Developmental presence and disappearance of postsynaptically silent synapses on dendritic spines of rat layer 2/3 pyramidal neurons

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Silent synapses are synapses whose activation evokes NMDA-type glutamate receptor (NMDAR) but not AMPA-type glutamate receptor (AMPAR) mediated currents. Silent synapses are prominent early in postnatal development and are thought to play a role in the activity- and sensory-dependent refinement of neuronal circuits. The mechanisms that account for their silent nature have been controversial, and both presynaptic and postsynaptic mechanisms have been proposed. Here, we use two-photon laser uncaging of glutamate to directly activate glutamate receptors and measure AMPAR- and NMDAR-dependent currents on individual dendritic spines of rat somatosensory cortical layer 2/3 pyramidal neurons. We find that dendritic spines lacking functional surface AMPARs are commonly found before postnatal day 12 (P12) but are absent in older animals. Furthermore, AMPAR-lacking spines are contacted by release-competent presynaptic terminals. After P12, the AMPAR/NMDAR current ratio at individual spines continues to increase, consistent with continued addition of AMPARs to postsynaptic terminals. Our results confirm the existence of postsynaptically silent synapses and demonstrate that the morphology of the spine is not strongly predictive of its AMPAR content.

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In early postnatal life, many brain regions contain silent synapses whose activation does not result in a postsynaptic current when the neuron is at its resting potential (Isaac et al., 1995, 1997; Liao et al., 1995; Durand et al., 1996). Silent synapses can be converted into fully functional synapses by appropriate stimulation (Voronin & Cherubini, 2004), a process that may play a role in sensory-dependent circuit refinement and synaptic plasticity (Feldman & Brecht, 2005).

Silent synapses have been proposed to be synapses that express NMDARs but not AMPARs and thus, due to the Mg2+ block of NMDARs, do not generate appreciable synaptic currents at resting potentials. This hypothesis is supported by experiments using minimal stimulation (Isaac et al., 1995, 1997; Liao et al., 1995; Durand et al., 1996) and paired recordings (Montgomery et al., 2001) that describe synapses with only NMDAR-mediated responses that are revealed at depolarized potentials. Additional support comes from immunolabelling and ultrastructural studies that describe synapses devoid of AMPARs (Nusser et al., 1998; Liao et al., 1999; Petralia et al., 1999; Pickard et al., 2000; Washbourne et al., 2002). Furthermore, in visual cortex slices from newborn rats, NMDAR-mediated spontaneous miniature EPSCs (mEPSCs) occur at higher frequency than AMPAR-mediated mEPSCs (Rumpel et al., 2004). Moreover, in hippocampal organotypic slice cultures, spines have been identified that display NMDAR-mediated Ca2+ transients without an associated synaptic potential (Ward et al., 2006).

An alternative hypothesis proposes that each postsynaptic terminal expresses both AMPARs and NMDARs and that silent synapses arise as a result of low glutamate concentration in the synaptic cleft that is only sufficient to activate NMDARs, which have higher glutamate affinity than AMPARs (Patneau & Mayer, 1990). Low glutamate concentration could result from impaired opening of vesicles into the synaptic cleft (Choi et al., 2000; Gasparini et al., 2000; Renger et al., 2001; Mozhayeva et al., 2002) or because glutamate diffuses into the cleft from a distant source (Kullmann et al., 1996; Gasparini et al., 2000). This hypothesis is supported by the findings that, in dissociated neurons, both AMPARs and NMDARs are found at newly formed synapses (Cottrell et al., 2000; Friedman et al. 2000).

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2000) and that, in hippocampal slices from newborn rats, AMPAR- and NMDAR-mediated spontaneous miniature EPSCs occur at similar frequencies (Groc et al. 2002).

