Silent synapses: what are they telling us about long-term potentiation?

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At several cortical synapses glutamate release events can be mediated exclusively by NMDA receptors, with no detectable contribution from AMPA receptors. This observation was originally made by comparing the trial-to-trial variability of the two components of synaptic signals evoked in hippocampal neurons, and was subsequently confirmed by recording apparently pure NMDA receptor-mediated EPSCs with stimulation of small numbers of axons. It has come to be known as the 'silent synapse' phenomenon, and is widely assumed to be caused by the absence of functional AMPA receptors, which can, however, be recruited into the postsynaptic density by long-term potentiation (LTP) induction. Thus, it provides an important impetus for relating AMPA receptor trafficking mechanisms to the expression of LTP, a theme that is taken up elsewhere in this issue. This article draws attention to several findings that call for caution in identifying silent synapses exclusively with synapses without AMPA receptors. In addition, it attempts to identify several missing pieces of evidence that are required to show that unsilencing of such synapses is entirely accounted for by insertion of AMPA receptors into the postsynaptic density. Some aspects of the early stages of LTP expression remain open to alternative explanations.

Keywords: silent synapses; AMPA; NMDA; expression; spillover

1. THE SILENT SYNAPSE PHENOMENON

Numerous experimental observations have been used to shed light on the early steps of NMDA receptor-dependent LTP expression. About 10 years ago, among the more robust findings (that is, reproduced in several laboratories) were two results that, at first sight, led to opposing conclusions. First, LTP was found to be associated with an increase in the average number of quanta of glutamate detected by the postsynaptic neuron in response to a presynaptic action potential (Bekkers & Stevens 1990; Malinow & Tsien 1990; Kullmann & Nicoll 1992; Larkman *et al.* 1992; Liao *et al.* 1992; Voronin 1994). By analogy with many other synapses in the nervous system, an increase in quantal content implies a presynaptic increase in transmitter release probability. Second, LTP was reported to be accompanied by a relatively selective increase in the signal mediated by AMPA receptors, with little change in the NMDA receptor-mediated component (Kauer *et al.* 1988; Muller & Lynch 1988; Perkel & Nicoll 1993). This finding, by contrast, is most easily explained by an enhancement in the function of AMPA receptors at the postsynaptic membrane. An important step towards a resolution of this paradox came with several observations that have become known as the 'silent synapse' phenomenon: evoked monoquantal events can be detected in a postsynaptic neuron, which are apparently exclusively mediated by NMDA receptors (Kullmann & Siegelbaum 1995). After LTP induction, these events are replaced by dual-

component (AMPA and NMDA receptor-mediated) signals.

Initial evidence for silent synapses came from a comparison of the trial-to-trial variability of the AMPA and NMDA receptor-mediated components of EPSCs in CA1 pyramidal neurons (Kullmann 1994; Selig *et al.* 1995). Briefly, the argument goes as follows. Because the CV (a dimensionless measure of variability) is principally determined by the stochastic release of different numbers of vesicles from presynaptic afferents, it can be used to compare the probabilistic behaviour of signals mediated by the different receptors. Before LTP induction, the CV of the AMPA component evoked by a stimulus repeatedly delivered to a population of afferent fibres is consistently larger than that of the NMDA component. Because the CV varies inversely with the quantal content, this observation implies that, on average, fewer AMPA receptor-mediated quanta mediate the EPSC than NMDA receptor-mediated quanta. (Differences in the stochastic behaviour of channels themselves can be shown not to affect this conclusion (Kullmann 1994).) After LTP, the CV of the AMPA component decreases to become more similar to that of the NMDA component.

An alternative approach to demonstrate more graphically the existence of silent synapses was subsequently reported. This relied on minimal stimulation to excite a small number of presynaptic axons (figure 1). Exclusively NMDA receptor-mediated EPSCs could be shown to occur, and AMPA receptor-mediated quanta emerged following LTP induction (Isaac *et al.* 1995; Liao *et al.* 1995; Durand *et al.* 1996). An even more direct demonstration of silent synapses comes from paired recordings of synaptically coupled pyramidal neurons, which removes residual

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Figure 1. The silent synapse phenomenon. An example of synaptic transmission mediated by AMPA and NMDA receptors. A granule cell was recorded at room temperature, initially voltage clamped at -70 mV to block the NMDA receptors (*a*), and then depolarized to $+50$ mV (*b*). The stimulation of a few lateral perforant path axons evoked AMPA receptor-mediated EPSCs with a high failure rate (*a*). The failure rate for the NMDA receptor-mediated component was much lower (*b*). Data from M.-Y. Min and D. M. Kullmann.

