DISTINCT TRANSMITTER RELEASE PROPERTIES DETERMINE DIFFERENCES IN SHORT-TERM PLASTICITY AT FUNCTIONAL AND SILENT SYNAPSES

Carolina Cabezas and Washington Buño.

INSTITUTO CAJAL, CSIC, AVENIDA DOCTOR ARCE 37, 28002-MADRID, SPAIN.

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CORRESPONDING AUTHOR: Washington Buño. Instituto Cajal, CSIC, Av. Dr Arce 37, 28002, Madrid, Spain. Phone: 34-91-5854711 Fax: 34-91-5854754. E-mail: wbuno@cajal.csic.es.
ABSTRACT

Recent evidence suggests that functional and silent synapses are not only postsynaptically different but also presynaptically distinct. The presynaptic differences may be of functional importance in memory formation because a proposed mechanism for long-term potentiation is the conversion of silent synapses into functional ones. However, there is little direct experimentally evidence of these differences. We have investigated the transmitter release properties of functional and silent Schaffer collateral synapses and show that on the average functional synapses displayed a lower percentage of failures and higher EPSC amplitudes than silent synapses at +60 mV. Moreover, functional but not silent synapses show paired-pulse facilitation (PPF) at +60 mV and thus, presynaptic short-term plasticity will be distinct in the two types of synapse. We examined whether intraterminal endoplasmic reticulum Ca\(^{2+}\) stores influenced the release properties of these synapses. Ryanodine (100 µM) and thapsigargin (1 µM) increased the percentage of failures, and decreased both the EPSC amplitude and PPF in functional synapses. Caffeine (10 mM), had the opposite effects. In contrast, silent synapses were insensitive to both ryanodine and caffeine. Hence, we have identified differences in the release properties of functional and silent synapses, suggesting that synaptic terminals of functional synapses express regulatory molecular mechanisms that are absent in silent synapses.

KEYWORDS: hippocampus; glutamatergic synapses; presynaptic mechanisms; synaptic potency; presynaptic Ca\(^{2+}\) stores.
INTRODUCTION

Glutamatergic synapses between Schaffer Collateral (SC) and CA1 pyramidal neurons are a classic model for the study of synaptic plasticity such as paired-pulse facilitation (PPF) and long-term potentiation (LTP). There are two types of SC-CA1 pyramidal neuron synapses. One type, called functional synapses, show postsynaptic α-amino-3-hydroxy-5-methyl-4-isoxazole propionato receptors (AMPAR) -mediated currents when the postsynaptic cell is at its resting potential and both AMPAR and N-methyl-D-aspartate receptors (NMDAR) -mediated currents when depolarized. The other synapse type, termed silent synapse (or conditionally silent), only displays postsynaptic NMDAR -mediated currents at depolarized potentials, but does not respond at the resting potential (Isaac et al. 1995; Liao et al. 1995; Durand et al. 1996; reviewed in, Atwood and Wojtowicz 1999; Isaac 2003; Nicoll 2003). The different synapse types are thought to be of functional importance in information processing leading to memory formation because a proposed mechanisms for LTP is the conversion of silent synapses into functional ones (Goda and Stevens 1996; Hasselmo 1999; Poncer and Malinow 2001; reviewed in, Atwood and Wojtowicz 1999; Isaac 2003; Nicoll 2003).

Cholinergic agonists inhibit transmitter release at the presynaptic terminals of functional synapses, but they do not affect silent synapses (de Sevilla et al. 2002), suggesting that functional and silent synapses are both post- and presynaptically different. However, the molecular mechanisms underlying the presynaptic functional differences between both glutamatergic types of synapse remain elusive.

In our previous publication we concentrated our analysis on silent synapses and on a group of functional synapses that showed changes in the percentage of failures but no modifications of the EPSC amplitude (i.e. averages excluding failures; see below) during PPF, a presynaptic form of short-term plasticity (Katz and Miledi 1968; Kamiya and Zucker 1994; de Sevilla et al. 2002; Zucker and Regehr 2002; Martín and Buño 2003). The effects of carbamilcholene chloride (CCh) that decreases the probability of release and manipulations that increase the probability of release
did not modify the EPSC amplitude in silent and in those functional synapses (de Sevilla et al. 2002). However, in that same publication we noted that a group (≈ 50 %) of functional SC synapses showed both a decrease of the percentage of failures and an increase of the amplitude of the second EPSC (R2) during paired-pulse stimulation, and that the presynaptic inhibition by CCh could increase the percentage of failures and decrease the EPSC amplitude (de Sevilla et al. 2002).

To establish the contribution of the different types of synapses in synaptic plasticity, information handling and maturation it is crucial to understand the differences between them and the link connecting their pre- and postsynaptic properties (reviewed in Dumas 2005). Therefore, we centred the present analysis on the differences in release properties and paired pulse plasticity between silent and functional synapses, including those functional synapses that showed larger EPSC amplitudes of R2 as compared with the first EPSC (R1) during PPF. Using “minimal stimulation”, that activates one or very few synapses (Raastad 1995), we analyzed the differences in the release properties of silent and functional SC synapses. Functional synapses exhibited lower percentage of failures and higher EPSC amplitude than silent synapses and functional synapses exhibited PPF. In contrast, PPF was absent in silent synapses. We analyzed if these discrepancies could be caused by differences in the regulation of intraterminal endoplasmic reticulum (ER) Ca\(^{2+}\) stores, and we found that ryanodine (100 µM) and thapsigargin (1 µM) increased the percentage of failures and decreased both the EPSC amplitude and PPF in functional synapses. Caffeine (10 mM) had the opposite effects in functional synapses. In contrast, silent synapses were insensitive to these manipulations, suggesting that functional and silent synapses are both post- and presynaptically different. These differences bear upon the functional importance of silent synapses in synaptic plasticity and maturation because they suggest that different molecular mechanisms control transmitter release in both synapse types.
METHODS

Animal care procedures, surgery, and slice preparation have been described previously (de Sevilla et al. 2002). All the procedures carried out in this study conformed to the International Guidelines on the ethical use of animals with every effort being made to minimize the suffering and number of animals used.

**Preparation and recordings.** Hippocampal slices were obtained from young Wistar rats (12- to 16-day-old) after they were decapitated and the brain removed and submerged in cold (≈ 4°C) artificial cerebrospinal fluid (ACSF; see below). Slices (350-400 µm) were cut with a vibratome (Pelco 101, St Louis, Missouri, USA) and maintained at pH 7.3 by bubbling with 95 % O₂ and 5 % CO₂ (> 1 h at room temperature of 22-25°C). Slices were transferred to a recording chamber on an upright microscope (Olympus BX50WI, Olympus Optical, Tokyo, Japan) equipped with infrared and differential interference contrast imaging devices, and with a 40x water immersion objective. Slices were maintained at room temperature, and superfused at a rate of 1-10 ml/min with gassed ACSF. Patch-clamp recordings from CA1 pyramidal neurons in the whole-cell voltage-clamp configuration were performed with 3-7 MΩ fire-polished pipettes connected to a PC-ONE amplifier (Dagan Corporation, Minneapolis, USA). Fast and slow capacitances were neutralized and series resistance was compensated (≈ 80 %). Patch recordings were rejected when the access resistance (7-15 MΩ) increased > 20 % during the experiment. To minimize the contribution of postsynaptically mediated plasticity, control recordings of SC EPSCs were obtained > 20 min after accessing the intracellular compartment (Malinow and Tsien 1990; Martín and Buño 2003).