Layer 2/3 pyramidal neurons in somatosensory cortex receive glutamatergic inputs from layer 4 neurons onto their basal dendrites (Lubke et al. 2000) and show synaptic refinement that is sensitive to whisker-based sensory exploration (Micheva & Beaulieu, 1996; Stern et al. 2001). In order to determine if postsynaptically silent synapses are formed onto these cells, we examined the AMPAR and NMDAR contents of basal dendritic spines in acute slices from P9–17 rats. Two-photon laser glutamate uncaging (2PLGU) was used to bypass the presynaptic terminal and directly activate glutamate receptors on each spine. We demonstrate the existence of AMPAR-lacking spines in animals younger than P12 that are morphologically indistinguishable from AMPAR-expressing ones. Moreover, AMPAR-lacking spines are associated with release-competent nerve terminals. Finally, the AMPAR content of individual spines continues to increase relative to the NMDAR content throughout the second and third postnatal week. Thus, our data directly demonstrate the existence of postsynaptically silent synapses formed onto basal dendrites of layer 2/3 neurons and indicate that the spine morphology is not strictly correlated with the maturational state of the associated synapse.

Methods

Slice preparation and electrophysiology

Animals were handled in accordance with Federal guidelines, and all protocols were approved by the animal welfare committee of Harvard Medical School. Sprague–Dawley rats were anaesthetized by inhalation of isoflurane and depth of anaesthesia was judged by lack of a righting reflex. Following decapitation, the cerebral hemispheres were quickly removed, placed in ice-cold dissection medium and reduced to blocks. The dissection medium consisted of (mM): 110 choline chloride, 25 NaHCO₃, 1.25 NaH₂PO₄, 2.5 KCl, 7 MgCl₂, 0.5 CaCl₂, 11.6 sodium ascorbate, 3.1 sodium pyruvate and 25 glucose, equilibrated with 95% O₂–5% CO₂. Coronal slices (300 μm thick) were cut from somatosensory cortex and collected in a holding chamber at 34°C for 30 min in artificial cerebrospinal fluid (ACSF) consisting of (mM): 127 NaCl, 25 NaHCO₃, 1.25 NaH₂PO₄, 2.5 KCl, 1 MgCl₂, 2 CaCl₂ and 25 glucose, equilibrated as above. Slices were subsequently transferred to a recording chamber perfused at 2 ml min⁻¹ with ACSF at 22–25°C. The following drugs were dissolved in the ACSF (μM): 20 bicuculline (Tocris) and 50 picrotoxin (Tocris) to block GABA/ receptors; 1 TTX to inhibit spontaneous activity; 100 LY341495 (Tocris) to block mGluRs; 10 serine to reduce NMDAR desensitization and fully occupy the glycine binding site; and 0.3 SNX-482 (Peptide Institute), 1 ω-conotoxin-MVIIC (Tocris), 10 mibebradil and 20 nimodipine (Tocris) to block R₅, N/P/Q-, T- and L-type Ca²⁺ channels, respectively. These antagonists, in conjunction with intracellular Cs⁺, improve voltage- and space-clamp and isolate synaptic signals mediated by AMPARs and NMDARs. To monitor uncaging-evoked excitatory postsynaptic currents (uEPSCs) mediated by AMPARs and NMDARs (uEPSCampar and uEPSCnmdar, respectively), 2.5 or 5.0 mM 4-methoxy-7-nitroindolinyl-caged l-glutamate (MNI-glutamate; Tocris) was dissolved in the ACSF. When appropriate, AMPARs or NMDARs were blocked with 10 μM NBQX or (±)-3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP), respectively.

