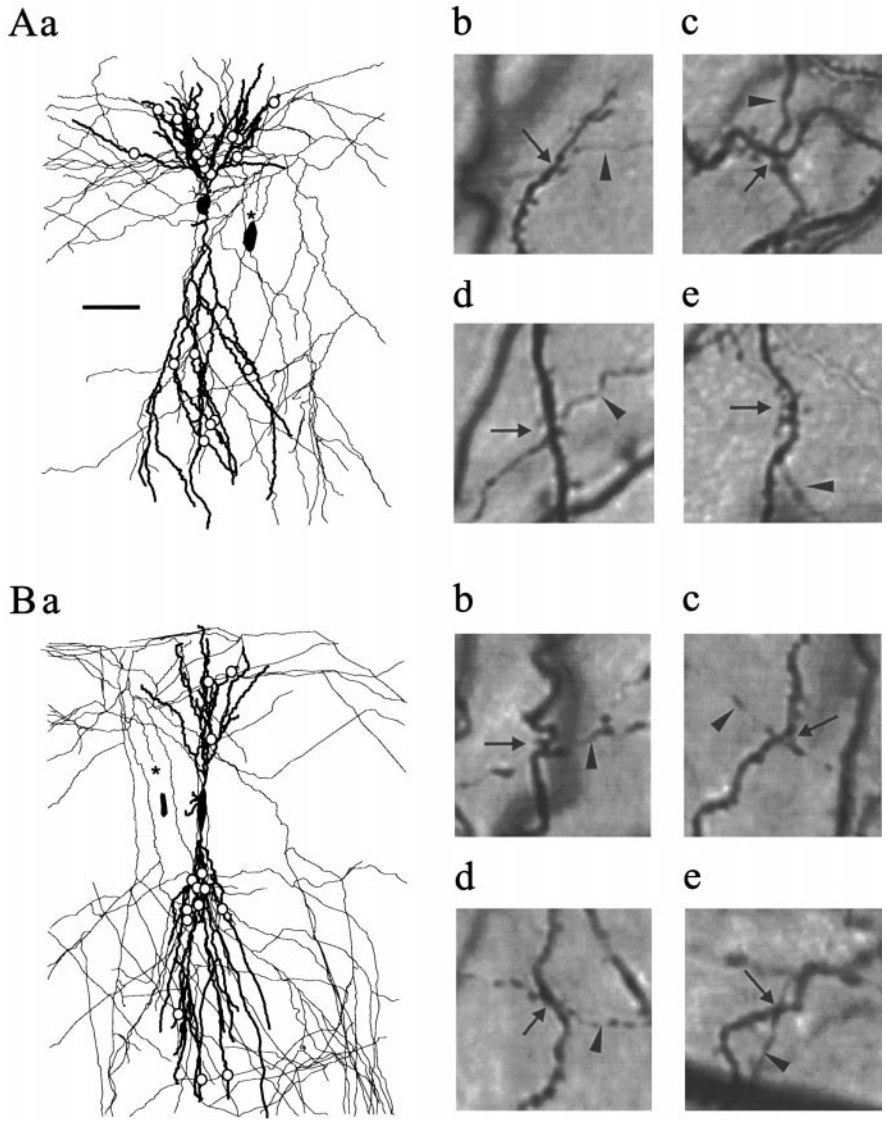

FIG. 4. Reconstructions of filled pairs. Only the postsynaptic dendrite and cell body (thick lines) and the presynaptic axon (thin lines) and cell body (shaded region) were reconstructed. Both pairs are oriented so the apical dendrites are pointing downward. Only a portion of the reconstructed presynaptic axon is shown, as many processes extend far from the postsynaptic cell, off the edge of the illustrated region, though the entire axonal arbor was reconstructed in both cases. Putative synaptic sites are indicated by small circles. Origins of the presynaptic axons are indicated by asterisks; presynaptic basal dendrite from which the axon emerged is not shown. *Aa*: pair in which very small synaptic currents were observed with frequent failures of transmission. There were 19 apparent contact sites. *A*, *b–e*: examples of putative synaptic sites. Axon is indicated by an arrowhead. Arrow indicates the site of close apposition to the postsynaptic dendrite. Orientations of these images are not identical to that of the reconstruction. *Ba*: pair in which the currents were larger. There were 14 apparent contact sites; examples are shown in *B*, *b–e*, labeled as in *A*. Scale bar: 100 μm for reconstructions; 7.25 μm for photomicrographs.

seen in the amplitude of EPSCs between pairs either alone or in combination.