**Solutions.** The ACSF contained (in mM): 119 NaCl, 2.5 KCl, 1.0 KH₂PO₄, 1.3 MgSO₄, 26.2 NaHCO₃, 2.5 CaCl₂, 30 sucrose, 0.05 picrotoxin, 0.01 glycine and 11 glucose at pH 7.3, and the internal pipette solution contained (in mM): 107.5 Cs-gluconate, 8 NaCl, 0.2 EGTA, 20 HEPES, 10 TEA-Cl, 4 Mg-ATP and 0.3 GTP at pH 7.3. Caffeine, CCh and DL-2-amino-5-phosphonovaleric
acid (APV) were dissolved in water, whereas ryanodine and thapsigargin were dissolved in DMSO (0.01%). DMSO at the concentrations used had no effects on synaptic responses or postsynaptic conductances (n=3). Ryanodine (100 and 50 µM), thapsigargin (1 µM), CCh (5 µM) and APV (50 µM) solutions were superfused, whereas caffeine was added directly to the chamber with an automatic calibrated micro syringe through a pipette (tip diameter: 400 µm) positioned with a mechanical micromanipulator close to the recording electrode tip (Martin and Buño 2003). A single volume of 100 µl of the caffeine solution was delivered (total delivery time was in ≈1 s). Chemicals were obtained from Sigma-Aldrich Quimica (Madrid, Spain) and Tocris (Avonmouth, United Kingdom).

**Synaptic stimulation.** Minimal bipolar stimulation of SCs was achieved using two silver chloride electrodes connected to a digital stimulator (Cibertec, Madrid, Spain), placed in the compartments of a pipette pulled as a patch electrode using theta capillaries (Ø of the tip ≈ 3-5 µm; WPI, Sarasota, USA), and filled with ACSF. The pipette was moved in the stratum radiatum, close to the apical dendrite (≈ 100 µm from the soma) of the cell recorded, and fixed when a single afferent was stimulated. There were no differences in the location of stimulation electrodes over the dendrites for functional or silent synapses. Only one afferent was analyzed in any neuron recorded. Stimulation was achieved with single pulses (300 µs duration) at 1.0 s⁻¹ or with pulse pairs at intervals of 50 ms (except when otherwise indicated) and repeated at 0.5 s⁻¹ and in a few cases (n=6) at 0.1 s⁻¹. No differences in the behavior of silent and functional synapses were found between results obtained at the different stimulation rates. The cell was depolarized to +60 mV to detect silent synapses and was then maintained at -60 mV during at least 5 min before analyzing the properties of silent synapses at +60 mV. The threshold stimulus intensity to evoke an EPSC was determined for functional (at -60 mV) or silent (at +60 mV) synapses and stimulus-response relationships were constructed with peak values of EPSCs averages (100 successive trials including failures) obtained at increasing stimulation intensities. Peak amplitudes of averaged EPSCs were
stable until a jump in EPSC amplitude was recorded (suggesting the recruitment of additional afferents); this usually occurred at intensities between 150 to 300 % of the initial threshold intensity. The stimulation intensity was then lowered to about 25%-50% above the initial threshold and EPSCs carefully checked for changes in either percentage of failures or amplitude throughout the experiment. These procedures minimized the possible activation of more than one afferent and of failures in the activation of the stimulated axon. Synapses which did not show amplitude stability of averaged EPSCs to stimulation during the time of recording were rejected. We found no significant differences in the threshold behaviors of functional and silent synapses, indicating no differences on the axon excitability of the two types of synapses. The experiment was started by delivering 100 successive stimulations (single or paired-pulses). When a putative silent synapse had been analyzed, 100 stimuli were delivered at -60 mV to verify that the synapse was silent and that there had been no changes in the properties of the synapse. We considered a synapse as silent when it responded exclusively at +60 mV, and did not respond at -60 mV, even with paired-pulse protocols. Therefore, we excluded a small proportion of synapses that at -60 mV did not respond to the first stimulus but did respond to the second pulse at very low probability (i.e., “mute synapses”; Hanse and Gustafsson 2001a; reviewed in Voronin and Cherubini 2004). Essentially identical procedures were used when a functional synapse was detected but stimulation was first at -60 mV and then at +60 mV (100 trials each). It should be emphasized that stimulation intensity (∼10-50 mA) was not modified during any of the experiments included here. However, at the end of the experiment stimulation intensity was again varied to determine if changes in the threshold or in the amplitude stability of unitary EPSCs, of silent or functional synapses, had occurred during the experiment and rejected when such changes were detected. These test also indicated the absence of long term plasticity such as LTP and LTD. In many cases (35/62) the effects of adding CCh (5 μM) to the ACSF was also tested at the end of the experiment because it has no effect on silent synapses but inhibits functional synapses (de Sevilla et al. 2002), thus providing a secure “fingerprint” of the
synapse type that had been analyzed. These tests were used to reduce the possibility of stimulating more than one SC fiber (Raastad 1995), and synapses that failed to meet these criteria were excluded from this analysis.