Whole-cell voltage-clamp recordings (Multiclamp 700A, Axon Instruments) were obtained from layer 2/3 pyramidal neurons (soma to pial surface distance: ~300 μm; range: 162–511 μm) visualized with infrared differential interference contrast optics and recognized as pyramidal neurons by their morphology under two-photon laser scanning microscopy (2PLSM; see below). Cells were voltage clamped at −70 or +40 mV to monitor uEPSCampar or uEPSCnmdar, respectively. Recording electrodes (3–5 MΩ) were made with borosilicate glass (Warner Instruments) and filled with (mM): 135 caesium methanesulphonate, 10 Hepes, 10 sodium phosphocreatine, 4 MgCl₂, 4 Na-ATP, 0.4 Na-GTP, pH 7.4 with CsOH. The intracellular solution contained the red fluorophore Alexa Fluor-594 (20 μM, Molecular Probes) to visualize the cell morphology, and the green-fluorescing Ca²⁺ indicator Fluo-5F (300 or 500 μM, Molecular Probes) to detect intracellular Ca²⁺ transients. All drugs were obtained from Sigma except when otherwise indicated. Currents were filtered at 2 kHz and digitized at 10 kHz. Series resistance (< 20 MΩ in all analysed recordings) and whole-cell capacitance were not compensated.

Imaging and uncaging

Two-photon laser scanning microscopy (2PLSM) and 2PLGU were performed using a custom microscope (Carter & Sabatini, 2004; Bloodgood & Sabatini, 2007). The laser for 2PLSM was tuned to 840 nm to excite both red and green fluorophores. This wavelength does not cause photolysis of the MNI-glutamate, as judged by the absence of NMDAR-mediated Ca²⁺ transients in spines of neurons held at 0 mV potential and visualized with 2PLSM at different wavelengths within the 810–860 nm range (data not shown). Spine morphology was measured from maximal projections of 3-dimensional image stacks.
Morphologically mature silent spines

(5–6 sections at 1.0 μm spacing, 256 × 256 resolution, image field of 7.6 × 7.6 μm). Spine length was measured from the junction with the dendritic shaft to the spine tip. To determine the apparent spine width, we measured fluorescence in a line across the head and determined the width of the distribution where fluorescence intensity fell to 50% of its maximum. This measurement is an overestimate of the true spine head width as it includes the distortion caused by the point-spread function of the microscope. To minimize voltage-clamp errors, spines located close to the soma (range 16–89 μm) were analysed.

Fluorescence transients were monitored in line (500 Hz) or frame (4 Hz) scans, and quantified as increases in green fluorescence divided by red fluorescence (∆G/∆R) (Sabatini et al. 2002). Application of hypertonic ACSF (500 mosmol l⁻¹ by sucrose addition) was accomplished by puffing onto the surface of the slice through a syringe connected to a pipette whose 5–10 μm tip was positioned above the spine of interest. 2PLGU was achieved by dissolving 2.5 or 5.0 mM MNI-glutamate in the bath and triggered by 0.3–0.8 ms (typically 0.5 ms) pulses of 720 nm laser light. For each spine, we determined the ‘best spot’ of uncaging around the spine head by systematically testing uncaging positions around the periphery of the spine head and finding the one where 2PLGU elicited the biggest uEPSC at −70 mV (Fig. 1B). Repeated uncaging at this spot was ensured using a stabilization routine that cancelled image drift during the interstimulus interval (Carter & Sabatini, 2004). uEPSC amplitude was measured at each potential from the average of 10 uncaging trials, delivered at 0.033 Hz. Spines whose uEPSCAMPAR amplitude was less than 2 standard deviations of the current during a 5 ms period before the stimulus were defined as silent.

Data are presented as means ± standard error of the mean. Student’s two tail t test was used (P < 0.05) to judge statistical significance.