To examine the possibility that there is a correlation between the size of the average EPSC in a pair and the number of synapses made between the two neurons, we have reconstructed the axonal arbor of the presynaptic cell and the dendritic arbor of the postsynaptic cell in two pairs, one with a very small response, and one with a larger response (Fig. 4). Analysis of these, and 10 other pairs not traced, reveals that the CA3 cells had mature morphologies with obvious basal and apical dendrites. Numerous synaptic spines covered the dendrites. What appeared to be thorny execrences were observed on many cells, extending from the region around the cell body. The cells had axonal projections much as would be expected in acute tissue, although perhaps more highly elaborated. The axon emerges from a single point on the basal side of the cell body or from the initial segment of a main basal dendrite and soon branches, sending projections to CA1 (Schaffer collaterals; not shown) as well as within CA3 (Fig. 4). Boutons were observed along the axons. The associative projections extended throughout both stratum oriens and s. lucidum/radiatum. Naturally, there was no extrahippocampal projection, but axons were observed extending nearly to the edge of the tissue. Thus the overall morphology is quite similar to that of CA3 cells in native tissue.

Close examination of the two reconstructed preparations reveal sites of close contact between the presynaptic axon and postsynaptic dendrites. At the light level, we cannot positively confirm that these are synaptic sites, though groups that have done analysis of similar preparations on the EM level have found a close correspondence between contact sites identified at the light level and synaptic sites (Gulyas et al. 1993; Markram et al. 1997). The number of contact points does not represent an upper limit for the number of synapses because some contacts could contain multiple active zones (Sorra and Harris 1993).

By comparing the number of potential synaptic sites in different pairs, the correlation between this and size of the synapse can be examined. One of the pairs we reconstructed had a very small EPSC, $<$10 pA on average, with failures of transmission in many sweeps. The other gave much larger responses, 29 pA on average. We predicted that ''weak'' pair

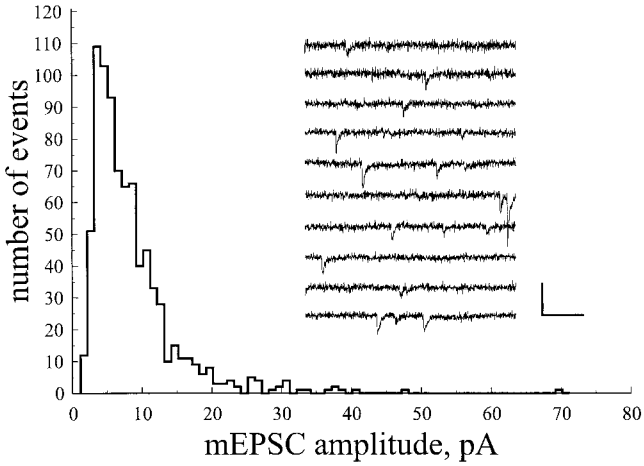

FIG. 5. Spontaneous miniature EPSCs in a CA3 pyramidal cell, recorded in the presence of TTX (1 μM) and picrotoxin (100 μM). *Inset*: selected sweeps illustrating typical mEPSCs. This is typical of observations from 3 cells. Scale: 20 pA/100 ms.

would have very few contacts, perhaps only one, whereas the ''strong'' pair would have more contacts. Surprisingly, the pair with the small response had 19 contact sites. The pair with the larger response had 14 contact sites. Thus there is no obvious relationship between number of contacts and the EPSC amplitudes observed, and the number of contacts sites for the weak pair was much larger than we predicted based on the electrophysiology. The contacts were distributed over both basal and apical dendrites. In the weak pair, 6 contacts were observed on apical dendrites, and 13 were on basal dendrites. In the strong pair, 3 contacts were on basal dendrites and 11 on apical dendrites.

Despite the relatively large number of potential contacts identified in the reconstructions, our electrophysiological data suggest that there are relatively few functional synaptic contacts in most pairs. Spontaneous miniature EPSCs (mEPSCs), recorded in the presence of 1 μM TTX and 100 μM picrotoxin had an average amplitude of 6.3 $\pm$ 3 pA, and ranged in amplitude from $<$3 up to 80 pA ($n = 3$ cells; Fig. 5). We note that the mEPSCs we recorded would include those originating from mossy fiber terminals because the granule cells are present in these cultures. It may be that some larger events originate from these synapses, which are close to the cell body. Still, even if the smaller mEPSCs ($\sim$5–10 pA) are taken to represent a typical associational synapse, the majority of our pairs, with mean EPSCs $<$30 pA, would consist of fewer than five active synapses.