**Data acquisition and analysis.** Data were low-pass filtered at 1.0 KHz and sampled at 10.0 KHz through a Digidata 1200B interface (Axon Instruments, Inc., Foster City, CA) and a Pentium-based computer. The pClamp programs (Axon Instruments) were used to generate stimulus-timing signals, transmembrane current command pulses, and to record and analyze data. For the analysis of the failures we used cells that showed EPSCs with an amplitude separation from noise that allowed visual discrimination between failure and success. Independent estimations of failures were made by two scientists who were not involved in this work and who did not know what to expect from the results, and there were no statistically significant differences between their estimations and the measurements made by one of us. In addition, the selected failures were averaged and the visual selection was rejected if the averages contained post-stimulus deflections as estimated by statistically significant (P<0.05) differences in the amplitude distribution of records at the baseline and at post-stimulus delays where the peak of successful responses occurred. During stimulation at -60 mV the EPSC amplitude was defined as the difference between the peak of the averaged EPSC and the preceding baseline. With stimulation at +60 mV, to compare the synaptic potency of functional with silent synapses, the peak amplitude of the NMDA component was estimated (see Results) and during paired-pulse stimulation the peak amplitude of the first EPSC of a pair R1 was estimated as at -60 mV, whereas the peak amplitude of the second EPSC R2 was the difference between the amplitude of the peak of NMDA component of the averaged EPSC and the exponential fits of the decaying phase of the preceding first EPSC R1 (Fig. 2A). The noise-free coefficient of variation (CV_{NF}) of the synaptic responses was calculated both for all 100 stimulations including failures and when failures were eliminated, with the formalism, \( CV_{NF} = (\sqrt{\delta^2_{EPSC} - \delta^2_{noise}})/m, \) where \( \delta^2_{EPSC} \) and \( \delta^2_{noise} \) are the variance of the baseline and the peak EPSC amplitude and \( m \) is the
mean EPSC peak amplitude, respectively (de Sevilla et al. 2002). The inverse of the square of the 
CV_{NF} \left(1/\text{CV}_{NF}^2\right) was used to estimate differences in release properties between two conditions 
where changes in release properties were expected, since the methodology provides an estimation of 
presynaptic function (Faber and Korn 1991; Debanne et al. 1995; Debanne et al. 1996; Foster and 
McNaughton 1991; Voronin et al. 1999). The CV_{NF} of responses excluding failures was used to 
estimate the variance of successful release, as an indicator of the variability of quantal responses. 
Results were expressed as mean ± SEM. Data was compared using the Student’s paired-or 
unpaired-\(t\)-test as appropriate (P<0.05 (*), P<0.01 (**), P<0.001 (***)). No age-related differences 
were found in the cells sampled.
RESULTS

Release properties are different in functional and silent synapses. Functional SC synapses produced glutamatergic EPSCs at both -60 and +60 mV (n=36/62 or 58 %: Functional Synapse, Fig. 1A). In contrast, silent synapses only displayed EPSCs at +60 mV (n=26/62 or 42 %: Silent Synapse, Fig. 1B). The EPSC rise time (estimated from fits to single exponential functions) was faster in functional than in silent synapses, and reached $\tau = 2.7 \pm 0.3$ ms (n=36, $\tau$ max. = 3.0 ms) in functional synapses compared to $\tau = 3.8 \pm 0.2$ ms (n=26, $\tau$ min. = 3.2 ms) in silent synapses (P<0.01; both at +60mV; Fig. 1A and B). These measurements confirmed that the faster AMPA components that evoked EPSC in functional synapses were absent in silent synapses that did not conduct at -60 mV because of the voltage-dependent Mg$^{2+}$-block of NMDA receptor channels.

We compared the percentage of failures of functional and silent synapses at +60 mV, and it was significantly larger in silent (73.5 ± 1.8 %; n=26) than in functional synapses (58.3 ± 2.7 %; P<0.001; n=36, Fig. 1C). We also compared the “synaptic potency” at +60 mV (i.e., the average peak amplitude of EPSCs when failures are excluded; Stevens and Wang 1994). Functional synapses show EPSCs with both an early AMPA and a late NMDA component, whereas silent synapses only display an NMDA EPSC. Therefore, to compare the amplitude of the NMDA component in both synapse types we estimated the amplitude of the NMDA component of EPSCs in functional synapses by measuring at delays of 30 ms from the stimulation artifact, where at +60 mV the AMPA component has terminated and there is only an NMDA component. We found that the synaptic potency of the NMDA component (at +60 mV, measured at 30 ms) was higher in functional (20.4 ± 1.5 pA; n=36) than in silent synapses (15.2 ± 1.3 pA; P<0.05; n=26, Fig. 1D). We also checked that the isolated AMPA component had terminated within 30 ms in seven functional synapses where averaged EPSCs evoked by 100 successive stimuli were recorded at +60 mV both in control ACSF (i.e., the “compound”, AMPA+NMDA EPSC) and after isolating the AMPA component by blocking the NMDA responses with APV (50 µM). The compound EPSC peaked at
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delays of 12.5 ± 0.6 ms with a mean peak amplitude of 26.0 ± 2.7 pA (n=7; data not shown). These values were not statistically different from those of the other functional synapses analyzed (28.4 ± 1.5 pA; 11.9 ± 1.3 ms; P>0.05; n=36). Therefore, these 7 functional synapses are representative of the sample of 36 functional synapses analyzed here. In these synapses the isolated AMPA component peaked at 3.0 ± 0.4 ms and ended at delays of 27.5 ± 0.8 ms.

Using the same amplitude estimation procedure we calculated the CV_{NF} of all 100 stimuli and only of successful NMDA responses for both types of synapses, which were 1.02 ± 0.1 and 0.52 ± 0.03 for functional, and 2.3 ± 0.7 and 0.29 ± 0.03 for silent synapses, respectively (P<0.001 in both cases; n=36 and n=26, respectively). The CV_{NF} of all 100 trials was significantly higher for silent than functional synapses, consistent with the higher percentage of failures of the former. In contrast, the CV_{NF} of successful responses was higher in functional than silent synapses, suggesting lower amplitude variability of the quantal responses in silent synapses.

Taken together, these results point to the existence of different presynaptic transmitter release properties in these two types of synapse and transform the notion that silent synapses are exclusively presynaptic or postsynaptic.

Functional synapses exhibit PPF; in contrast silent synapses lack PPF. To verify these differences, we analyzed the PPF, a presynaptic form of short-term plasticity present in our sample of functional synapses. With conventional stimulation, PPF is characterized by an increased second “compound” EPSC when it is elicited shortly after an initial EPSC (Creager et al. 1980; de Sevilla et al. 2002). With “minimal stimulation”, PPF may not be expressed in response to single paired-pulses due to the probabilistic nature of release, however it can be observed in averages when the percentage of failures of the second response R2 is lower than the first R1 (Creager et al. 1980; Dobrunz and Stevens 1997; Hanse and Gustafsson 2001a; 2001b; 2001c; de Sevilla et al. 2002; Zucker and Regehr 2002).
In functional synapses, the averages of EPSCs evoked by stimulation with pulse pairs (50 ms delay), including the failures, showed higher mean peak amplitudes of R2 than R1, both at +60mV (R2 = 15.9 ± 1.4 pA and R1 = 11.1 ± 1.0 pA; P<0.001; n=36) and at -60mV (R2 = -11.1 ± 0.9 pA and R1 = -6.8 ± 0.5 pA; P<0.001; n=36). This was consistent with a PPF being present (Fig. 2A) and implied that the synaptic efficacy of R2 was higher than that of R1 (Fig. 2B). We calculated a PPF index ((R2-R1)/R1) (de Sevilla et al. 2002; Martín and Buño 2003), which was 0.5 ± 0.1 at +60 mV and 0.7 ± 0.1 at -60 mV (P>0.05; n=36), indicating that there was no significant difference at either membrane potential (Fig. 2C).