Results

Whole-cell voltage-clamp recordings of pyramidal neurons were obtained in cortical slices of 2-week-old rats (P14–17). Neurons were filled with the red fluorophore Alexa Fluor-594 (Fig. 1A). Dendrites and spines were visualized with two-photon laser scanning microscopy (2PLSM) and brief pulses of 720 nm light were used to trigger two-photon laser glutamate uncaging (2PLGU). The uncaging laser beam was directed at the position on the periphery of the selected spine head that evoked the largest response. The evoked currents at this optimal location were ~2- to 3-fold larger than those evoked at the diametrically opposed spot, relative to the spine head (Fig. 1B and C). This strong location dependence is consistent with the high spatial resolution of 2PLGU and suggests direct stimulation of synaptic AMPARs (Matsuzaki et al. 2001; Sobczyk et al. 2005; Carter et al. 2007). By bypassing the presynaptic terminal, we avoid complications of the presence of presynaptically silent synapses and of changes in the probability of vesicular release or quantal content during development. We focused on spines of basal dendrites, the main site of innervation by excitatory inputs from neurons located in cortical layer 4 (Lubke et al. 2000). Furthermore, we only considered spines whose head was clearly wider than the spine neck, and we refer to such spines as ‘morphologically mature’ below. We did not examine structures with no discernible head that might have represented either filopodia or long spines with small heads.

At a holding potential of −70 mV, uEPSCs were inward, short-lived (Fig. 1D), and suppressed by the
application of 10 μM NBQX in the bath (Fig. 2C). For these reasons, they were consistent with activation of AMPARs. Depolarization to +40 mV revealed the presence of long-lived outward currents (Fig. 1D) whose slowly decaying component was suppressed by 10 μM CPP (Fig. 4B) and thus were consistent with the activation of NMDARs. The average current amplitude at −70 mV in a 2 ms window about the peak was used to quantify uEPSC\textsubscript{AMPA}. The average amplitude of the current at +40 mV during a 20–40 ms window after the uncaging pulse was used to quantify uEPSC\textsubscript{NMDA}. In a separate set of spines, the dependence of uEPSC\textsubscript{NMDA} on the uncaging position was determined (online supplemental material, Supplemental Fig. 1). In 5 of 7 spines, the location that produced the largest uEPSC\textsubscript{NMDA} coincided with that which produced the largest uEPSC\textsubscript{AMPA}. In the remaining two spines, uncaging at the spot that was optimal for uEPSC\textsubscript{AMPA} evoked current at +40 mV that was at least 75% of the maximal uEPSC\textsubscript{NMDA}. These results are consistent with the low dependence of NMDAR activation of the location of the uncaging spot (Noguchi et al. 2005; Sobczak et al. 2005; Carter et al. 2007).

In order to compare uEPSC\textsubscript{AMPA}/uEPSC\textsubscript{NMDA} ratios across cells and development, the laser power was set to obtain a ~20 pA uEPSC\textsubscript{NMDA} when uncaging at the optimal spot determined at −70 mV (on average uEPSC\textsubscript{NMDA} = 20.4 ± 0.94 pA, n = 37/28/15 spines/cells/animals, age P14–17). This large amplitude uEPSC\textsubscript{NMDA} at +40 mV was chosen to ensure that the level of glutamate released by the uncaging pulse was sufficient to activate AMPARs on the spine. At P14–17, every analysed spine showed clear uEPSC\textsubscript{AMPA} (on average −8.5 ± 0.61 pA) (Fig. 1F), indicating surface expression of AMPARs and NMDARs at each spine (Kharazia et al. 1996; Takumi et al. 1999) and consistent with reports of low silent synapse density at these ages (Durand et al. 1996; Isaac et al. 1997; Rumpel et al. 2004). Furthermore, this uEPSC amplitude is similar to that of spontaneous miniature EPSCs at −70 mV in layer 2/3 pyramidal neurons of P11–22 rat somatosensory cortex (Bender et al. 2006).

Figure 2. AMPAR-lacking spines are found in immature pyramidal neurons

A, left panel, image of a layer 2/3 pyramidal neuron from a P9 rat highlighting the recording electrode (*). Right panel, higher magnification image of the basal dendrite indicated by the arrow in the left panel, showing mature spines (arrowheads) and a filopodium (#). B, uEPSCs from a P9 spine at holding potentials of −70 and +40 mV; 5 individual trials (grey) and the corresponding averages (black) are shown. C, uEPSC\textsubscript{AMPA} plotted versus uEPSC\textsubscript{AMPA} in control conditions (△, n = 72 spines, P9–13) or in the presence of NBQX (▲, n = 13 spines, P9–15). The dashed line marks the position expected for spines lacking AMPARs. Many control spines fall along this line and show uEPSC\textsubscript{AMPA} similar to that recorded in the presence of NBQX.