Because the estimate of contact sites does not appear to correlate with response size it is possible that the wide range in amplitudes represents connections with roughly constant numbers of synapses, but with widely ranging release probabilities or differences in quantal size between pairs. One measure of the relationship between EPSC amplitude and the underlying properties of the quantal synaptic responses is the coefficient of variation (CV) of the EPSC amplitude (Faber and Korn 1991). We found a significant positive correlation between $1/CV^2$ and the mean EPSC amplitude (Fig. 6; correlation coefficient 0.64; $P < 0.01$; $n = 27$). Pairs with small mean EPSCs tended to have small $1/CV^2$ values while pairs with large EPSCs had larger values. Although interpretation of such data is not straightforward (Faber and Korn 1991), it is consistent with the idea that the release probability and/or the number of release sites is higher for pairs with larger responses.

If it is true that differences in probability of release are a major component of the differences in $1/CV^2$, then it should be possible to increase the amplitude of the response of a weak pair to the vicinity of a strong pair by increasing release probability. We tested this possibility by raising calcium and lowering magnesium in the ACSF to increase the probability of release (Fig. 7). In seven of nine pairs, changing the calcium/magnesium ratio in the ACSF from 2.5 mM Ca/1.3 mM Mg to 3.5 mM Ca/0.7 mM Mg or 5 mM Ca/1 mM Mg did not result in an increase in the maximal EPSC amplitude observed. In other words, there was a ''ceiling'' that could not be passed simply by increasing calcium (Fig. 7*C*). In two pairs there was a small increase in the maximal response size after calcium elevation but only to a maximum of $\leq$120% of control levels. On average, the maximal EPSC in high calcium was 105 $\pm$ 10% of that in control conditions. This lack of change was not due to a lack of effect of raising calcium, as there were clear effects of increasing calcium on transmission. In high calcium, the average EPSC was 146 $\pm$ 45% of that in control conditions, and the PPR decreased by 25 $\pm$ 13% of the control values. Furthermore there was a large increase in $1/CV^2$ (375 $\pm$ 349%; range, 114–1,200%), when calcium was raised, as expected if the probability of release had been increased. We also noted that in high calcium, the positive relationship between EPSC amplitude and $1/CV^2$ was maintained (correlation coefficient 0.76; $P < 0.05$; Fig. 6*B*). The corresponding data for these experiments under control calcium conditions were included in Fig. 6*A* and also were correlated significantly with mean EPSC amplitude when considered independently (0.87; $P < 0.05$). In two pairs, we tested the effects of lowering calcium/magnesium to 1.3 mM/2.5 mM (Fig. 7*A*). In both experiments, there was a large decrease in the mean EPSC (67% average) and an increase in the PPR of 21% on average.

Pharmacological manipulation of the presynaptic cell

One of the advantages of using pair recordings is that the cytoplasm of the presynaptic cell is directly accessible to experimental manipulation by including exogenous compounds in the presynaptic recording electrode. We observed transmission between pairs of connected cells was quite stable over long periods of time (upward of 3–4 h) when using a

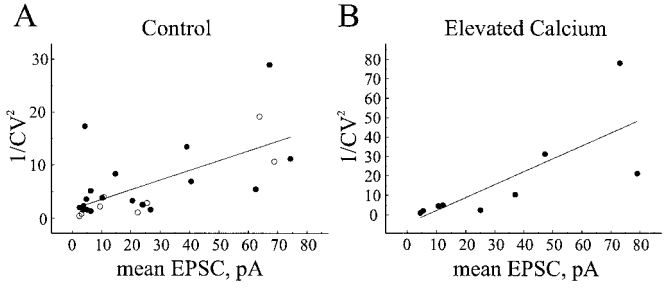

FIG. 6. Relationship of $1/CV^2$ to mean EPSC amplitude. *A*: under control calcium conditions. Each point represents a different pair recording. Line is the least-squares fit to the data. ○, data from experiments in which bath Ca/Mg was later changed to 3/0.75 or 5/1. Data for those experiments under elevated calcium conditions are plotted in *B*.