During PPF, the percentage of failures of R1 was higher than at R2. At +60 mV, the percentage of failures of R1 was 58.3 ± 2.7 % and for R2 it was 48.1 ± 2.8 % (P<0.001; n=36), while at -60 mV, R1 failed 61.5 ± 2.1 % and R2 45.2 ± 3.2 % (P<0.001; n=36). Hence, the percentage of failures of R1 and R2 were essentially identical at -60 mV and +60 mV (P>0.05; n=36; Fig. 2E), supporting the assumption that the differences in the percentage of failures between R1 and R2 resulted from a presynaptic influence and minimizing the possibility that we were stimulating more than one type of synapse. In addition, the 1/CV² calculated for all 100 stimulations was higher for R2 than R1 (10.3 ± 1.6 and 1.5 ± 0.1, respectively, P<0.001; n= 36), consistent with a presynaptic mediated effect.

On the average in functional synapses the synaptic potency of R2 was higher than that of R1 at both -60 and +60 mV during PPF (Figs. 2D and F). At +60 mV the synaptic potency was 28.4 ± 1.5 pA for R1 and 32.7 ± 1.9 pA for R2 (P<0.001; n=36) and at -60 mV, it was -16.0 ± 0.9 pA for R1 and -19.5 ± 1.2 pA for R2 (P<0.001; n=36). We also calculated a synaptic potency index ((P2-P1)/P1) where P1 and P2 are the potency of the first and second EPSC, respectively. A potency index of zero indicates that on the average the peak amplitude of the R2 should be identical to that of the R1 EPSC, a result consistent with the same number of quanta being released by the first and
second action potentials. However, the potency index was > zero for the greater part of the functional synapses considered (P<0.001; n=26), a result probably caused by an increased release probability of R2 relative to R1 combined with a greater number of synaptic contacts per afferent (Hsia et al., 1998; reviewed in Dumas 2005), that was either caused by activation of a single terminal with multiple release sites, or by the recruitment of several terminals. In this scenario, the number of quanta released by a single action potential reaching the terminal will be given by the number of release sites formed by the SC afferent with the postsynaptic CA1 neuron, which is invariant in our conditions, and the release probability at each release site which will vary during paired pulse stimulation. In addition, there was a group of functional synapses (n=10) in which the potency index was not statistically different from zero (cf., de Sevilla et al., 2002). In all of the functional synapses the potency index was practically identical at -60 mV and +60 mV (P>0.05; n=36) and the $1/CV^2$ calculated for successful responses was higher for R2 than R1 (8.5 ± 1.3 and 5.4 ± 0.7, respectively; P>0.05; same synapses), supporting the assumption that the differences in synaptic potency of R1 and R2 most likely resulted from a presynaptic change. In these studies, the voltage insensitivity of the above described behaviors minimizes the possible contribution of recruitment of silent synapses and of “spillover” (Choi et al. 2000; Maggi et al. 2003).

To test that the higher amplitude of R2 was not due to a postsynaptic effect, we calculated the synaptic potency of R2 EPSCs both when the R1 response failed and succeeded. When R1 fails, the action potential that reaches the terminal is unable to elicit release, and there is no possible postsynaptic action of the first stimulation that could modify the R2 EPSC. We found that the synaptic potency of R2 was similar when calculated for responses with or lacking an R1 EPSC (P>0.05; n=36) (Figs. 3A-4B2). The cumulative distributions of the R2 EPSC peak amplitudes were also practically identical in the presence or absence of R1 EPSCs (P>0.05, n=36; Figs. 3B1 and B2).
Therefore, in our sample these results imply that the increased synaptic potency of R2 associated with PPF depends on presynaptic release mechanisms and minimizes a possible postsynaptic contribution.

The EPSC rise time and the time to peak were similar (P>0.05; n=36) at both membrane potentials (-60 mV and +60 mV) for these functional synapses. In addition, the waveform of the average of R1 and R2 EPSCs (Fig. 3C) were also similar, a result that reduces the possible stimulation of several presynaptic fibers that would result in a slower rise time and a wider duration of R2 EPSCs due to the unsynchronized release caused by the simultaneous activation of additional fibers with dissimilar conduction velocities. The same was true for EPSCs evoked by single pulses where rise times did not correlate with EPSC amplitude (P>0.05).

Superfusion with CCh (5 µM) increased the PPF, increased the percentage of failures and reduced the synaptic potency both of R1 and R2 in this type of functional synapse (the synaptic potency of R1 decreased 21.7 ± 2.7 % and that of R2 31.7 ± 3.5 %; (P<0.01; n=7; data not shown). This result is consistent with a reduction of the probability of release in synapses that have several contacts per afferent.

In the same type of analysis on silent synapses, the mean peak amplitudes of R1 and R2 EPSCs were similar (R2 = 7.1 ± 0.8 pA and R1 = 7.0 ± 0.8 pA; P>0.05; n=26; Fig. 4A), implying that there was no PPF (the mean PPF index was 0.01 ± 0.04; Figs. 4B and C). In addition, we found no difference in the percentage of failures of R1 (73.5 ± 1.8 %) and R2 (72.5 ± 2.2 %; P>0.05; n=26; Fig. 4E), and the synaptic potency of R1 (22.6 ± 1.2 pA) and R2 (24.2 ± 1.3 pA; P>0.05; n=26) were also similar (Figs. 4D and F). Moreover, the 1/CV^2 calculated for all 100 stimulations was essentially identical for R2 and R1 (0.63 ± 0.1 and 0.74 ± 0.08, respectively), and the same was true for 1/CV^2 calculated for successful responses that was 26.1 ± 7.0 for R1 and 40.8 ± 15.3 for R2, respectively (P>0.05; same cells).
Therefore, consistent with the essentially identical percentage of failures, amplitude and \(1/CV^2\) of R1 and R2, PPF was absent. In addition, both the PPF and the potency indexes were practically zero for all silent synapses (P > 0.05; n = 26). We also analyzed the effects of paired-pulses at different intervals between 20 and 200 ms, and PPF was absent in all the silent synapses analyzed (n = 4; data not shown). Moreover, as described previously (de Sevilla et al. 2002), CCh did not affect silent synapses (P > 0.05; n = 7, data not shown).

Taken together, these results suggest that at functional synapses the PPF arises from the combined effects of a lower percentage of failures and an increased release of transmitter at R2 in comparison with R1 (Debanne et al. 1996). The decreased percentage of failures of R2 with respect to R1 is thought to result from a Ca\(^{2+}\) signal that remains in the terminal after the first action potential and accumulates with the Ca\(^{2+}\) inflow during the second action potential (Katz and Miledi 1968; Kamiya and Zucker 1994; Dobrunz and Stevens 1997; Zucker and Regehr 2002). The increased release probability of R2 as compared with R1 combined with more than one synaptic contact per afferent could explain the increased release at R2 relative to R1 (see above, Debanne et al. 1996). In contrast, the percentage of failures and the synaptic potency of R1 and R2 were similar and PPF was absent in all silent synapses, suggesting both that the activity-dependent presynaptic control during PPF is not engaged in silent synapses.