AMPAR-lacking, morphologically mature spines in immature rats

In spines from rats younger than 2 weeks (P9–13), uEPSC\textsubscript{NMDA} of similar amplitude (average 20.6 ± 0.67 pA, n = 72/52/27 spines/cells/animals) and time course (Supplemental Fig. 2) to that in older animals was obtained. However, spines could be identified that demonstrated uEPSCs at +40 mV but not at −70 mV (Fig. 2B and C). In these spines, uEPSC\textsubscript{AMPA} (on average −0.4 ± 0.10 pA, n = 15/14/11 spines/cells/animals) was similar to that seen in the presence of the AMPAR antagonist NBQX (on average −0.1 ± 0.20 pA, n = 13/5/3 spines/cells/animals; P > 0.1), consistent with a lack of functional surface AMPARs. Furthermore, uEPSC\textsubscript{AMPA} averaged across all spines (−3.9 ± 0.45 pA) and across only non-silent spines (−4.9 ± 0.49 pA, n = 57/38/16 spines/cells/animals) from P9–13 animals were significantly decreased compared to data from P14–17 animals. To confirm that spines of similar morphology had been analysed across ages, we measured the apparent length and head-width of spines in the study (Fig. 3). These parameters were constant across the age range (Fig. 3B), confirming that the increase in uEPSC\textsubscript{AMPA} reflected developmental changes. Furthermore, no significant correlations were observed between uEPSC\textsubscript{AMPA} and spine length (Fig. 3C, R^2 = 0.08) or width (R^2 = 0.04, data not shown).

Despite nearly constant morphology of analysed spines, AMPAR-lacking spines were found only in young animals. Thus, the percentage of spines with uEPSC\textsubscript{AMPA} indistinguishable from noise was 39% at P9, 32% at P10, 7% at P11 and 0% in older animals (Fig. 3D). Moreover, uEPSC\textsubscript{AMPA} increased consistently with age,
including during the week following the disappearance of AMPAR-lacking spines (Fig. 3E). This is consistent with the described increase in AMPAR/NMDAR synaptic current ratios in the same neurons early in development (Mierau et al. 2004).

**Silent spines are associated with release-competent presynaptic terminals**

2PLGU probes the glutamate receptor content of individual spines but cannot confirm the presence of a functional synapse, which requires a competent presynaptic terminal. To determine if an individual spine analysed by 2PLGU was associated with a release-competent presynaptic terminal, we used puffs of hypertonic solution to evoke the release of the readily releasable pool of glutamatergic vesicles (Fig. 4). In young animals, the mEPSC rate is low and the holding current at $-70 \text{ mV}$ shows small fluctuations. In contrast, during application of hypertonic ACSF, the mEPSC rate and holding current fluctuations are greatly increased (Fig. 4A). To detect glutamate release from the presynaptic terminal onto the analysed spine, the green-fluorescing Ca$^{2+}$ indicator Fluo-5F was included in the recording pipette, and Ca$^{2+}$ transients in the spine head were monitored during application of hypertonic solution. In order to minimize movement of the imaged spine during puffing, the hypertonic solution was applied to the surface of the slice, resulting in activation of only a single or few spines within the field of view (Supplemental Fig. 3).