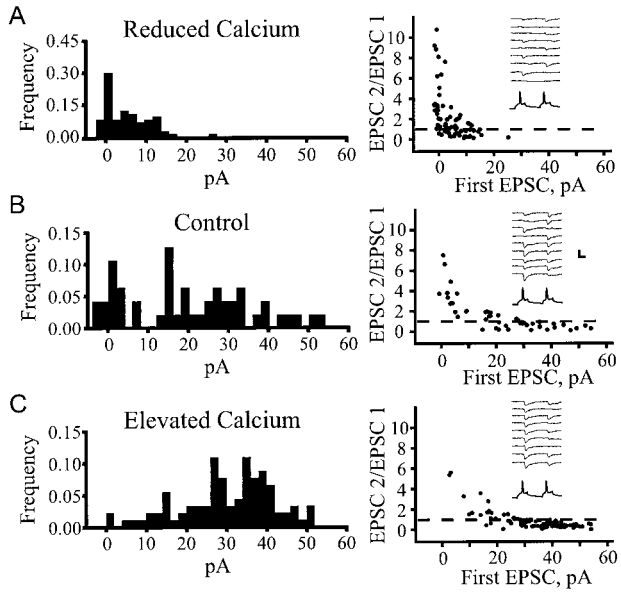

FIG. 7. Effects of changing bath divalent cation concentration on synaptic responses in a pair recording. *Left*: amplitude histograms of EPSCs after single presynaptic action potentials under different bath Ca/Mg conditions. *Right*: paired-pulse results for the same condition, plotting the paired-pulse ratio against the amplitude of the 1st EPSC, as in Fig. 3*C*. *Inset*: sweeps are consecutive individual trials under each condition; scale bar indicates 10 ms/50 pA. *A*: in low calcium/high magnesium (1.3 mM/2.5 mM). Note that much smaller EPSCs dominate the amplitude histogram, as opposed to just an increase in failures, and paired-pulse responses (*right*) show more facilitation though many sweeps still exhibit depression. *B*: in control conditions (2.5 mM/1.3 mM), a wide range of response sizes and paired pulse ratios (*right*) were obtained. *C*: in elevated calcium (5 mM/1 mM). Maximal response size in C does not exceed that in *B*, despite a substantial increase in the mean response. Paired-pulse responses are dominated by depression (*right*).

standard whole cell recording. Thus synaptic transmission apparently is unaffected by prolonged dialysis of the presynaptic cytoplasm under our experimental conditions (Fig. 8*A; n* = 34). Nonetheless we have found it possible to introduce substances into the presynaptic cell and have those substances reach the axon terminals at effective concentration. As an initial demonstration of our ability to manipulate the presynaptic cell, we included high concentrations of calcium chelators in the presynaptic electrode. We selected BAPTA and EGTA, two calcium chelators whose effects on transmission have been characterized in other studies, in hippocampal synapses (by bath application of membrane-permeant analogues of these compounds) as well as at other synapses.

When the presynaptic electrode contained BAPTA (10 mM; potassium salt), transmission declined rapidly (Fig. 8*B*). Typically there was a period of several minutes during which transmission was stable, followed by a period of decline, which proceeded until transmission nearly was blocked. During this time, there was no significant differences in presynaptic action potential height and width between control and injected pairs over the course of the experiments [height ratio ($mV_{t=20\ min}$/$mV_{t=0\ min}$) for control: 0.95 ± 0.17 (mean ± SD; n = 15); BAPTA: 0.94 ± 0.18 (n = 10); difference not significant. Width ratio (ms_{t20}/ms_{t0}; measured at the base) control: 0.96 ± 0.23; BAPTA: 1.09 ± 0.12; difference not significant].

Maximal block was characterized predominantly by failure of transmission in most trials although occasional small EPSCs continued to appear (Fig. 8*B*). The time course of BAPTA block was variable with half-maximal block being observed within minutes in some cases and taking as long as 30 min in other experiments. On average, the block reached a half-maximal level after 15 min. A similar extent of block was observed in all experiments (n = 16). There was some indication that block was more rapid when the recording locations were close together (<100 μm, using visualized recording), but we have not systematically investigated this. In a previous study on the effects of presynaptic BAPTA injection on transmission at the squid giant synapse, it was calculated that 1 mM BAPTA resulted in 50% block of transmission (Adler et al. 1991). To get a better idea of the sensitivity of the release mechanisms in our preparation, we tested the effects of a lower BAPTA concentration, 1 mM (n = 5). We found that the effects of 1 mM BAPTA were quite similar to 10 mM with a progressive block of transmission observed soon after break-in. Although

