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Postsynaptically Silent Synapses in Single Neuron Cultures

Stephen N. Gomperts,* Anuradha Rao,§ Ann Marie Craig,§ Robert C. Malenka,†‡ and Roger A. Nicoll*†‖

*Department of Cellular and Molecular Pharmacology
†Department of Physiology
‡Department of Psychiatry
University of California
San Francisco, California 94143
§Department of Cell and Structural Biology
University of Illinois
Urbana, Illinois 61801

Summary

We have used the synapses that isolated hippocampal cells in culture form onto themselves (autapses) to determine if some synapses lack functional AMPA receptors (AMPARs). A comparison of the synaptic variability of the AMPAR- and NMDAR-mediated evoked responses, as well as of miniature synaptic responses, indicates that a population of events exists that only contains an NMDAR component. Spillover of glutamate from adjacent synapses cannot explain these results because in single cell cultures all synaptic events mediated by AMPARs should be detected. Immunocytochemical analysis of these cultures clearly reveals a population of synapses with puncta for NR1 (NMDAR) but not for GluR1 (AMPAR). These results provide strong anatomical and physiological evidence for the existence of postsynaptically silent synapses.

Introduction

The majority of synapses in the CNS release the excitatory transmitter glutamate, which acts on a variety of receptor subtypes. Classically, it is proposed that at most of these synapses glutamate binds to AMPA receptors (AMPARs) and NMDA receptors (NMDARs), which are colocalized at excitatory synapses. However, recent physiological evidence has suggested that a population of synapses may possess NMDARs but lack functional AMPARs and, therefore, are silent at the resting membrane potential. Furthermore, it has been proposed that the conversion of these silent synapses to ones containing functional AMPARs may be critically important for the expression of long-term potentiation (LTP) (Isaac et al., 1995; Liao et al., 1995; Durand et al., 1996; but see Niu et al., 1998), as well as for the activity-dependent modifications of neural circuitry during development (Durand et al., 1996; Wu et al., 1996; Isaac et al., 1997).

However, an alternative "spillover" hypothesis has been proposed to explain the existence of these postsynaptically silent synapses (Kullmann et al., 1996). Specifically, it has been argued that glutamate released from neighboring synapses can spill over and activate receptors at synapses that have not, themselves, released glutamate. Since the affinity of glutamate for NMDARs is approximately 100-fold higher than it is for AMPARs, the selective activation of NMDARs at these synapses is possible.

While it has been difficult experimentally to rule out the "spillover" hypothesis, some anatomical evidence is consistent with the notion of silent synapses. Thus, immunohistochemistry of synapses in culture has revealed that some synapses can stain for NMDARs but not for AMPARs (Rao and Craig, 1997; Lissin et al., 1998). In addition, immunogold labeling has revealed a large heterogeneity in the number of AMPARs at excitatory synapses (Nusser et al., 1998).

To address more directly the existence of silent synapses, we have carried out a study of miniature excitatory postsynaptic currents (mEPSCs) (Bekkers and Stevens, 1989) in single cell cultures of hippocampal neurons (Segal and Furshpan, 1990) together with the immunocytochemical localization of glutamate receptors. Specifically, we have identified a population of mEPSCs that are mediated solely by NMDARs (NMDAR-only mEPSCs) and show immunohistochemically in these cultures the presence of synapses that contain NMDAR puncta but not AMPAR puncta. Most importantly, the presence of NMDAR-only mEPSCs in single cell culture rules out the possibility of spillover as the basis for silent synapses, since all synaptic responses mediated by AMPARs are recorded in such a preparation. These results provide direct evidence for the existence of a population of synapses that contain the normal complement of NMDARs but are silent in terms of AMPAR function.

Results

If the spillover of glutamate explains the existence of NMDAR-only EPSCs and all synapses contain both NMDARs and AMPARs, an anatomical arrangement must exist in which there are synapses in close proximity of one another but on separate neurons, since release of glutamate from any synapse on the same neuron would always activate AMPARs and yield an AMPAR EPSC (Figure 1). Thus, we reasoned that if we could design a condition in which such spillover between neurons could not occur, the presence of NMDAR-only EPSCs would be strong support for the existence of silent synapses. When neurons are cultured in complete isolation from one another, they form synapses onto themselves, referred to as autapses (Segal and Furshpan, 1990). Under these conditions, spillover of glutamate cannot explain NMDAR-only EPSCs because all synaptic responses to released glutamate will be detected by the recording electrode. This arrangement does not exclude the possibility that spillover could occur between synapses, but it does exclude spillover as a basis for an NMDAR-only EPSC (see Figure 1). We therefore cultured neurons in isolation and looked for the existence of NMDAR-only EPSCs. All experiments

‖To whom correspondence should be addressed (e-mail: nicoll@phy.ucsf.edu).

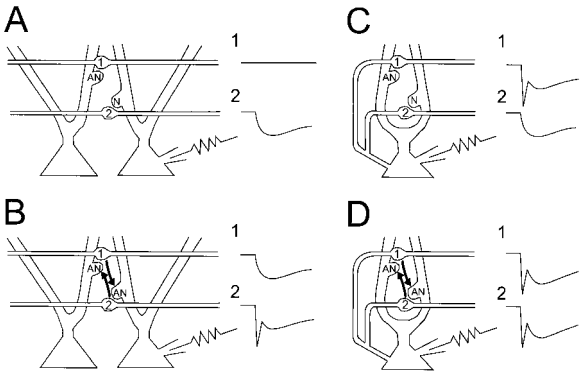

Figure 1. In the Autapse, Detectable NMDAR-Only Events Do Not Derive from Spillover

With multiple postsynaptic cells, NMDAR-only events may derive from direct activation of synapses that have only functional NMDARs (A2) or from spillover of glutamate from one synapse to another, resulting in the activation of NMDARs but not AMPARs due to the higher affinity of NMDARs for glutamate (B1). Traces 1 and 2 depict the response in the recorded cell to glutamate release at synapses 1 and 2, respectively. In the autapse, NMDAR-only events due to synapses expressing functional NMDARs but not AMPARs should be visible as discrete independent events (C2), but NMDAR-only events due to spillover would be masked by the concomitant activation of synaptic AMPARs and NMDARs at the releasing synapse (D).