It is noteworthy that in a group of functional synapses that showed PPF it was not paralleled by an increased synaptic potency of R2 relative to R1. In those functional synapses PPF was exclusively caused by a decreased proportion of failures of R2 as compared with R1 (n = 10). The synaptic potency was also unchanged by other manipulations that modified release probability in those functional synapses (de Sevilla et al. 2002). Therefore, these functional synapses probably have a single synaptic contact and release site per afferent. Consequently, functional synapses are not homogeneous in their release properties. Although the differences in release properties between functional synapses may be of physiological relevance, their investigation would require further
analysis that should be treated independently and is out of the scope of the present work that centers on the global differences between the properties of functional and silent synapses.

The differences in release properties between silent and functional synapses suggest that the amount of transmitter released by an action potential reaching the terminal is a more finely regulated process in functional than in silent synapses. Therefore, a key conclusion from the above results is that presynaptic terminals of both types of synapse are functionally different.

Transmitter release is regulated by presynaptic ER Ca\(^{2+}\) stores in functional but not in silent synapses. The contribution of ER Ca\(^{2+}\) stores and Ca\(^{2+}\)-induced Ca\(^{2+}\)-release (CICR) to neurotransmitter release in central synapses is a matter of debate (see Discussion). In hippocampal synapses CICR may contribute to PPF by amplifying the Ca\(^{2+}\) signal induced by the arrival of the second action potential (Emptage et al. 2001), thus the differences in PPF of silent and functional synapses could reside in the properties of CICR at the corresponding terminals or in the control of the intraterminal Ca\(^{2+}\) concentration by the regulation of Ca\(^{2+}\) sequestration and release from the ER (Galante and Marty 2003). Therefore, we tested the effects interfering with CICR and the regulation of ER Ca\(^{2+}\) stores.

Bath applied ryanodine (100 \(\mu\)M), that blocks ryanodine receptors, increased the percentage of failures and reduced the synaptic potency and PPF in functional synapses in a gradual manner (Figs. 5A-5D). The effect of ryanodine reached a maximum after \(\approx 25-30\) min and was identical at -60 and +60 mV (P>0.05; only the data at +60 mV will be shown for simplicity). The percentage of failures increased for R1 32% (from 55.4 \(\pm\) 6.9 to 73.2 \(\pm\) 4.7; P<0.05; n=6) and for R2 56% (from 45.2 \(\pm\) 8.5 to 70.6 \(\pm\) 5.4; P<0.05; n=6), while the synaptic potency decreased 21% for R1 (from 35.8 \(\pm\) 2.2 to 28.1 \(\pm\) 1.1; P<0.01; n=6) and 24% for R2 (from 41.4 \(\pm\) 2.4 to 31.2 \(\pm\) 0.6; P<0.01; n=6).

Ryanodine also reduced the PPF index from 0.44 \(\pm\) 0.1 to 0.16 \(\pm\) 0.05 (P<0.05, same cells). In this case, a reduction of the release probability caused a decreased PPF, thus contradicting the usually accepted inverse relationship between release probability and PPF (Dobrunz and Stevens...
1997; Dobrunz 2002; but see Hanse and Gustafsson 2001). However, a similar unexpected effect of ryanodine on PPF has been interpreted to indicate that CICR amplifies the Ca\textsuperscript{2+} signal induced by the arrival of the first action potential at the presynaptic terminals, which adds with the Ca\textsuperscript{2+} inflow activated by the second action potential (Emptage et al. 2001). The effects of ryanodine were identical at -60 and +60 mV, suggesting a presynaptic site of action and minimizing postsynaptically mediated modifications. A lower concentration of ryanodine (50 µM) showed a tendency to similar effects but changes were not statistically significant (data not shown; n=6). The effects of ryanodine on functional synapses was reduced (∼20%; n=6) following prolonged ∼50 min washout.

To verify that the regulation of ER Ca\textsuperscript{2+} stores or CICR were involved in transmitter release by functional synapses, we applied thapsigargin to deplete calcium stores by inhibiting Ca\textsuperscript{2+} uptake by the Ca\textsuperscript{2+}-ATPase. Initially, superfusion with thapsigargin (1 µM, ≈ 15 min of superfusion) decreased the percentage of failures and increased the synaptic potency and PPF, consistent with the initial transient increase of intraterminal Ca\textsuperscript{2+} due to depletion of the presynaptic ER stores (Clementi et al. 1992). However, after this initial period thapsigargin gradually increased the percentage of failures and reduced the synaptic potency and PPF. For R1 the percentage of failures increased 29% (from 68.1 ± 1.4 to 87.9 ± 1.7) and the synaptic potency decreased 35% (from 24.6 ± 1.8 to 16.01 ± 1.2), whereas for R2, the percentage of failures increased 28% (from 60.3 ± 2.1 to 84.8 ± 2.6), the synaptic potency decreased by 47% (from 33.1 ± 1.8 to 17.5 ± 1.6) and the PPF index was reduced by 134% (from 1.24 ± 0.5 to -0.42 ± 0.6), respectively (in all cases P<0.05; n=3). These effects of thapsigargin reached a maximum after ≈ 30-40 min and were identical at -60 and +60 mV, suggesting a presynaptic effect. There was no recovery from effects of thapsigargin following a prolonged washout >60 min.

Bath applied caffeine (10 mM), that increases the Ca\textsuperscript{2+}-sensitivity of ryanodine receptors, thus amplifying CICR, decreased the percentage of failures, increased the synaptic potency, and reduced
PPF in functional synapses (Figs. 5A-5D). The percentage of failures and the synaptic potency of R1 changed more than those of R2; for R1 the percentage of failures decreased 84%, (from 65.8 ± 8.2 to 9.6 ± 4.9; P<0.001; n=8), and for R2 45% (from 52.4 ± 8.6 to 20.6 ± 8.1; P<0.05; n=8). The synaptic potency of R1 EPSCs increased 74% (from 29.6 ± 3.0 to 49.2 ± 6.0; P<0.05; n=8), and the potency of R2 32%, (from 33.6 ± 4.5 to 44.7 ± 6.2; P<0.05; n=8). The PPF index was also reduced from 0.45 ± 0.2 to -0.19 ± 0.1 (P<0.01, same cells). The effects of caffeine were insensitive to a prolonged washout (>60 min), an effect that can be attributed to the caffeine–induced persistent potentiation of glutamate release in SC synapses (Martín and Buño 2003).