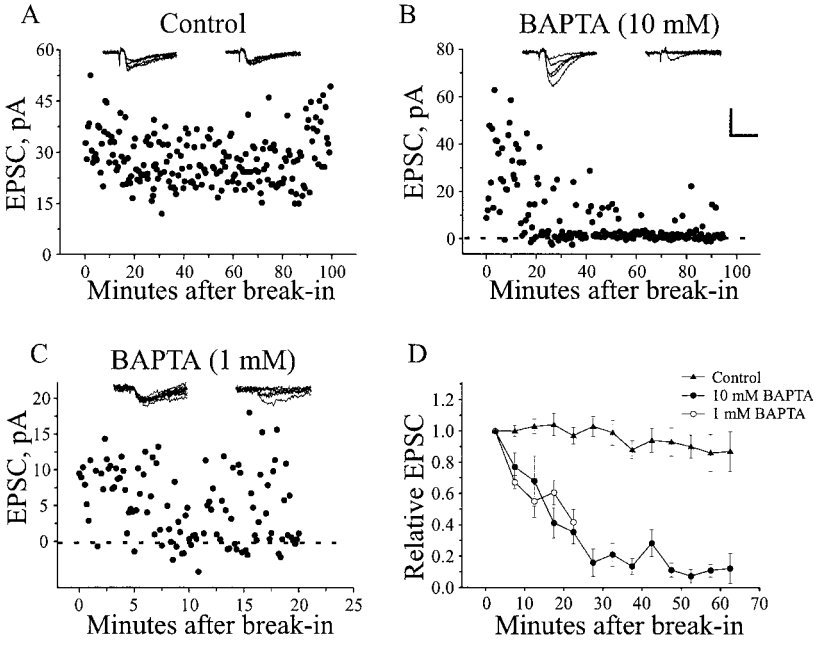

FIG. 8. Effects of injecting bis-(*o*-aminophenoxy)-*N,N,N′,N′*-tetraacetic acid (BAPTA) into the presynaptic cell of synaptically coupled pairs. *A*: control experiment with no added calcium buffer in the presynaptic electrode. *B*: 10 mM BAPTA presynaptic. *C*: 1 mM BAPTA presynaptic. *D*: summary of experiments indicating the mean and SE of the relative EPSC amplitude at various time points after break-in. Number of experiments represented at each time point decreases with time as not all experiments were maintained for a full hour. Scale bar: 10 ms; 50 pA for *A* and *B;* 25 pA for *C*.

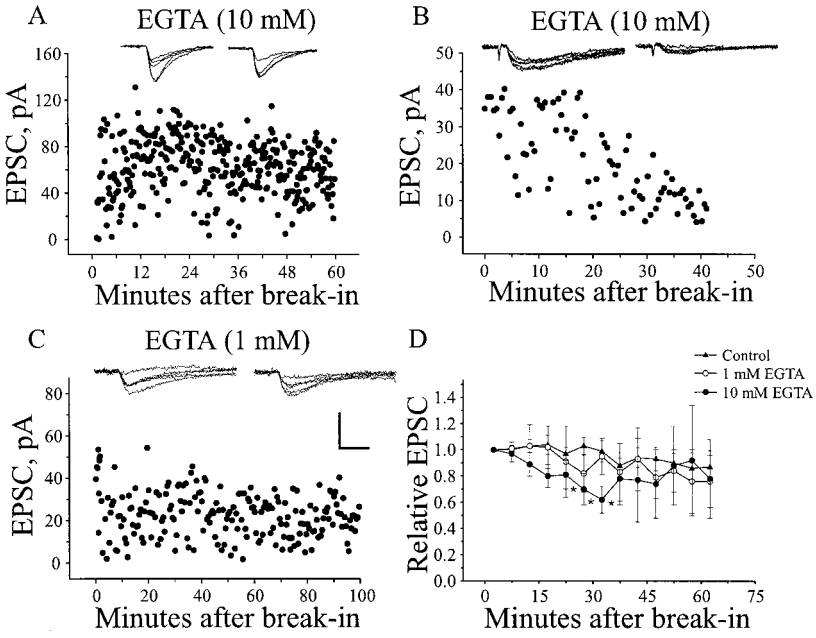

FIG. 9. Effects of injecting EGTA into the presynaptic cell of synaptically coupled pairs. *A*: example of an experiment where 10 mM EGTA had no apparent effect on transmission when included in the presynaptic electrode. *B*: experiment where a decline in transmission occurred with 10 mM EGTA in the electrode. *C*: experiment where 1 mM EGTA had no effect on transmission. *D*: summary of experiments indicating the mean and SE of the relative EPSC amplitude at various time points after break-in. Control data are the same as that presented in Fig. 8, and as in Fig. 8, the number of experiments represented decreases with time. *, significant difference from control ($P < 0.05$). For the averaged data with 10 mM EGTA, the block of transmission appears transient because the experiments maintained for the longest time points did not show significant block. Scale bar: 10 ms; 100 pA for *A;* 50 pA for *B* and *C*.

these recordings were maintained only for 20 min, in some experiments, the effects of 1 mM BAPTA appeared to stabilized within the recording period at a level of 20–70% of control levels (Fig. 8*C*). The BAPTA data are summarized in Fig. 8*D*.