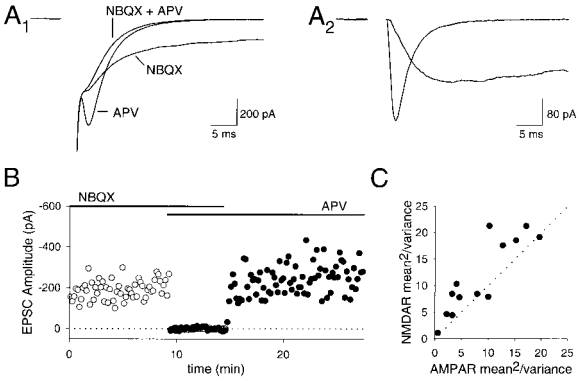

Figure 2. The NMDAR EPSC Has a Greater Mean^2/Variance than the AMPAR EPSC

(A) Representative average traces of pharmacologically isolated NMDAR and AMPAR EPSCs superimposed on the action potential artifact (A1) and after subtraction of the action potential artifact (A2). Traces are the average of 15 responses in each pharmacological condition: NBQX (5 μM) to isolate the NMDAR EPSC, D-APV (100 μM) to isolate the AMPAR EPSC, and both to isolate the action potential artifact.

(B) Typical experiment to measure mean^2/variance of NMDAR and AMPAR EPSCs. A minimum of 30 trials was collected in each pharmacological condition.

(C) Summary graph of the AMPAR and NMDAR mean^2/variance for 13 cells. The NMDAR mean^2/variance is greater than the AMPAR mean^2/variance for the majority of the cells and is greater on average ($p < 0.0l$). The average mean^2/variance ratio of NMDAR EPSCs to AMPAR EPSCs is 1.6 $\pm$ 0.2. A line of slope l (dotted line) illustrates the predicted values for equal mean^2/variance of AMPAR and NMDAR EPSCs.

were done in the absence of external Mg^{2+} so that the NMDAR EPSCs could be recorded at the resting membrane potential.

We first compared the variation, calculated as the mean^2/variance, of the evoked AMPAR EPSC to that of the NMDAR EPSC. If there are synapses that have only NMDARs in addition to synapses that have both AMPARs and NMDARs, then the variation of the NMDAR EPSC should be less (mean^2/variance will be greater) than that of the AMPAR EPSC (Kullmann, 1994). Evoked autaptic responses from a typical cell are shown in Figure 2A in which the selective AMPAR antagonist NBQX and the selective NMDAR antagonist APV are applied sequentially. The stimulus artifact recorded in the presence of both antagonists has been subtracted from the records shown in Figure 2A2. The entire experiment is plotted in Figure 2B, and it is clear that the variation of the size of the AMPAR EPSCs is greater than that of the NMDAR EPSCs. The relative fluctuation of the two components is plotted for a number of cells in Figure 2C, and for the majority of cells (10 of 13), the points fall above the line of unity, indicating that the mean^2/variance is greater for the NMDAR EPSC ($p < 0.01$). The average mean^2/variance ratio of NMDAR EPSCs to AMPAR EPSCs is 1.6 $\pm$ 0.2 (n = 13), which corresponds to 38% of synapses containing only NMDARs. These results are consistent with the observations made in hippoccampal slice preparations (Kullmann, 1994; Selig et al., 1995; but see Niu et al., 1998) and suggest that there is a substantial population of synapses that have NMDARs but no detectable AMPARs.

Further evidence suggesting the existence of NMDAR-only EPSCs comes from the comparison of AMPAR-containing mEPSCs to evoked EPSCs in the same cell. For this analysis mEPSCs were identified by the fast rising AMPAR component of the response. An average of these AMPAR-containing mEPSCs reveals a slow tail, which is due to the activation of NMDARs presumably colocalized at the same synapse (Figure 3A). When the relative contribution of the NMDAR component and AMPAR component is compared between the mEPSC and the evoked dual component response in the same cell, it is clear that the slow NMDAR EPSC contributes considerably less to the mEPSC than it does to the evoked response (Figure 3A). A summary of a number of cells with such a within cell comparison is graphed in Figure 3B. This discrepancy was also seen when amplitudes of the two components were compared rather than charge transfer. The NMDAR/AMPAR amplitude ratio was 0.35 for the mEPSCs but was 0.65 for evoked EPSCs, corresponding to a proportion of NMDAR-only events of approximately 44% $\pm$ 5% to make up the difference. Interestingly, there was a direct correlation between the size of the AMPAR and the NMDAR components of the AMPAR-containing mEPSCs. Thus, mEPSCs having large AMPAR EPSCs on average have large NMDAR EPSCs (Figure 3C). This was true for all cells examined (n = 5). This finding makes it unlikely that the failure to identify and include some mEPSCs with small AMPAR EPSCs could explain the difference between the mEPSC and the evoked EPSC, since such events are likely to have correspondingly small NMDAR components. Thus, the larger contribution of NMDARs to the evoked response when compared to dual component mEPSCs suggests the existence of a population of

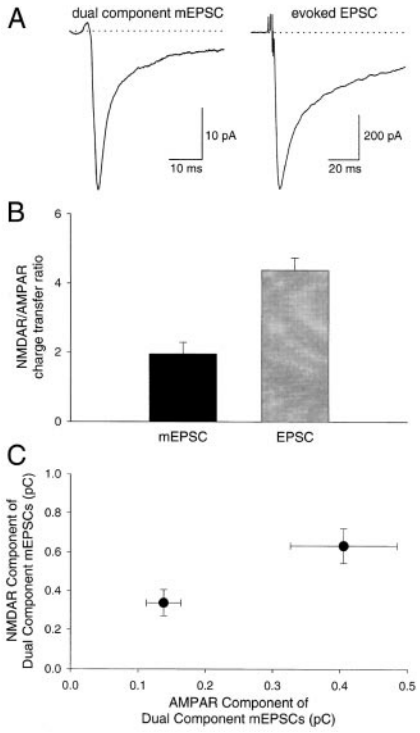

Figure 3. The NMDAR/AMPAR Charge Transfer Ratio of the Evoked EPSC Is Larger than That of the Dual Component mEPSC

(A) Representative averaged traces of the dual component mEPSC and evoked EPSC obtained in one isolated autaptic hippocampal neuron. The mEPSC trace is an average of 400 events. The dual component EPSC trace was derived from the sum of the individual receptor components, each the average of 15 events.