Individual spines were first analysed electrophysiologically as above using 2PLGU (Fig. 4B), and uEPSC$_{\text{AMPA}}$ and uEPSC$_{\text{NMDA}}$ were measured. The cell was subsequently held at $0 \text{ mV}$, and a line scan was performed across the spine head and parent dendrite while puffing the hypertonic solution. In a subset of spines, Ca$^{2+}$ transients were seen in the spine head during the puff that were absent in the adjacent dendrite (Fig. 4C and D). These transients were blocked by CPP (Fig. 4D), together with uEPSC$_{\text{NMDA}}$ (Fig. 4B), and were consistent with the known time course and spatial confinement of synaptically evoked NMDAR-mediated Ca$^{2+}$ transients in active spines (Sabatini et al. 2002; Carter & Sabatini, 2004). Similar Ca$^{2+}$ transients were seen in $\sim 50\%$ of spines, indicating that they were associated with a release-competent terminal. Ca$^{2+}$ transients were detected in both AMPAR-expressing and AMPAR-lacking spines, supporting the notion that the latter represent the postsynaptic component of postsynaptically silent synapses (Fig. 4E). Spines that did not respond to hypertonic puffs may not have been exposed to effective levels of sucrose or they may represent spines not associated with release-competent terminals.

![Figure 3. Spines lacking or expressing AMPARs are morphologically indistinguishable](image_url)

- **A**: images of spines from relatively immature (left panel) and mature (right panel) rats. **B**: mean length ($\bullet$, left ordinate) and width ($\circ$, right ordinate) of spines analysed electrophysiologically as a function of animal age. The numbers of analysed spines at each age are indicated. **C**: uEPSC$_{\text{AMPA}}$ plotted versus spine length ($n = 74$ spines). Dashed line marks the predicted position of AMPAR-lacking spines. **D**: percentage of AMPAR-lacking spines as a function of animal age. **E**: developmental curve of uEPSC$_{\text{AMPA}}$ ($\bullet$) and uEPSC$_{\text{NMDA}}$ ($\circ$). The average uEPSCs calculated from the subset of spines that are non-silent in P9–11 animals are shown by grey circles. The numbers of analysed spines contributing to each data point are also given.
Discussion

We used a combination of 2PLSM, 2PLGU and whole-cell recordings to examine AMPAR- and NMDAR-mediated currents at single spines during development. We examined layer 2/3 pyramidal neurons in acute slices of rat somatosensory cortex and focused on spines of basal dendrites, which receive glutamatergic inputs from layer 4. Our results demonstrate the existence of postsynaptically silent synapses and show that, before age P12, these synapses can be found on morphologically mature spines. At later ages, all studied spines had functional surface AMPARs, but the AMPAR/NMDAR current ratio at individual spines continued to increase with age, consistent with increased synaptic expression of AMPARs.

Figure 4. Spines lacking AMPARs are associated with functional presynaptic terminals

A, the rate of mEPSCs detected at −70 mV is increased by puffs of hypertonic solution (right panel) relative to baseline levels (left panel) (age P9). B, image of a parent dendrite (dn) and spine (sp.) that showed no uEPSCs at −70 mV and large currents at +40 mV (white traces) that are blocked by the NMDAR antagonist CPP (red trace) (age P10). C, green fluorescence collected in the line scan indicated by the dashed line in (B). Inset: quantification of the green fluorescence transient (ΔG/R) in the spine head (sp) and neighbouring dendrite (dn) indicating that Ca$^{2+}$ influx was limited to the spine. D, quantification of fluorescence transients in the spine (sp, green trace) and dendrite (dn, black trace) on a longer time scale than C, displaying several evoked Ca$^{2+}$ transients in the spine. Over this longer time scale, evidence of Ca$^{2+}$ diffusion in the parent dendrite is seen. The asterisk (*) marks the event shown in (C). Ca$^{2+}$ influx into the spine was blocked by CPP (red trace). E, uEPSC$^{\text{AMPAR}}$ plotted versus uEPSC$^{\text{NMDAR}}$ for spines that were exposed to hypertonic ACSF. In some cases a Ca$^{2+}$ increase restricted to the spine was evoked (green circle, n = 9), whereas in others ΔCa$^{2+}$ was not detectable or was not restricted to the spine (●, n = 8) (age P9–15). A red contour marks AMPAR-lacking spines.