Figure 2. Possible explanations of silent synapses. (*a*) A 'deaf' synapse (i) expresses NMDA but not functional AMPA receptors. By contrast, a 'vocal' synapse (ii) expresses both receptors. (*b*) The slow exocytosis of glutamate at a 'whispering' synapse reaches a concentration in the cleft that is sufficient to activate high-affinity NMDA receptors but not AMPA receptors. (*c*) The glutamate spillover activates NMDA but not AMPA receptors at an 'eavesdropping' and presynaptically silent synapse (i). The releasing varicosity (ii) is likely to be in synaptic contact with a different neuron, explaining why no AMPA receptor-mediated component is detected.

uncertainty about the number of axons stimulated under different recording conditions (Montgomery *et al.* 2001).

2. POSSIBLE UNDERLYING PHENOMENA

The first, and still most compelling, interpretation of silent synapses is that, under baseline conditions, functional AMPA receptors are absent at a subset of synapses (Kullmann 1994) (figure 2*a*). This view has received important support from immunogold labelling of AMPA and NMDA receptors in the hippocampus: although AMPA receptors are undetectable at a proportion of synapses, NMDA receptors are more uniformly distributed (Nusser *et al.* 1998; Racca *et al.* 2000). Although this approach is sensitive to assumptions about the sensitivity of antibodies, it is qualitatively consistent with the existence of postsynaptically silent ('deaf') synapses. LTP is

assumed to be accompanied by the appearance of such clusters, either by insertion into the postsynaptic density from a cytoplasmic store, or by translocation from an extrasynaptic location (Malenka & Nicoll 1999). The evidence for AMPA receptor trafficking mechanisms, both constitutive and in response to LTP induction stimuli, is overwhelming (Malinow & Malenka 2002). However, before assessing how completely this model accounts for the phenomenology of LTP, it is worth considering several alternative possibilities. These all rely on the fact that NMDA receptors have very different kinetics than AMPA receptors (Lester *et al.* 1990; Patneau & Mayer 1990; Jonas *et al.* 1993).

With prolonged application of glutamate, the apparent affinity of NMDA receptors is approximately 100-fold higher than that of AMPA receptors (Patneau & Mayer 1990). This does not mean that they are necessarily 100-

Figure 3. Unsilencing with LTP induction: the possible mechanisms. (a) The insertion of a vesicle of AMPA receptors into a deaf synapse. (b) Movement of a raft of perisynaptic AMPA receptors into the synaptic cleft. (c) A change in the mode of exocytosis could increase the occupancy of AMPA receptors. (d) Conversion of a mute eavesdropping synapse by an increase in presynaptic release probability. (e) A structural change in a postsynaptic spine could cause it to approach a release site.

fold more sensitive to glutamate transients such as occur in response to presynaptic exocytosis, because the occupancy and opening probability of receptors also depend on the time course of the glutamate transient. At one extreme, if NMDA and AMPA receptors are exposed to an extremely brief glutamate transient, they might even have similar occupancies, because in this situation their opening probabilities are principally determined by their binding rates, which are generally thought to be similar for the two receptors (Kullmann 1999). This may account for some studies that have yielded similar estimates for AMPA and NMDA receptor occupancy at hippocampal synapses (McAllister & Stevens 2000). However, as the glutamate transient becomes slower, the unbinding and ultimately desensitization rates take on a greater importance. If the glutamate wave is relatively slow, NMDA receptors might be relatively selectively activated by glutamate, because their unbinding and desensitization rates are considerably slower than those of AMPA receptors. This could occur through at least two mechanisms.