(B) The NMDAR/AMPAR charge transfer ratio is greater for the evoked EPSC than for the mEPSC ($n = 5$, $p < 0.01$), as expected if both NMDAR-only mEPSCs and dual component mEPSCs contribute to the evoked response.

(C) The AMPAR and NMDAR components of dual component mEPSCs correlate in size. Dual component events with the smallest and the largest 20–50 AMPAR components were each averaged, and the AMPAR and NMDAR charge transfers were determined. Mean NMDAR charge transfer differs significantly between events with large and small AMPAR components ($n = 5$, $p < 0.05$). This correlation between AMPAR and NMDAR EPSC size suggests that neither component of the dual component mEPSC is selectively affected with respect to the other by the resolution limit for detection of mEPSCs.

mEPSCs that lack AMPARs and were not included in the mEPSC average. We therefore did a detailed study of the spontaneous synaptic activity in these cells to determine if some of these spontaneous events might lack the AMPAR component of the response.

Such an analysis of spontaneous synaptic currents indicated that there are fast rising and decaying events, almost always followed by slowly decaying, noisy events, as well as slowly rising and decaying events that lack the fast rising component. Examples of these responses are shown in Figure 4A on a slow time scale (A1, see asterisks) and at an expanded time scale for dual component mEPSCs (A2) or slow mEPSCs (A3). Pharmacological analysis confirmed that the fast component is mediated by AMPARs and the slow component by NMDARs. Thus, application of APV completely blocked the slow events (Figures 4B1 and 4B2), and NBQX blocked the early fast component (Figures 5 and 7A). Figure 4C shows averages of the dual component mEPSCs, the NMDAR-only mEPSCs, and the mEPSCs recorded in the presence of APV.

A more quantitative analysis of the mEPSCs in these cells is shown in Figure 5. mEPSCs were collected from the same cell ($n = 4$) in three conditions (control conditions, APV, or NBQX) and cumulative probability distributions of the rise times plotted. The cumulative rise time distribution of normal events shares features found in the distributions of mEPSC rise times recorded in either APV or NBQX. Importantly, a population of events that are slower than those recorded in APV is clearly evident. The distribution of the normal events can be fit with a linear combination of the distributions of events recorded in APV and in NBQX. This fitting estimates that approximately 36% $\pm$ 4% ($n = 4$) of events in the normal condition represent NMDAR-only mEPSCs. In selecting events that appeared to have only the slow component, it is possible that a small fast component could have been overlooked because of the noisy nature of the individual traces. We do not think that this is the case, because when a large number of these slow events are averaged together, there is little evidence of a fast component, and a small AMPAR component would have to be below our detection limit to remain undetected in the trace. Furthermore, the rise times of the averaged NMDAR-only mEPSCs are very similar to those of averaged pharmacologically isolated evoked and miniature NMDAR synaptic currents (Figures 5B–5D). It is interesting to note that the decay of the mEPSCs is considerably more rapid than the decay of the evoked NMDAR response. We believe that this difference is due in large part to the asynchronous release of transmitter that occurs following the synchronous release (Goda and Stevens, 1994) and the summation of these asynchronous events on the falling phase of the evoked response.

The experiments thus far are all consistent with there being a population of synapses that contain NMDARs but do not contain functional AMPARs. While it is possible that AMPARs may be present at these synapses but not contribute to the recorded synaptic currents, alternatively, these synapses may lack AMPARs. In an attempt to distinguish between these two possibilities and to provide independent, nonelectrophysiological evidence for a population of NMDAR-only synapses, we used immunocytochemical staining of AMPARs (anti-GluR1) and NMDARs (anti-NR1) on our single cell cultures. The NR1 subunit is an essential subunit for all functional NMDARs, and GluR1 has been shown to colocalize precisely with the other AMPAR subunits (GluR2/3) expressed in hippocampal cultures (Craig et al., 1993). Figure 6 shows results from a typical autapse in which synapses were identified by the presynaptic marker synaptophysin (A). Two areas of interest are identified by the boxes and shown below. Virtually all the synapses stained for NR1 (compare the red NR1 puncta to the white synaptophysin puncta). Such a close congruence would appear to rule out the formal possibility that glutamate may spill over from active terminals that are not apposed to any functional receptor. As shown in (B), a substantial fraction of synapses, while staining for NR1,

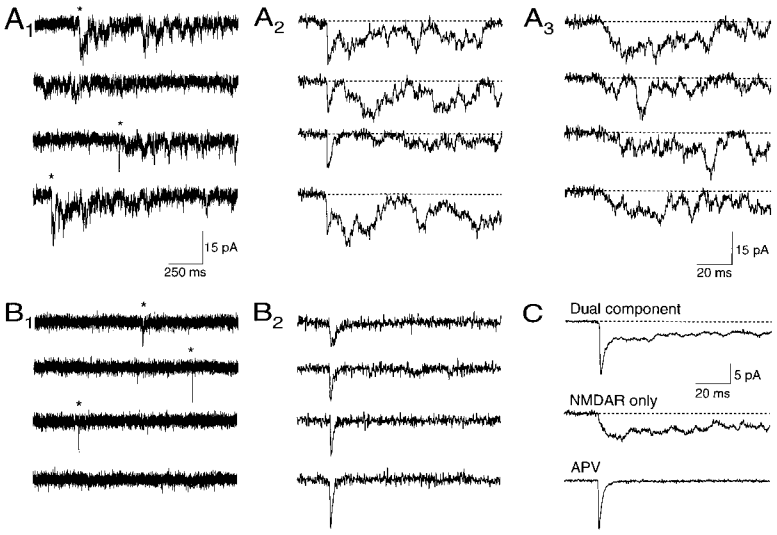

Figure 4. Two Classes of Spontaneous Events Are Evident in Isolated Autaptic Cells

(A1) Continuous record demonstrating, on a slow time scale, the variability of spontaneous currents in 0 Mg^{2+} (marked with asterisks). Events separate into two broad classes: those with a fast initial component, dual component mEPSCs (A2) and those that lack a fast initial component, NMDAR-only mEPSCs (A3). (B) APV eliminates the slow component, leaving fast spontaneous events, characteristic of AMPAR mEPSCs (B1 and B2).