Postsynaptic nature of silent synapses

Silent synapses were initially described as synapses that display currents at +40 mV but not at −70 mV and were interpreted as containing NMDARs but lacking AMPARs (Isaac et al. 1995; Liao et al. 1995; Durand et al. 1996; Montgomery et al. 2001). However, other studies suggested that silent synapses might reflect synapses exposed to low levels of glutamate that are sufficient to activate NMDARs but not the lower affinity AMPARs. The postsynaptic terminals of silent synapses might be exposed to a reduced level of glutamate released by a competent nerve terminal (Choi et al. 2000; Renger et al. 2001; Mozhayeva et al. 2002) or to glutamate that enters the cleft due to spillover from neighbouring synapses (Kullmann et al. 1996; Gasparini et al. 2000).

2PLGU bypasses the presynaptic terminal and, when used to stimulate spines longer than ~1 μm, allows the selective activation of the postsynaptic terminal of a single spine (Matsuzaki et al. 2001; Sabatini et al. 2002; Carter & Sabatini, 2004; Beique et al. 2006). The dependence of uEPSC amplitudes on the location of the uncaging spot (Fig. 1B and C) suggests that a highly localized group of receptors is activated, such as that associated with the postsynaptic-density. Nevertheless, the volume in which glutamate is released is larger than that of the synaptic cleft, and extrasynaptic glutamate receptors are undoubtedly stimulated. Thus, spines without uEPSC$^{\text{AMPAR}}$ lack functional surface AMPARs both in the synapse and in the perisynaptic membrane. These spines may lack surface AMPARs or may express a nonfunctional or silenced receptor (Xiao et al. 2004).

Our results indicate that AMPAR-lacking spines are found in young animals (Fig. 2) and that NMDARs located on these spines detect glutamate released from presynaptic terminals (Fig. 4). Thus, we conclude that postsynaptically silent synapses do exist and, in basal dendrites of layer 2/3 pyramidal neurons, are associated with morphologically mature dendritic spines. Nevertheless, it is impossible to disprove that the glutamate which activates NMDARs on the AMPAR-lacking spines diffuses (‘spillover’) from a neighbouring synapse. The degree of spillover may have

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been enhanced in our experiments, which were performed at room temperature (Aztely et al. 1997). Lastly, we cannot rule out that presynaptically silent synapses also exist, and it is possible that some of the AMPAR-lacking spines that could not be stimulated by hypertonic sucrose puffs represent the postsynaptic component of such synapses.

Development of layer 2/3 synapses

As has been previously described for silent synapses (Durand et al. 1996; Isaac et al. 1997), we find that AMPAR-lacking spines are present in developing animals and disappear (or become extremely rare) by age P12 (Fig. 3D). Rumpell and colleagues examined the development of silent synapses in layer 2/3 pyramidal neurons of the visual cortex and found them to be present until P14 (Rumpel et al. 2004). The two findings are in general good agreement; the slightly longer time of expression of silent synapses in the Rumpell paper may arise from experimental differences such as the activation of apical and basal synapses in their study and differences between visual and somatosensory cortex. Furthermore, the silent synapses found by Rumpell and colleagues may include synapses made onto filopodia or directly onto the dendritic shaft, classes of synapses that we did not examine. Lastly, the spines that we describe as lacking AMPARs lack them in the postsynaptic density and are therefore silent. However, the converse may not be true: it is possible that some spines that have functional surface AMPARs may lack them in the synaptic cleft and be silent when exposed to synaptically released glutamate. For all of these reasons, the fraction of spines that lack uncaging-evoked AMPARs currents is likely an underestimate of the fraction of synapses that are silent.