In contrast, ryanodine did not significantly modify the percentage of failures of either R1 or of R2 EPSCs in silent synapses (from 76.4 ± 1.8 to 80.0 ± 2.1; P>0.05; n=8 for R1, and from 75.6 ± 3.0 to 81.3 ± 2.5; P>0.05; n=8 for R2; Fig. 5G). The synaptic potency was also unaffected by ryanodine (from 21.1 ± 1.7 to 18.5 ± 1.4, for R1; and from 23.1 ± 2.1 to 18.7 ± 1.5, for R2; P>0.05; n=8 in both cases, Fig. 5F). In addition, silent synapses were also insensitive to caffeine since neither the percentage of failures of R1 or R2 EPSCs changed (from 70.3 ± 4.9 to 64.0 ± 8.7, for R1; and from 69.6 ± 3.4 to 68.3 ± 7.4, for R2; P>0.05; n=7 in both cases), and exposure to caffeine did not modify the synaptic potency of silent synapses (from 24.8 ± 3.1 to 27.2 ± 3.2, for R1; and from 27.6 ± 3.4 to 26.8 ± 2.9, for R2; P>0.05; n=7 in both cases; Fig. 5F). Moreover, neither ryanodine nor caffeine modified the PPF index of silent synapses (P>0.05; n=8 and 7 respectively; Fig. 5H).
DISCUSSION

Release properties are different in functional and silent synapses. We show that presynaptic terminals of functional and silent SC-CA1 pyramidal neuron synapses express different transmitter release properties that are regulated by distinct presynaptic mechanisms. Firstly, the percentage of failures is higher and the synaptic potency is lower in silent than functional synapses. Secondly, PPF is detected in functional synapses but is absent in silent synapses. Thirdly, release is regulated by presynaptic ER Ca\(^{2+}\) stores in functional synapses, regulatory mechanisms that are not active in silent synapses. Finally, manipulations that modify release probability; such as PPF and caffeine (present results) or increasing the extracellular Ca\(^{2+}\)/Mg\(^{2+}\) ratio or CCh (de Sevilla et al. 2002), do not convert one type of synapse into the other, suggesting that the type of synapse depends primarily on the nature of the postsynaptic glutamatergic receptors expressed and on the voltage-dependent Mg\(^{2+}\) block of NMDA channels (Isaac et al. 1995; Liao et al. 1995).

It should be emphasized that artifacts caused by the possible simultaneous stimulation of more than one SC fibre or of failures in the activation of SC fibres, are unlikely because we have performed several tests and controls to minimize these possibilities.

Schaffer collateral synapses are thought to have a single release site in neonate animals (Bolshakov and Siegelbaum 1995; Dobrunz and Stevens 1997; Hanse and Gustafsson 2001a; 2001c; 2002) but may show a greater number of synaptic contacts and release sites per afferent, later (> 1 week) during development (Kullmann and Nicoll 1992; Larkman et al. 1992; Sorra and Harris 1993; Liao et al. 1995; Hsia et al. 1998) and as a result of presynaptic changes with LTP (Malinow and Tsien 1990; Malinow 1991; Bolshakov et al. 1997; Voronin et al. 1999). Moreover, in neonatal rats (<1 week) the AMPA/NMDA ratio is low (≈ 0.2), suggesting that most synapses are silent (Hsia et al. 1998). Those synapses lack PPF, a result that agrees with our present findings. However, the reason underlying this neonatal behavior is uncertain because the absence of PPF has either been related with a high release probability in these synapses (Bolshakov and Siegelbaum
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which was not the case in our experiments, or to other presynaptic mechanisms (Hsia et al. 1998). The discrepancy between our results and those of Bolshakov and Siegelbaum (1995) could indicate that silent synapses undergo changes related to maturation in our older age sample, as occurs in thalamocortical synapses (Yanagisawa et al. 2004) and in hippocampal synapses around the second and third postnatal week (reviewed in Dumas 2005).

It has been shown that SC buttons may contain one or several release sites or that single SC axons may contact CA1 pyramidal neurons with more than one synapse at the post-natal age of our sample (Harris and Stevens 1989; Bolshakov and Seigelbaum 1995; Oertner et al. 2002; Conti and Lisman 2003; reviewed in Dumas 2005). Consequently, two different groups of functional synapses with different presynaptic but similar postsynaptic properties coexist in our sample. Although the functional meaning of both types of functional synapses remains to be determined, it is conceivable that the different release properties are in an intermediate step in the process of conversion to the late phase of LTP (Bolshakov et al. 1997; Palmer et al. 2004) or of maturation, when a greater number of synaptic contacts per afferent may be present (Hsia et al., 1998; reviewed in Dumas 2005), either because SC buttons may express multiple release sites or a single SC axon may establish several synapses with a CA1 pyramidal neuron. The intermediate step may be related to the different discrete synaptic states that have been proposed to exist (Montgomery and Madison 2004).

Fluctuations in synaptic potency in SC synapses during PPF, have also been found using Ca^{2+} imaging techniques that guarantee recordings from a single spine (Oertner et al. 2002; Reid et al. 2004) and with paired-recordings of monosynaptically connected CA3-CA1 pyramidal neurons that ensure the activation of a single axon (Malinow and Tsien, 1990; Foster and McNaughton 1991; Malinow 1991; Debanne et al. 1996; Chen et al. 2004).

The differences in synaptic potency of the NMDA component between silent and functional synapses deserve attention, and could reflect: (i) presynaptic differences in the number of release
sites in individual buttons or of the number of synapses made by single SC fibres (Kullmann and Nicoll 1992; Larkman et al. 1992; Sorra and Harris 1993; Liao et al. 1995; Hsia et al. 1998); (ii) distinct presynaptic Ca$^{2+}$ channel types (Iwasaki et al. 2000; Scheuber et al. 2004); (iii) different intraterminal second messenger cascades; (iv) a postsynaptic divergence linked to changes in the number of NMDA receptors present in the corresponding spine; (v) to differences in the type of NMDA receptor expressed in both synapse types (Kirson et al. 1999; Yanagisawa et al. 2002; reviewed in Dumas 2005).