(C) Average of dual component mEPSCs, of NMDAR-only mEPSCs, and of the AMPAR events recorded in APV. Note that much of the variance inherent in the NMDAR current is lost in the average.

The calibration in (A3) also applies for (A2) and (B2). The calibration in (A1) also applies for (B1).

lacked punctate immunoreactivity for GluR1 (green puncta). Interestingly, on another segment of dendrite shown in (C), most of the synapses contained both GluR1 and NR1 puncta. In this cell the NR1-only puncta were not randomly distributed throughout the dendritic processes, although on other cells there was intermingling of NR1-only puncta with the colocalized NR1 and GluR1 puncta. On average, NR1-only synapses were found at a density of 9/100 μm of dendrite, while synapses that contained both GluR1 and NR1 puncta occurred at a density of 49/100 μm of dendrite. A minority of synapses contained only GluR1 puncta, and these were found at a density of 2/100 μm of dendrite. The anatomical fraction of synapses that have only NR1 puncta is 16%. These results complement our physiological data and suggest that the NMDAR-only mEPSCs that we recorded are generated by synapses that contain high levels of NMDARs but not AMPARs.

In all of the preceding experiments, the neurons were grown on glial cells. While these cultures are ideally suited for electrophysiological recordings, they are suboptimal for immunocytochemistry because the underlying glial cells decrease the resolution of the optical imaging and it is difficult to find neuronal processes that have minimal underlying glia. We therefore also examined hippocampal neurons that were grown directly on polylysine-coated glass suspended above a feeder layer (Goslin et al., 1998), a preparation that was used in previous studies on the cellular distribution of glutamate receptor clusters (Rao and Craig, 1997; Rao et al., 1998). Spontaneous synaptic events were common in these neurons, and as in the single cell cultures, there were events that had fast and slow components and also events that had only the slow component (Figure 7A). The finding that the rise time of the average of the slow events is the same as the rise time of events recorded in the presence of NBQX confirms that these slow events are NMDAR-only mEPSCs. Immunocytochemical staining of sister cultures from which these recordings were made demonstrates a substantial proportion of synapses that express NMDAR puncta but lack AMPAR puncta (Figure 7B, and see Rao and Craig, 1997). One possible trivial explanation for the presence of NR1 puncta that do not colocalize with GluR1 puncta would be a population of synapses with faint puncta for both NR1 and GluR1, with GluR1 puncta simply falling below the detection limit. This idea is not consistent with the finding that synapses classed as containing or lacking GluR1 puncta on the basis of strong or faint GluR1 staining showed no difference in fluorescence intensity for NR1 within the NR1 puncta (Figure 7C). Thus, the synapses with the lowest levels of AMPAR staining, comparable to the diffuse extrasynaptic staining of dendritic shafts, show a normal complement of NMDARs and presumably account for the physiologically silent synapses recorded from these cells. The immunocytochemical and physiological evidence in support of silent synapses now extends to both the single cell culture preparation and the multiply innervated low density cultures. Thus, the evidence in support of silent synapses is not a consequence of the single cell culture preparation.

Discussion

Recent electrophysiological studies in hippocampal slices (Isaac et al., 1995; Liao et al., 1995; Durand et al., 1996), as well as other preparations (Wu et al., 1996; Isaac et al., 1997; Bardoni et al., 1998; Li and Zhuo, 1998), have suggested that a population of synapses exists that contain functional NMDARs but not functional AMPARs. Furthermore, it is proposed that during LTP, functional AMPA receptors rapidly appear at these synapses. However, a plausible alternative explanation for these NMDAR-only synaptic responses is that glutamate can spill over from neighboring synapses and because of the 100-fold higher affinity of glutamate for NMDARs over AMPARs, only an NMDAR response is generated (Kullmann et al., 1996). To address the issue of spillover, we used single cell cultures, a condition in which spillover could not explain an NMDAR-only synaptic response.

Using these single cell cultures, we first compared the variability of the AMPAR EPSC to that of the NMDAR EPSC and found that the variability of the AMPAR EPSC was more than that of the NMDAR EPSC. This finding agrees with previous work in the hippocampal slice

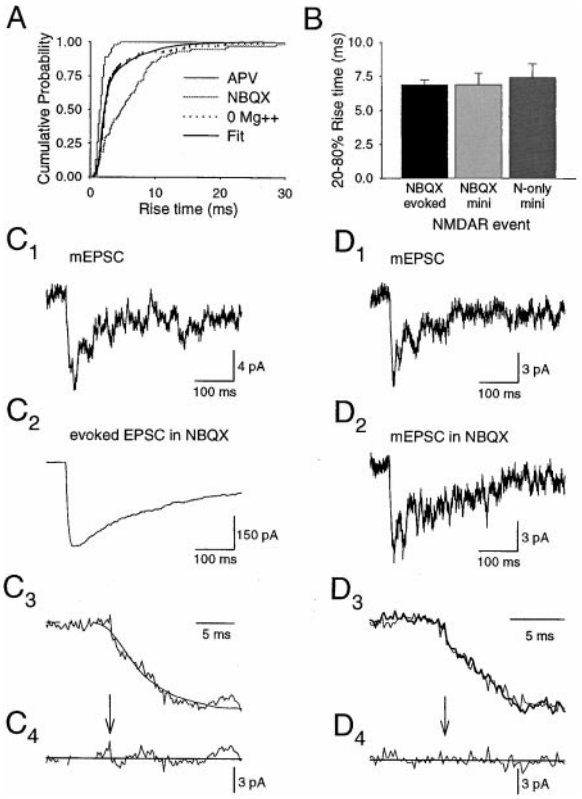

Figure 5. NMDAR-Only mEPSCs Can Be Distinguished from Dual Component Events on the Basis of Rise Time

(A) The proportion of NMDAR-only mEPSCs can be measured from a comparison of rise times of normal events and of pharmacologically isolated AMPAR and NMDAR mEPSCs. The cumulative rise time distribution of normal events shares features of both the APV and the NBQX distributions. Notably, it has a prominent population of events that are slower than those seen in APV. The distribution of normal events is well fit with a linear combination of these two distributions. The proportion of NMDAR-only mEPSCs, derived as the weight of the NBQX distribution, is 38%.