We also tested the effects of including EGTA in the presynaptic electrode (Fig. 9). With 1 mM EGTA in the pipette, no effect was seen on transmission (Fig. 9*C; n* = 4). Similarly, in many experiments with 10 mM EGTA, there was no significant effect on transmission (Fig. 9*C*). However, in some cases (7/14), there was an appreciable block of transmission, ranging ≤75%. An example of such an experiment is shown in Fig. 9*B*. On average, 10 mM EGTA blocked transmission ~20% (Fig. 9*D*).

DISCUSSION

These data represent an initial characterization of pair recordings of CA3 pyramidal cells in the interface cultures, and can be compared with work done with roller cultures (Debanne et al. 1995) and acute slices (Miles and Wong 1986). Besides demonstrating that these cultures are a good model system and outlining the properties of synaptic transmission between single connected neurons, we have shown that pharmacological manipulation of the presynaptic cell is feasible for small molecules. In doing so, we have tested hypotheses concerning the sensitivity of the release machinery to exogenous calcium buffers.

Comparison to acute slices

The synaptic responses we obtained are quite similar to those obtained in acute hippocampal slices by (Miles and Wong 1986). In this study, it was reported that the quantal content of pairs was low, consistent with our findings, at least for most pairs. Second, although most of our experiments were conducted in voltage clamp, the responses we have examined in current clamp showed depolarizations of similar magnitude to those seen in the acute slice. Miles and Wong (1986) also observed polysynaptic inhibition that could be elicited by a single action potential in the presynaptic pyramidal cell, as we have (Fig. 2).

On average, our recordings were dominated by paired-pulse depression. This is in contrast to the ~25% facilitation seen by Miles and Wong (1986) in CA3-CA3 pair recordings in acute slices (in 2 mM Ca and 1.6 mM Mg). Debanne et al. (1996) found a slight facilitation (8%) in CA3-CA3 pairs in roller tube cultures with an Ca/Mg composition of 2.8/2.0 mM. The difference in our results might be accounted for by the fact that we routinely used 2.5/1.3 Ca/Mg, which may yield a slightly higher probability of release than the Ca/Mg compositions used by Miles (1986) and Debanne et al. (1996). On the other hand, using field stimulation in 2.5/1.3 Ca/Mg, Zalutsky and Nicoll (1990) observed paired-pulse facilitation in acute slices. Thus the predominance of depression could indicate that the probability of release in cultures is higher on average than in acute slices. The finding that raising calcium did not result in the uncovering of low-probability release sites is consistent with this idea (Fig. 6). The probability of release in acute slices has been reported to be high in young rats (<2 wk) and to decrease with age (Bolshakov and Siegelbaum 1995). Because our cultures were prepared from 1-wk-old animals and cultured for 1–2 wk, our results may reflect this developmental difference. An alternative explanation is that the synapses in our cultures do not fully express the facilitation mechanism.

Variability between pairs

We observed a great deal of variability in the response amplitudes between different pairs. This could arise from a number of sources: differences in the number of active synapses between pairs, differences in the probability of release, or in quantal size. Additional variability could arise from differences in voltage-clamp errors in measuring EPSCs arising from distant synaptic sites or from different distributions of synapses on apical and basal dendritic trees. Each factor in theory could account for the observed variability alone or in combination

with the others. For purposes of discussion, we first will consider the possibility that one of these factors alone might account for most or all of the observed variability.

The first possibility that we considered is that large variability in the average EPSC amplitude between pairs arises simply because the number of active synapses simply varies from pair to pair. However, the number of potential synaptic contacts we found anatomically did not correlate with the EPSC sizes observed in a pair, and furthermore the pair that had a very small response had many more potential contacts than would be predicted from the physiology. There is no obvious difference in the distance of the contacts from the cell body in the two pairs we reconstructed that could account for the difference in the observed current amplitude, though the weak pair had a larger proportion of contacts on basal dendrites (Fig. 4). We note that other investigators who have reconstructed pairs also observed little correlation between the number of contacts and response size (Deuchars et al. 1994; Markram et al. 1997), although the numbers of contacts observed in these cases were smaller than in our pairs, and the synapses were in acute slices of neocortex rather than cultured hippocampal slices.