In addition, we show that the synaptic potency of R2 EPSCs was similar when calculated for responses with or lacking an R1 EPSC and that the cumulative distributions of the R2 EPSC peak amplitudes were also practically identical in the presence or absence of R1 EPSCs. We interpreted this result to indicate the absence of a postsynaptic mediated effect in the regulation of synaptic potency during PPF. This result contradicts reports which show that when there is a R1 EPSC the reduction of the ready releasable pool (RRP) of vesicles leaves less releasable vesicles when the second action potential reaches the terminal thus reducing the probability of release during R2 (Stevens and Wang 1994; Debanne et al. 1996; Dobrunz and Stevens 1997). This discrepancy may indicate that release does not critically affect the size of the RRP in our experimental conditions. A possibility that may explain the disagreement is that activation of a single SC afferent may recruit several release sites with low release probability at each site that would not appreciably deplete the RRP at each site. Indeed, the relationship between release and the RRP is a matter of debate and both a decrease and no change of R2 as a result of R1 has been reported (Stevens and Wang. 1994; Debanne et al. 1996; Dobrunz and Stevens 1997; Hanse and Gustafson 2001a-c; 2002). Although saturation of postsynaptic receptors could also explain the argument, saturation is unlikely in our sample because the synaptic potency increased during the PPF and the increase was similar for AMPA and NMDA EPSC components which have very different sensitivity to released glutamate.
It is significant that other presynaptic mechanisms may control synaptic potency. Indeed, such changes may involve either a regulation in the “size” of the vesicles (i.e., the amount of transmitter each vesicle holds) (Atwood and Wojtowicz 1999) or the amount of transmitter released by a single vesicle may be controlled in an activity-dependent manner by the mechanism known as “kiss-and-run” (Stevens and Williams 2000; Aravanis et al. 2003; Becherer et al. 2003; Gandhi and Stevens 2003; Chen et al. 2004). However, present results do not enable discrimination among the possible mechanisms. In addition, to what extent a single release site releases at most one or several vesicles at a time is a matter of debate (Auger and Marty 2000; Wadiche and Jahr 2001).

**Presynaptic ER Ca\(^{2+}\) stores regulate release in functional but not in silent synapses.** The contribution of ER Ca\(^{2+}\) stores and Ca\(^{2+}\)-induced Ca\(^{2+}\)-release (CICR) to neurotransmitter release in central synapses is a matter of debate, and positive (Llano et al. 2000; Emptage et al. 2001; Galante and Marty 2003; Martín and Buño 2003) and negative (Carter et al. 2002) results have been reported. In addition, contradictory results on the contribution of CICR in transmitter release have also been reported for SC-CA1 pyramidal neuron contacts where either no effect of ryanodine was observed at low concentrations or –as reported here- a decrease of the EPSC was recorded with high concentrations (100 µM) of ryanodine (Carter et al. 2002). Consistent with our results, the inhibitory effect of ryanodine (and thapsigargin) on the EPSC had been reported previously in inhibitory cerebellar synapses and interpreted as caused by a decreased Ca\(^{2+}\) sensitivity of the mechanism of vesicle exocytosis possibly produced by a local reduction of the resting Ca\(^{2+}\) concentration (Galante and Marty 2003).

The data available in the literature was obtained with “conventional” stimulation or by analyzing mEPSCs and mEPSPs and with the postsynaptic neuron held at the resting membrane potential. In that situation only non-NMDA EPSC components of functional synapses are recorded and silent synapses are not because of the voltage-dependent Mg\(^{2+}\) block of NMDA receptors. However, to our knowledge no attempt has been made to separately analyze CICR in functional and
silent SC synapses which may show differences in the regulation of ER Ca\(^{2+}\) stores. In addition, the differences in the effects of ryanodine and the negative results should be taken with care because the metabolic state of the neuron may have profound consequences on the function of intracellular Ca\(^{2+}\) stores (reviewed in Collin et al. 2005).

Ryanodine (50-100 µM) blocks ryanodine receptors and inhibits CICR. In contrast, caffeine at mM concentrations increases the Ca\(^{2+}\)-sensitivity of ryanodine receptors, thus amplifying CICR (Llano et al. 2000; Galante and Marty 2003; Martín and Buño 2003). Caffeine also increases Ca\(^{2+}\) influx through presynaptic N-type channels by inhibiting presynaptic adenosine receptors, thus relieving the adenosine-mediated block of N-type channels (Qian and Saggau 1997; Martín and Buño 2003). Therefore, caffeine effects are complicated because at mM concentrations it increases the intraterminal Ca\(^{2+}\) both by raising the influx of Ca\(^{2+}\) followed by an increase in CICR. However, we had previously shown that SC NMDA and non-NMDA EPSC components were blocked to a similar degree by adenosine (de Sevilla et al. 2002), reducing the possibility of a contribution of differences in the regulation of Ca\(^{2+}\) influx by a divergence in the caffeine sensitivity of presynaptic adenosine receptors. Therefore, the dissimilar effects of caffeine on silent and functional synapses described in the results are most likely due to a specific effect on the ER Ca\(^{2+}\) stores and the CICR mechanism.

Others have shown that blockade of CICR can effectively reduce the Ca\(^{2+}\) signal in the synaptic terminals of SC synapses evoked by the arrival of both action potentials during paired-pulse stimulation (Emptage et al. 2001). These same authors demonstrated that although ryanodine decreased intraterminal Ca\(^{2+}\) on the first stimulus it did not affect transmitter release, presumably because CICR did not occur fast enough. However, upon arrival of the second stimulus ryanodine both reduced Ca\(^{2+}\) signal and decreased release. In view of these results the authors argue that in normal conditions the Ca\(^{2+}\) signal from the CICR caused by the first stimulus persists long enough to increase PPF by adding with the Ca\(^{2+}\) influx caused by the second action potential or by
sensitizing the release apparatus to a subsequent stimulus. However, the effects of ryanodine and thapsigargin reported here may not only reflect the consequences of blocking CICR. Rather, the drugs could empty the Ca\(^{2+}\) stores in the ER, ultimately leading to a reduction in presynaptic cytoplasmic Ca\(^{2+}\), which would explain the effect on release by single action potentials (Galante and Marty 2003). This could also explain the reduced facilitation, although a block of CICR may also be involved. Likewise, the effects of 10 mM caffeine on release by one spike are almost certainly due to an increase in resting presynaptic Ca\(^{2+}\), which would be expected if ER Ca\(^{2+}\) stores are present. If release to the first spike is saturated, this would reduce facilitation.

We show that both ryanodine and thapsigargin increased the percentage of failures and decreased both the synaptic potency and PPF in functional synapses. In addition, we show that caffeine decreased the percentage of failures, increased the synaptic potency, and reduced PPF in this group of functional synapses. In contrast, the drugs are inactive in silent synapses.

We should like to emphasize that whatever the mechanism of action of ryanodine, thapsigargin and caffeine on these functional synapses, the key point is that those effects were absent in silent synapses, suggesting that regulation of presynaptic Ca\(^{2+}\) by ER stores plays a role in controlling release in functional but not in silent synapses.

Different presynaptic Ca\(^{2+}\) channel types or dissimilarities in the density of presynaptic Ca\(^{2+}\) channels (Wu and Saggau 1994; Iwasaki et al. 2000; Scheuber et al. 2004) may contribute to the mechanisms that differentiate both kinds of synapse, without ruling out the contribution of CICR. However, the analysis of those differences was out of the scope of the present work and will be carried out in the future.