(B) Average evoked EPSCs recorded in NBQX, mEPSCs recorded in NBQX, and NMDAR-only mEPSCs share similar rise times. The 20%–80% rise times were measured from the average of 15 evoked NMDAR EPSCs (n = 5), 8 to 11 NBQX mEPSCs (n = 3), and 9 to 22 NMDAR-only mEPSCs (n = 5).

(C) Average NMDAR-only mEPSCs resemble evoked NMDAR EPSCs. Averages of 15 NMDAR-only mEPSCs (C1) and of 15 evoked EPSCS in NBQX (C2) are shown for a representative cell. (C3) Superimposed average traces of the evoked and miniature EPSCs demonstrate the similarity of their rising phases. (C4) The difference between the NMDAR-only mEPSC and the scaled evoked NMDAR EPSC is small.

(D) Average NMDAR-only mEPSCs resemble miniature NMDAR EPSCs. Averages of ten NMDAR-only mEPSCs (D1) and of eight mEPSCS recorded in NBQX (D2) of a representative cell are displayed. (D3) Superimposed average traces of the NMDAR-only and NBQX mEPSCs are shown. (D4) The difference between the NMDAR-only mEPSC and the NMDAR mEPSC derived in NBQX is also small. Arrows mark the points of alignment for averaging NMDAR mEPSCs.

preparation (Kullmann, 1994; Selig et al., 1995; Kullmann et al., 1996; but see Niu et al., 1998) and suggests that more synapses contribute to the NMDAR EPSC than to the AMPAR EPSC.

We next compared the proportion of the NMDAR component in AMPAR-containing mEPSCs to the proportion

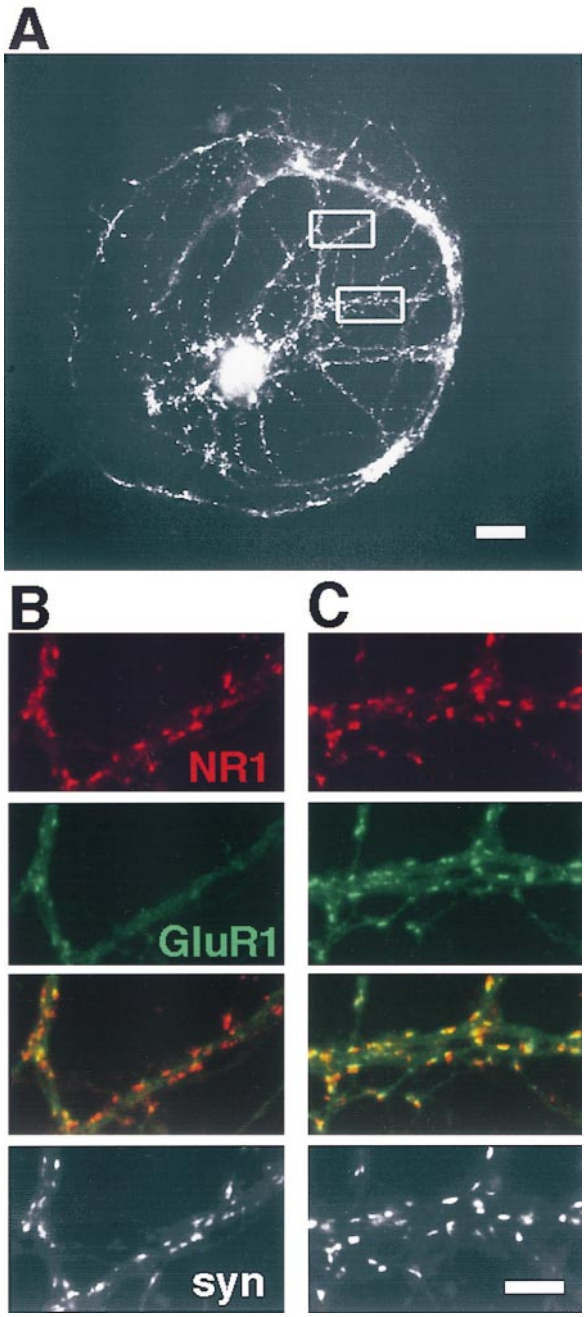

Figure 6. Autapse Immunocytochemistry Shows Prominent NMDAR-Only Puncta

(A) Low magnification view of an isolated neuron and its processes labeled with an antibody for the presynaptic marker synaptophysin. Scale bar = 20 μm. The two boxed regions are shown at higher magnification in ([B], upper box) and ([C], lower box). In (B) and (C), the immunocytochemistry for the NR1 subunit of the NMDAR is shown in red, the GluR1 subunit of the AMPAR in green, and synaptophysin in the bottom panel in greyscale. The overlaid NR1 and GluR1 images in the third panel show yellow puncta at regions where the two are colocalized. In the region shown in (B), a stretch of dendrite contains synapses that have clusters of NMDAR but no clusters of AMPAR. Nineteen cells from this culture (three isolated, the rest innervated) were imaged and scored for their percentage of such potential silent synapses. Of the total number of synapses that contained one or both receptors that were scored, 16.4% ± 4.9% had only NMDAR clusters. In (C), the scale bar in the bottom panel = 5 μm.