A second possibility is that there is large variability in the average probability of release between pairs. There was a positive correlation between the coefficient of variation and the average size of the EPSC in a pair, suggesting that stronger pairs might possess a higher release probability or have more release sites than weaker pairs. Because paired-pulse facilitation is thought to be due to a temporary increase in the probability of release (Zucker 1989), the PPR should decrease with increasing initial probability of release as demonstrated by Dobrunz and Stevens (1997). Thus if all other parameters are equal, pairs with a relatively low PPR would be expected to have a higher probability of release, and thus exhibit relatively large average EPSCs to a single action potential. The lack of any such relationship between PPR and average EPSC size suggests that any differences in the probability of release cannot entirely explain the range of response sizes we observed (Fig. 3*B*). Furthermore if smaller responses occurred because release probability in weaker pairs was consistently lower than that in stronger pairs, it would be expected that the amplitude of average small EPSCs could be increased to a size approaching that in strong pairs by increasing release probability. Raising the probability of release with calcium did not readily convert small responses to large responses (Fig. 6). This suggests that there is no subpopulation of very-low-probability synapses between pairs that can be revealed by raising probability of release. In addition, taken with the fact that most pairs were dominated by paired-pulse depression, these data suggest that the probability of release in most pairs is fairly high.

A third explanation for the differences in mean EPSC amplitude among pairs is that the quantal size is larger in pairs with larger responses. Thus pairs with large responses might consist of the same number of active synapses as those with small EPSCs, but with a larger quantal size. This possibility is supported by the broad distribution of the mEPSCs, though mossy fiber synapses may account for some of the larger events. There is some evidence against differences in quantal size as the sole explanation for interpair variability. First, there was a positive correlation between $1/CV^2$ and EPSC amplitude. Although this type of analysis can be problematic (Faber and Korn 1991), in the simplest case, the inverse square of the CV is predicted to increase with increasing probability of release or increasing number of release sites but should not change when only the quantal size is increased. Second, under conditions where the probability of release is lowered, strong pairs readily become weak. For example, when stimulating pairs with trains of action potentials, which caused depression, much smaller events and failures appeared in the same pair that gave rise to large events (Fig. 1*C*). We also found that loading of the presynaptic cell with BAPTA or EGTA causes a graded decline of transmission, and we observed possible quantal events after BAPTA block was maximal (Fig. 7*B*). A similar effect was seen after lowering extracellular calcium (Fig. 6*A*). These small events are comparable in size with the smallest events observed in any pairs ($<$10 pA). Thus the large EPSCs in these pairs are apparently made up of the sum of smaller events with a quantal size similar to those making up the events in weak pairs.

An alternative hypothesis is that the synapses in weak pairs are located further from the cell body than those in strong pairs, resulting a larger voltage-clamp errors and thus underestimation of EPSC amplitudes for distal synapses. If this is the case, it is not evident from our reconstructions because the putative synapses in both pairs appear to be distributed widely over the dendritic tree. The same is likely to be true for the other stained pairs that were not reconstructed because the presynaptic axons always projected widely among the dendritic layers. Thus there is no obvious segregation of synapses to proximal or distal dendrites that could readily account for the difference in response amplitudes, although this easily could be a contributing factor.

On the basis of the whole of our evidence, including paired-pulse analysis, manipulations of calcium/magnesium ratio, and $1/CV^2$ analysis, we conclude that differences in quantal size and release probability do not appear to vary sufficiently to fully account for the observed differences in amplitude. Thus it seems most likely that the variability in EPSC amplitude arises primarily from variation in the number of synapses formed between different pairs despite the results of the reconstruction of two pairs. It is likely that the limited anatomic analysis of potential contacts we performed does not provide an accurate representation of the number of active synapses. This may be because some synaptic contacts are effectively nonfunctional either presynaptically (due to a very low or a zero probability of release) or postsynaptically (due to the lack of functional AMPA receptors but not necessarily a lack of NMDA receptors) (Malenka and Nicoll 1997).