**Final considerations.** The concept that silent synapses lack AMPA receptors is actively debated and alternative mechanisms have been proposed to explain silent synapses. It has been argued that silent synapses may contain both AMPA and NMDA receptors but do not conduct, either: (i) due to having a release probability close to zero (“mute synapses”; Hanse and Gustafsson
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2001a; reviewed in Voronin and Cherubini 2004); or (ii) because the concentration of glutamate in the cleft activates their NMDA receptors yet is insufficient to stimulate AMPA receptors (Choi et al. 2000); or (iii) of “spillover”, where glutamate diffusing out of the synaptic cleft into neighboring synapses is sufficient to activate the postsynaptic high affinity NMDA receptors, but not the less sensitive AMPA receptors (Kullmann and Asztely 1998). Although our results are consistent with the classical scenario where silent synapses lack AMPA receptors, and hence are “conditionally silent” or “deaf”, they also suggest presynaptic differences in both synapses types, and hold up the notion that these silent synapses are both pre- and postsynaptically different from functional ones.

It is likely that the mechanisms of synaptic plasticity are not exclusively pre- or postsynaptic, but rather involve a balance and integration of reciprocal influences on the development and maturation of synaptic machinery on both sides of the synaptic cleft. Therefore, a possible functional consequence of our results is that the conversion of silent to functional synapses with maturation and LTP must be associated with modifications of the presynaptic terminals as determined both with electrophysiological and imaging techniques (Malinow 1991; Bolshakov et al. 1997; Voronin et al. 1999; Palmer et al. 2004; Stanton et al. 2005; reviewed in Dumas 2005). The link connecting pre- and postsynaptic properties may need an unknown signal that modifies the terminal and that could either arise in the pre- or postsynaptic neuron or in a third element that could be the surrounding astrocytes.
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FIGURE LEGENDS

Figure 1. Functional synapses show higher release probability and synaptic potency than Silent synapses. A: left records, superimposed EPSCs and failures (10 sweeps) evoked by “minimal” stimulation at +60 and -60 mV in a representative Functional synapse. A: right records, representative example of averaged EPSCs (100 successive trials excluding failures) recorded at +60 and -60 mV in a functional synapse. The onset slopes were fit with single exponentials (superimposed) and the corresponding τ are shown. B: as in A, but Silent synapse. C: summary of average percentage of failures (%) at +60 mV in Functional (n=36) and Silent synapses (n=26), respectively. D: summary of Synaptic Potency (average peak amplitude of successful responses in pA) at +60 mV in Functional (n=36) and Silent synapses (n=26).

Figure 2. PPF in functional synapses results from the combination of a higher release probability and the higher synaptic potency of the second response (R2). A: averaged EPSCs (100 successive trials, including failures) evoked by paired-pulse minimal stimulation (50 ms) at -60 and +60 mV showing first (R1) and R2 responses in a representative functional synapse. B: summary of Synaptic Efficacy (mean EPSC peak amplitudes including failures) of R1 and R2 at +60 and -60 mV. C: summary of the PPF index ((R2-R1)/R1) at both potentials. D: superimposed EPSCs and failures (10 sweeps) evoked by paired-pulse minimal stimulation at +60 and -60 mV in a representative functional synapse. E: summary of average percentage of failures (%) of R1 and R2 at +60 and -60 mV. F: summary of average Synaptic Potency (pA) of R1 and R2 at +60 and -60 mV. In all cases where summary data is shown n=36.

Figure 3. In functional synapses the synaptic potency of the R2 EPSC is similar whether the R1 EPSC fails or succeeds. A: superimposed representative EPSC averages obtained both at -60 and +60 mV when the R2 EPSC was evoked in the absence of the R1 EPSC (black traces; n=32 responses), and when the R2 EPSC was evoked in the presence of the R1 EPSC (grey traces; n=26 responses). B: cumulative probability plot of the R2 EPSC peak amplitude (Synaptic Potency)
when R1 failed (●) and succeeded (○) at +60 mV. B2: same as in B1 but at -60 mV. C: left, representative example showing averaged R1 (black traces) and R2 EPSCs (gray traces; excluding failures) at -60 and +60 mV during PPF. C: right, the EPSC were scaled to the amplitude of R1 and superimposed to show the similarity of their waveforms.

Figure 4. PPF is absent in silent synapses. A: average EPSCs (100 successive responses, including failures) evoked by paired-pulse stimulation at -60 and +60 mV in a representative silent synapse. B: summary of Synaptic Efficacy of R1 and R2 EPSC at +60 mV. C: summary of PPF index ((R2-R1)/R1) at +60 mV. D: superimposed EPSCs and failures (10 sweeps) evoked by paired-pulse minimal stimulation at -60 and +60 mV in a representative silent synapse. E: summary of average percentage of failures (%) of R1 and R2 at +60 mV. F: summary of Synaptic Potency (pA) of R1 and R2 at +60 mV. In all cases where summary data is shown n=26.

Figure 5. Modifications in CICR affect functional but not silent synapses. A: representative EPSC averages (100 sweeps including failures) of R1 and R2 at +60 mV and -60 mV in control conditions (black trace), and in the presence of caffeine (red trace), or ryanodine (blue trace), in representative functional synapses. B: cumulative probability plot of R1 (●) and R2 (▼) EPSC peak amplitudes at +60 mV, under control conditions (black), in the presence of caffeine (red), or ryanodine (blue) in functional synapses. (n=6, with ryanodine, and n=8 with caffeine). C: summary of average percentage of failures (%) of R1 and R2 at +60 mV under control conditions and in the presence of caffeine or ryanodine in functional synapses. (n=8 and n=6, respectively). D: summary of PPF index at +60 mV in control conditions, in the presence of caffeine or ryanodine in functional synapses. (n=8 and n=6, respectively). E: representative EPSC averages (100 sweeps), including failures of R1 and R2 at +60 mV and -60 mV under control conditions (black trace), in the presence of caffeine (red trace) or ryanodine (blue trace) in a representative silent synapse. F: same as in B, but in silent synapses (n=8, with ryanodine, and n=7 with caffeine). G: summary of average percentage of failures (%) of R1 and R2 at +60 mV under control conditions and in the presence of
caffeine or ryanodine (n=7 and n=8, respectively). *H*: summary of PPF index at +60 mV in control conditions, and in the presence of caffeine or ryanodine in silent synapses. (n=7 and n=8, respectively).
Figure 1

Figure 2
Release properties at glutamatergic synapses

Figure 3

Figure 4
Release properties at glutamatergic synapses

**A** Functional Synapses

![Graph A](image)

**B** Cumulative Probability

![Graph B](image)

**C** Failures (%)

![Graph C](image)

**D** PPF Index

![Graph D](image)

**E** Silent Synapses

![Graph E](image)

**F** Cumulative Probability

![Graph F](image)

**G** Failures (%)

![Graph G](image)

**H** PPF Index

![Graph H](image)

Figure 5