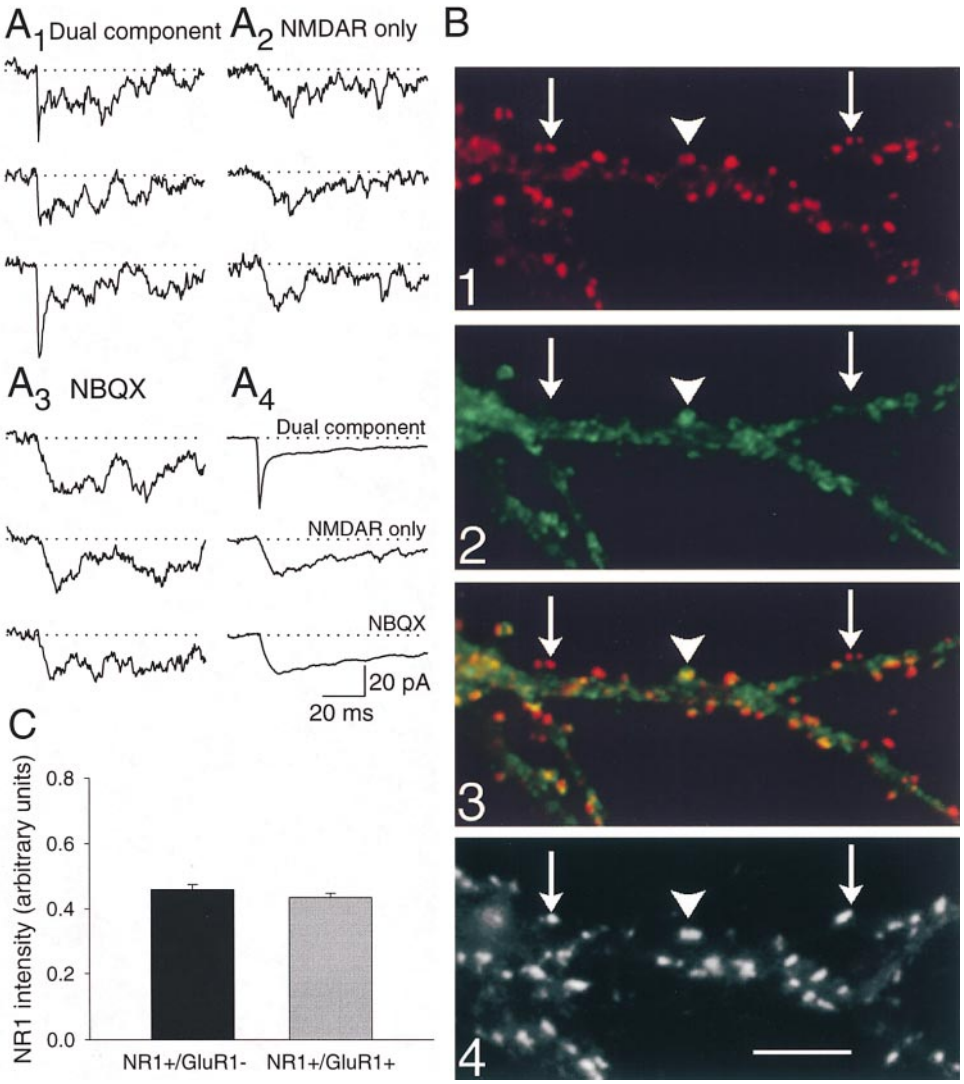

Figure 7. NMDAR-Only mEPSCs and NMDAR-Only Synaptic Puncta Are Evident in Low Density Cultures of Hippocampal Neurons

(A) The presence or absence of a fast initial component distinguishes dual component mEPSCs from NMDAR-only mEPSCs, but the kinetics of the slow component is common to both. (A1) shows example traces of events with both components, while (A2) shows events with only slow components. (A3) shows traces of events collected in the presence of NBQX to block the AMPAR-dependent fast initial component. (A4) displays average traces of these populations of events ($n = 81$ for the dual component response, $n = 24$ for the NMDAR-only response, and $n = 49$ for the responses obtained in NBQX). The NMDAR-only and NBQX-averaged traces have 20%–80% rise times of 4.5 ms and 4.2 ms, respectively.

(B) Immunocytochemical localization of NR1, GluR1, and synaptophysin identifies some synapses with NR1 puncta but lacking GluR1 puncta. (B1), (B2), (B3), and (B4) show staining for NR1 (red), GluR1 (green), double color overlay, and synaptophysin (greyscale), respectively, in a sister culture of the cell recorded in (A). Arrows mark two synapses with NR1 puncta but no GluR1 puncta, while the arrowhead identifies a synapse with both NR1 and GluR1 puncta. Scale bar = 5 μm.

(C) The average staining intensity of NR1 is similar in synaptic puncta with strong and faint staining for GluR1. Synapses were selected by the presence of high average NR1 intensity, and at these synapses, average GluR1 intensity was measured. We compared two classes of synapses. The first had the brightest 10% of GluR1 intensities and were clear NR1+/GluR1+. The second had the lowest 10% of GluR1 intensities. This population had GluR1 intensities no greater than the adjacent extrasynaptic dendritic regions and were nominally designated NR1+/GluR1−.

of the NMDAR component in the evoked response in the same cell. The evoked EPSC contained a substantially larger contribution of NMDAR component than did the AMPAR-containing mEPSC, suggesting that there are NMDAR responses that are not associated with AMPAR mEPSCs but which nevertheless contribute to the evoked EPSC. It is unlikely that the failure to detect small AMPAR-containing mEPSCs explains the difference between mEPSC and evoked EPSC because we found that there was a direct correlation between the size of the AMPAR and the NMDAR components.

We then went on to perform a detailed analysis of mEPSCs from the single cell cultures. We found that while most events had a fast rising component followed by a slowly decaying component, a fraction of events lacked the fast component. Pharmacological analysis

indicated that the fast component was mediated by AMPARs and the slow component was mediated by NMDARs. Two previous reports using confluent cultures have observed mEPSCs (Bekkers and Stevens, 1989) or spontaneous action potential evoked EPSCs (Kiyosue et al., 1997) that lack AMPAR responses. However, since both of these studies were done in confluent cultures, spillover of glutamate could account for these findings.

These physiological data for NMDAR-only synaptic currents could be explained in one of four ways: (1) NMDAR-only synapses have the normal number of AMPARs, but they are not functional, (2) these synapses have little, if any, detectable AMPARs, (3) some synaptic vesicles contain small concentrations of glutamate sufficient to activate NMDARs but not AMPARs, or (4) the NMDAR-only synapses might have a small AMPAR component that went undetected because of dendritic filtering. The immunocytochemical identification of a population of synapses that possess normal NMDAR puncta but no AMPAR puncta clearly supports alternative 2 but does not exclude a contribution by the other alternatives.