First, slow release of glutamate through a fusion pore might allow the agonist to reach a relatively low concentration, which activates NMDA receptors relatively selec-

tively (giving rise to a 'whispering' synapse, figure 2b). The evidence that such slow release occurs at hippocampal synapses *in situ* is incomplete (Choi *et al.* 2000). Nevertheless, some findings argue that different modes of vesicle cycling do occur (Murthy & Stevens 1998), and that a slow mode of release can be converted to a faster mode (Renger *et al.* 2001). For such a mechanism to occur in LTP (Choi *et al.* 2000) a signal would need to travel to the presynaptic terminal from the postsynaptic spine, which is agreed to be the site of coincidence detection.

Second, a slow wave of glutamate might activate NMDA receptors relatively selectively if the latter are relatively remote from the release site. Diffusion of glutamate could result in sufficient dilution and slowing of the transient to ensure that NMDA receptors are relatively selectively activated, even if AMPA receptors are also present. The evidence for extrasynaptic glutamate escape is overwhelming:

- (i) Several subtypes of glutamate receptor (including metabotropic and kainate receptors) are present on axons relatively far from the site of release, and can detect synaptically released glutamate in the hippo-

campus (Yokoi *et al.* 1996; Shigemoto *et al.* 1996, 1997; Semyanov & Kullmann 2000, 2001; Cossart *et al.* 2001; Jiang *et al.* 2001).

- (ii) The principal transporters that clear glutamate from the extracellular space are extrasynaptic (Danbolt 2001).
- (iii) The time course of the astrocytic currents associated with their action indicates that glutamate persists in the extracellular space for a relatively long time, therefore allowing the neurotransmitter to diffuse a relatively long distance from the site of release (Bergles *et al.* 1999).

These considerations lead to the expectation that NMDA receptors relatively far from the release sites may be activated. Numerical simulations, moreover, show that, as the distance from the release site increases, the occupancy of NMDA receptors decreases more slowly than that of AMPA receptors (Rusakov & Kullmann 1998; Barbour 2001). This raises the possibility that synaptic signals mediated by NMDA receptors, with little activation of AMPA receptors, may arise from such a spillover (figure 2c). A potential interpretation of this finding is that cross-talk among neighbouring glutamatergic synapses might even account for much of the silent synapse phenomenon (Kullmann *et al.* 1996; Kullmann & Asztely 1998). Thus, synapses may not be as private as previously assumed, at least for the activation of NMDA receptors. In support of this hypothesis, manipulations of glutamate uptake with changes in temperature and uptake blockers alter the proportion of silent synapses in a pattern that agrees with changes in the extent of glutamate spillover (Asztely *et al.* 1997).

These findings have prompted a re-evaluation of the spatial extent of spillover signalling. Considerable uncertainty surrounds several parameters underlying the release, diffusion and uptake of glutamate (Rusakov & Kullmann 1998; Barbour 2001). Nevertheless, some degree of cross-talk at 'eavesdropping' synapses that occur in close proximity (<200 nm) is inescapable. This is especially so for those not-infrequent cases where there is no interposed astrocytic process (Spacek 1985; Lehre & Danbolt 1998; Ventura & Harris 1999). Another situation where NMDA receptor-mediated cross-talk is likely to occur is where a single bouton makes two synapses onto different spines (Sorra & Harris 1993): stochastic release at one of the release sites may well result in significant activation of NMDA receptors at both synapses. Finally, abundant extrasynaptic NMDA receptors also occur, many of which are likely to detect extrasynaptic glutamate escape (Charton *et al.* 1999). Whether some of these occur at nascent synapses is open to debate.

3. UNSILENCING WITH LONG-TERM POTENTIATION

It must be pointed out that the explanations listed in § 2 for silent synapses are not mutually exclusive: it is highly likely that several, if not all, of these possibilities coexist. Nevertheless, how can each model be related to the increase in quantal content that occurs with NMDA receptor-dependent LTP? (All of the following interpretations coexist happily with an increase in quantal ampli-

tude mediated by phosphorylation and/or recruitment of additional AMPA receptors into existing clusters at synapses.)