Our results also show that the transition from silent to non-silent during development does not involve a binary, step-like increase in AMPAR content. Before P12, the size of uEPSC_{AMPAR} in spines that show such current is small with many responses clustered below 5 pA (Fig. 2C). AMPAR content increases with age, including after age P12 when silent spines are no longer found (Fig. 3E). Similar increases in synaptically evoked AMPAR/NMDAR current ratios have been described in layer 2/3 somatosensory cortical neurons (Mierau et al. 2004) and may account for their weak response to whisker stimulation before P14 (Stern et al. 2001). Gradual AMPAR expression, in addition to an increased number of synapses, may contribute to the strengthening input/output relation seen in hippocampal CA3–CA1 connections during development (Hsia et al. 1998).

During the developmental time period investigated in the present paper, NMDAR subunit composition changes (Carmignoto & Vicini, 1992) from a subtype containing predominantly NR1/NR2B subunits to a subtype containing NR1/NR2A/NR2B in variable combinations (Sheng et al. 1994). Decreasing contribution of NR2B subunit during the second postnatal week has been demonstrated in layer 2/3 pyramidal neurons of somatosensory cortex (Mierau et al. 2004). Notwithstanding the shift in subunit composition, the unitary current amplitudes of recombinant NR1/NR2A and NR1/NR2B channels are the same (Stern et al. 1992; Cull-Candy et al. 2001) in the presence of 10 μM glycine (replaced here with 10 μM of its analogue serine). An additional complication of the NR2B to NR2A subtype switch is that the opening probability of recombinant NR2A containing receptors is higher than those containing NR2B (Chen et al. 1999; Erreger et al. 2005) (although see Prybylowski et al. 2002), who report that the opening probability of NMDARs does not differ depending on the NR2 subunit that is incorporated). For this reason it is possible that less uncaged glutamate was needed to produce a 20 pA uEPSC in old animals than in young animals. If this case, we may have underestimated the AMPAR/NMDAR current ratio in older animals as well as the rate of increase of this ratio during development.

Spine morphology

The definition of a silent synapse is strictly functional. A recent study performed in organotypic hippocampal slice cultures has visualized the dendritic spines associated with silent synapses (Ward et al. 2006). The authors discuss that those silent spines were ‘no different in appearance’ from the active ones, although no supporting data were given. In accordance with this paper, we find that both AMPAR-lacking and AMPAR-containing spines are found within the class of morphologically mature synapses. Similarly, 2PLGU analysis of single spines of CA1 pyramidal neurons of PSD-95 knock-out mice uncovered a high percentage of AMPAR-lacking spines that were morphologically similar to spines with AMPARs (Beique et al. 2006). In the same report, no postsynaptically silent spines were found in age-matched (P13–16) control animals. In separate studies, hippocampal spines immunonegative for AMPARs have been shown to have smaller synaptic area than spines with synaptic AMPARs (Nusser et al. 1998; Takumi et al. 1999). Similarly, 2PLGU analysis of single hippocampal spines in P15–22 animals demonstrated a tight correlation between AMPAR-current amplitude and spine morphology such that small or thin structures had little sensitivity to glutamate (Matsuzaki et al. 2001). In the present paper, we found a large variability in AMPAR-current amplitudes within a relatively morphologically homogeneous population of spines. Our study examined only spines with clear necks and heads. Therefore, the results may not be applicable to small spines that are below the resolution of 2PLSM or to stubby spines that, because of their short length, cannot be
selectively activated by glutamate uncaging without also activating glutamate receptors located on the dendritic shaft.

Conclusion

We used combined two-photon microscopy and glutamate uncaging to directly demonstrate the existence of dendritic spines on basal dendrites of layer 2/3 pyramidal neurons that express functional NMDARs but not AMPARs. At least a subset of these AMPAR-lacking spines is associated with release competent presynaptic boutons, indicating the existence of postsynaptically silent synapses. Furthermore, AMPAR-lacking spines on these cells were found only prior to P12, and the AMPAR/NMDAR current ratio at individual spines continued to increase through the following postnatal week.

References


Morphologically mature silent spines


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Supplemental material

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