Similar physiological and anatomical data were also obtained from low density hippocampal neurons grown according to Goslin et al. (1998), a preparation in which the resolution of the immunochemical staining is enhanced. Thus, we are confident that the silent synapses expressing only NMDARs do not contain high concentrations of AMPARs. Some of these synapses exhibited no detectable GluR1 immunoreactivity, but many exhibited a low level of GluR1 immunofluorescence. This immunoreactivity, which was higher than the level seen in axons in the field, and thus possibly specific, was no greater than that seen in adjacent extrasynaptic regions of dendritic shaft. We therefore cannot resolve accumulation of GluR1 at the synapse in these cases. To what extent this diffuse staining reflects surface receptor or intracellular pools of receptor requires further characterization.

In our immunocytochemical studies, we detected approximately 15% of synapses as containing only NMDAR puncta, while the electrophysiological data suggested that approximately 35%–45% of the recorded events were NMDAR-only mEPSCs. If all of the synapses have, on average, an equal probability of releasing a quantum of glutamate, then approximately 35%–45% of synapses generate NMDAR-only responses. This difference might reflect a difference in the sensitivity of the two techniques for detecting AMPARs. Alternatively, it is possible that some of the GluR1 staining may not represent functional surface AMPARs. It would be of considerable interest to examine the effects of LTP on the silent synapses we have characterized in these single neuron cultures. However, thus far we have been unsuccessful in inducing LTP in these neurons.

Electrophysiological evidence for the existence of silent synapses that lack functional AMPARs has been provided from the study of a variety of structures in addition to the hippocampus, including thalamocortical synapses in somatosensory cortex (Isaac et al., 1997), retinal inputs to the frog optic tectum (Wu et al., 1996), and spinal cord synapses (Bardoni et al., 1998; Li and Zhuo, 1998). Thus, silent synapses may be ubiquitous throughout the CNS. It is important to note that while referred to as silent, NMDAR-only synapses are in fact conditionally silent and would have the interesting property that they selectively transmit their synaptic input only when the postsynaptic cell is already depolarized by other inputs. This depolarization gate would provide a very select context in which these synapses could contribute to synaptic integration. Such synapses would also serve as coincidence detectors, amplifying the signals that led to their recruitment. In addition, NMDAR-only synapses may well provide an important substrate for shaping neural circuitry in response to experience. However, a major problem for this hypothesis has been the difficulty of ruling out spillover of glutamate as the explanation for the electrophysiological observations (Kullmann and Asztely, 1998). Here, we have provided direct anatomical and electrophysiological evidence from the same preparation for the existence of a population of synapses that contains the normal level of NMDARs but little or no detectable AMPARs. Furthermore, this analysis was performed in a preparation in which spillover cannot account for the synaptic responses mediated only by NMDARs. Thus, the findings provide strong support for the existence of postsynaptically silent synapses and direct attention to the molecular processes responsible for the differential regulation of the expression of synaptic AMPARs and NMDARs.

Experimental Procedures

Tissue Culture

Microdot cultures were prepared from hippocampal neurons of the CA1 and CA3 regions of the hippocampus of P0 Sprague-Dawley rat pups. The dentate gyri were grossly dissected away, and cells derived from the remaining tissue were prepared as described in Tong and Jahr (1994), except that papain was not used and B27 (GIBCO BRL) was supplemented. The growth medium was exchanged fully one day after plating and weekly in part thereafter. Autaptic recordings were obtained from isolated neurons grown on collagen/poly-D-lysine microdots. Low density cultures were prepared from E18 rats as in Goslin et al. (1998). Neurons were maintained in MEM with N2 supplements above a glial feeder layer. APV (100 μM) was added at 1 week after plating and renewed every 2 to 4 days.

Whole-Cell Experiments

Recordings were made at room temperature from 4- to 15-day-old isolated autaptic neurons and 14- to 24-day-old neurons of APV-treated low density cultures, using an Axopatch-1D amplifier with low resistance patch pipettes (2–5 MΩ). Pipette solutions contained (in mM) 122.5 K-gluconate, 8 NaCl, 10 HEPES, 0.2 EGTA, 2 MgATP, 0.3 NaGTP, adjusted to pH 7.4 with KOH. For autapse recordings, 20 mM K_2-creatine phosphate and 50 U/ml phosphocreatine kinase were added. The extracellular solution contained (in mM) 140 NaCl, 3.5 KCl, 10 HEPES, 20 glucose, 0.3–3 $CaCl_2$, and 20 μM glycine adjusted to pH 7.3 with NaOH. For acquisition of miniature EPSCs, 3 mM Ca^{2+} was used, and in low density multiply innervated cultures, 1 μM TTX and 50 μM picrotoxin were added to the extracellular solution. Evoked recordings were performed in 7–15 day neurons. Since the frequency of mEPSCs in these older autaptic cells was often too high to permit us to readily distinguish individual events, we generally restricted our analysis of mEPSCs to younger cells (4–7 days).

Cells were held at −60 mV and were stimulated once every 10–20 s with a 2.5 ms 70 mV depolarizing current pulse. AMPAR-mediated currents were isolated by the addition of 100 μM D-APV (Tocris Neuramin); NMDAR-mediated currents were isolated by the addition of 5 μM NBQX (Tocris Neuramin). Synaptic currents were completely

abolished with addition of both D-APV and NBQX. Series resistance ranged from 10 to 30 MΩ and was compensated (80%) in all experiments. The series and input resistances were monitored throughout each experiment with a 3 mV calibration pulse given 40 ms before each stimulation. Junction potentials were not corrected. Evoked EPSCs were acquired and analyzed on line using custom software (D. Selig). Currents were low-pass filtered at 2 kHz and digitally sampled at 5 kHz. AMPAR and NMDAR EPSC peak amplitudes were measured from the average of 15–30 traces. mEPSCs were acquired using Axoscope (Axon Instruments) and were analyzed using Mini (J. H. Steinbach) and Quanta (S. Borges). Threshold mEPSC amplitude was set at 4 pA, and events were collected in each pharmacological condition.