Variation of responses within pairs

In addition to the variability among pairs, EPSC amplitudes within a given pair recording fluctuated considerably (i.e., Fig. 3*C*). Although we have not conducted a formal quantal analysis of our EPSCs, it is likely that the major source of this variation is fluctuation in the number of quanta released from trial to trial as at other synapses. In addition, the average quantal content in most pairs appears to be fairly low, because failures of transmission were observed (i.e., Fig. 7) and most EPSCs in pairs were not more than four to six times as large as typical mEPSCs (Fig. 5). One consequence of fluctuations in transmission seems to be that when paired pulses are delivered, there is what can be termed ''competition'' for synaptic re-

sources between the first and second EPSCs (Fig. 3*D*) as has been observed previously in organotypic slices (Debanne et al. 1996) and in motor cortex (Thomson et al. 1993). The magnitude of the effect was quite variable although we detected competition in most but not all pairs. The limiting resource may be presynaptic, as shown by Debanne et al. (1996) and might reflect readily releasable synaptic vesicles. In addition, the fact that $1/CV^2$ for the second pulse compared with the first pulse is correlated with PPR in our pairs is also suggestive of a presynaptic change. However, from our data we cannot rule out other explanations such as postsynaptic receptor desensitization (Arai and Lynch 1998) or rapid feedback presynaptic inhibition mediated by glutamate (Chittajallu et al. 1996).

Effects of presynaptic calcium chelator injection

In most studies, in a variety of preparations, BAPTA has been demonstrated to block transmission (Adler et al. 1991; Borst and Sakmann 1996; Niesen et al. 1991), whereas EGTA variously has been reported to have no effect (Adams et al. 1985; Adler et al. 1991; Atluri and Regehr 1996; Delaney et al. 1991; Spigelman et al. 1996; Swandulla et al. 1991) or to partially block transmission (Borst and Sakmann 1996; Kretz et al. 1982; Salin et al. 1996). To our knowledge, there have been no tests of the effects of calcium chelators in hippocampus using direct intracellular injection into presynaptic cells.

Our experiments with BAPTA demonstrated a high degree of sensitivity to this chelator at concentrations no higher than 1 mM. This is a comparable sensitivity to that observed in the squid (Adler et al. 1991), although it is likely that in our experiments the concentration of BAPTA in the terminal after 20 min is still substantially lower than that in the pipette.

In our hands, EGTA was capable of blocking transmission in at least some experiments, despite its slower binding of calcium than BAPTA, in agreement with the data of others who used presynaptic injection to administer the chelator to mammalian neurons (Borst and Sakmann 1996). While the effects of EGTA, when observed, may be analogous to those of BAPTA, that is, via buffering of calcium before release can be triggered, we cannot rule out alternatives to this explanation. First, EGTA could be having a toxic effect perhaps related to its release of protons when binding calcium. Another possibility is that other events mediated by calcium, besides the rapid triggering of release, might be perturbed. For example, if a slow calcium signal is required for the refilling of synaptic vesicle docking sites and EGTA blocked this signal, then eventually transmission would be blocked.

At CA3 associational synapses, effects of EGTA (using bath application of EGTA-AM) on baseline transmission also have been observed by (Salin et al. 1996) (40% block) but not by Spigelman et al. (1996). The variability of EGTA effects on our study likely reflects the fact that only a few synapses are recorded in each experiment. One reason EGTA had no apparent effect in some experiments might be differences in the concentration of EGTA attained at the terminal. Arguing against this, if we assume that EGTA diffuses into the cell as readily as BAPTA, it is likely that EGTA reached a high concentration in the terminal within 20 min in most cases, even when block was not observed. However, without an independent measure of EGTA concentration, the possibility remains that in some experiments, an insufficient concentration was reached in the terminal. The more interesting possible reason for the variability of the EGTA effect is a underlying difference in the properties of synapses. For example, in some terminals, release sites may not be as closely linked to calcium entry sites as in others (Smith and Augustine 1988).

These data demonstrate the feasibility of performing pharmacological manipulations of presynaptic neurons in hippocampal slice cultures. The effects of BAPTA and EGTA were very rapid with noticeable effects within 10 min being typical. This speed suggests that such manipulations need not need to be limited to small molecules such as BAPTA. Indeed, we have indications from experiments with fluorescently labeled dextrans that reasonably rapid ($<$1 h) access to terminals $\leq$200 μms away from the recording site might be obtained for substances with molecular weights $<$3,000 (unpublished data). This opens the possibility that types of analysis of the synaptic vesicle release machinery that has been performed at the squid giant synapse (DeBello et al. 1995) might be extensible to the hippocampal slice.

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Present address of P. Pavlidis: Center for Neurobiology and Behavior, Columbia University, New York, NY 10032.

Address for reprint requests: D. V. Madison, B115 Beckman Center, Stanford University School of Medicine, Stanford, CA 94305-5345.

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