Mean^2/Variance Analysis

The mean and variance of EPSCs in each pharmacological condition were measured using a minimum of 30 events. To insure good voltage clamp for the mean^2/variance analysis, Ca^{2+} was reduced to keep AMPAR EPSC amplitudes below 400 pA. The amplitude of each EPSC component was measured with a 2–3 ms window at the peak of the response. The variation in size of evoked synaptic currents dominated the measured mean^2/variance of each receptor component. Other, secondary sources of variation are the action potential artifact, recording noise, and background glutamate receptor channel noise derived from mEPSCs. These sources of noise were removed from the calculated mean^2/variance (see Kullmann, 1994) but contributed $<$5% to the total variation. Although intrinsic differences between NMDARs and AMPARs may lead to their differential contributions to the variance, an error due to neglect of intrinsic differences is in the wrong direction to explain our results (see Kullmann, 1994).

Comparison of mEPSC and Evoked NMDAR/AMPAR Charge Transfer Ratios

Charge transfer was measured by integrating from EPSC onset to 100 ms in the presence of NBQX or APV for evoked responses and in normal conditions or APV for mEPSCs. The charge transfer ratio of mEPSC recorded in APV to dual component mEPSC was evaluated and transformed arithmetically into the NMDAR/AMPAR charge transfer ratio. The NMDAR/AMPAR charge transfer ratio for the evoked response was taken directly from the isolated components. To obtain the proportion of NMDAR-only synapses with this method, we compared the amplitudes of the evoked components and the unitary components and derived the ratio of the NMDAR to AMPAR synapses in the evoked response. Because the evoked AMPAR amplitude is more sensitive to jitter in the summation of individual synaptic events, the proportion of NMDAR-only synapses calculated with this method is likely to represent an overestimate. We obtained similar results between small and large mEPSCs when comparing NMDAR and AMPAR peak amplitudes (data not shown).

Rise Time Analysis

The rise times of mEPSCs were measured from the point of deviation from baseline to the end of the smooth rising phase. Measurement to the peak of mEPSCs was avoided in order to maximize detection of small, rapid events buried in the slow, noisy rising phase of the NMDAR component of the mEPSC. To optimize discrimination between AMPAR and NMDAR mEPSCs, average NMDAR-only mEPSCs were only measured in cells with fast, sharply distributed AMPAR mEPSC rise times. mEPSCs were stringently defined as NMDAR-only if their rise times exceeded the slowest rise time derived in APV by 2 ms. These NMDAR-only mEPSCs were aligned at their point of initial rise and averaged. The mean NMDAR mEPSCs derived in NBQX with the same criteria used in selecting NMDAR-only events had identical 20%–80% rise times and similar kinetics to those of the average NMDAR-only mEPSCs thus derived (see Figures 5 and 7). The largest AMPAR mEPSC that could be buried in our average NMDAR-only mEPSC would be below our limit of resolution even in the presence of APV ($n = 5$).

For rise time distribution fits to estimate the actual proportion of NMDAR onlys, NBQX and APV mEPSC distributions composed of approximately 100 events were fit using Table Curve (Jandel Scientific) to derive analytical functions that described them (r^2 always exceeded 0.98). The normal distribution of mEPSCs, composed of approximately 200 events, was then fit with a linear combination of these functions, allowing the relative weights of the APV and NBQX functions to vary. A small offset in rise time values (300 μs) optimized superposition of the fit in APV with rapid events in normal conditions (without APV). An offset is to be expected since the NMDAR component was found to slow the AMPAR component by 450 ± 114 μs ($n = 5$).

Immunocytochemistry

Triple label immunocytochemistry was performed on microisland cultures as described previously (Rao and Craig, 1997). Double label immunocytochemistry with GluR1 and NR1 antibody does not change the pattern of staining or decrease the number of clusters detected when compared to labeling with the GluR1 antibody alone (Rao and Craig, 1997; compare Figures 4 and 6). Therefore, the detection of NMDAR-only synapses by immunocytochemistry cannot be due to any kind of competitive interaction between the two antibodies that results in an inability to detect GluR1 at sites where it is colocalized with NR1. The microisland cultures were evaluated for putative silent synapses by overlaying the three labels in three different color channels of an Adobe Photoshop image and scoring for the presence or absence of a cluster of staining of the appropriate size apposed to a synaptophysin puncta. Low density cultures were triple stained either as described previously or using goat anti-NR1 (Santa Cruz Biotechnology, 1:2000), rabbit anti-GluR1 (Upstate Biotechnology, 1:8000), and mouse anti-SV2 (1:50). The monoclonal anti-SV2 developed by Kathleen Buckley, Harvard University, was obtained from the Developmental Studies Hybridoma Bank maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA 52242, under contract NO1-HD-7–3263 from the NICHD. Secondary antibodies used were: donkey FITC-conjugated anti-goat (Jackson Labs, 1:200), donkey Texas Red-conjugated anti-rabbit (Jackson Labs, 1:200), and horse biotin-conjugated anti-mouse (Vector Labs, 1:600) followed by AMCA-conjugated streptavidin (Vector Labs, 1:50). The staining intensity for NMDAR and AMPAR at synapses in the low density cultures were compared using 12 bit images and Oncor imaging software. Clusters of NR1 were selected by interactively setting a threshold intensity that defined the borders of the cluster to the observer. All clusters above this intensity threshold that were apposed to synaptophysin puncta were analyzed. The average intensity within each cluster was obtained for NR1, and then a mask was made of the image and applied to the paired GluR1 image to find the average intensity of GluR1 staining in the same region. Background staining was estimated by the level of fluorescence in a stretch of axon in the field and subtracted from all intensity values for that image. Intensity values were obtained from 811 clusters from six cells each from two independent cultures. All cluster intensity values for a culture were normalized by setting the highest intensity value to 1.

Data Analysis

Results are presented as means ± SE. Data were compared statistically using the Student's t test, and significance was defined at $p < 0.05$. To insure that the nonlinear scale inherent in a distribution of ratios did not falsely generate significance, we also performed statistical analyses with the log of NMDAR/AMPAR ratios of mEPSCs and evoked EPSCs. This did not alter the result, and the p values for this comparison are those shown in the text.

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