4. AMPA RECEPTOR INSERTION

In the postsynaptically silent synapse model ('deaf synapses') the simplest explanation for unsilencing with LTP is the insertion of AMPA receptor clusters into postsynaptic densities where they are initially absent (Kullmann 1994) (figure 3a). An abundance of evidence for both constitutive and regulated AMPA receptor trafficking to and from the membrane now exists (reviewed by Malinow & Malenka (2002)). Although there is little doubt that AMPA receptor insertion takes place during LTP, and that newly functional receptors contribute to synaptic transmission, there are several apparent discrepancies and missing pieces of evidence.

First, close attention to the pattern of distribution of AMPA receptors among synapses studied with immunogold labelling reveals that only relatively small synapses are devoid of these receptors (Nusser *et al.* 1998; Takumi *et al.* 1999). It has been argued that small synapses (assessed by the dimensions of the postsynaptic density, spine or bouton) have relatively low release probabilities (Harris & Sultan 1995; Schikorski & Stevens 1997). Thus, it is puzzling that silent synapses as identified by electrophysiological recordings are so abundant.

Second, where is the store of latent AMPA receptors? For the quantal content to increase, it is not sufficient for individual receptors to be recruited in a graded manner. This leads to the prediction that vesicles containing multiple receptors (approximately the same number as present in already 'vocal' synapses) should occur under baseline conditions. As yet, no such vesicles have been convincingly revealed with immunogold methods, even though multiple AMPA receptors can be reliably detected in serial sections of the postsynaptic density itself. Some evidence for clustering of AMPA receptors within dendrites has been reported with fluorescent microscopy (Shi *et al.* 1999), but this approach lacks the spatial resolution to determine whether they are pre-packaged in an organelle suited for rapid all-or-none delivery to the synapse. The alternative possibility, that latent receptors are actually loosely scattered in multiple cytoplasmic vesicles before assembly into a cluster, is obviously less attractive, but it may be necessary to meet the challenge presented by this model if cytoplasmic clusters continue to evade detection.

Third, a similar argument can be made about intracellular membrane specializations that might be suited for trafficking receptors to the synapse. Although a spine apparatus that might be involved in such trafficking has been reported at some synapses, this is preferentially found at large synapses, that is, those that probably already have AMPA receptors, and not at small synapses that may lack AMPA receptors (Spacek & Harris 1997).

An alternative possibility, that latent clusters of AMPA receptors are already in the membrane, but in an extrasynaptic location (figure 3b), suffers from similar problems: why have such extrasynaptic clusters not been revealed by immunogold methods?

Finally, methods that have been used to reveal activity-dependent incorporation of AMPA receptors (either

physiological or anatomical) have a limited temporal resolution. This leaves open the possibility that AMPA receptor insertion requires several minutes to take place. However, LTP induction takes place only over a few seconds (Gustafsson & Wigstrom 1990). It is difficult to argue that receptor phosphorylation accounts for the very earliest stages of LTP (seconds–minutes), only later being supplemented or replaced by receptor insertion, because the increase in quantal content appears to take place extremely rapidly (Petersen *et al.* 1998).

In summary, although AMPA receptor insertion no doubt occurs during LTP induction, the subcellular location of the reserve store of such receptors remains to be identified unambiguously, and it will be important to narrow the time window during which the phenomenon occurs. Nevertheless, insertion of receptors does not rule out other mechanisms taking place during the earliest stages of LTP.

5. OTHER POSSIBLE EXPLANATIONS FOR UNSILENCING

Compared with the abundance of evidence for receptor trafficking, alternative models for LTP expression that can also explain why synapses become unsilenced are relatively less well supported by experimental data. One simple hypothesis is that a retrograde signal switches on presynaptically silent ('mute') synapses (figure 3*d*). NMDA receptors at such synapses may initially be activated by the spillover of glutamate from neighbouring synapses. The coincidence of such NMDA receptor activation with postsynaptic depolarization would cause a retrograde signal (chemical or mechanical) to switch on the presynaptic synapse. This model, although supported by some indirect evidence (Kullmann & Siegelbaum 1995; Kullmann *et al.* 1996), is difficult to reconcile with the finding that synaptic glutamate transporter currents recorded in astrocytes do not change after LTP (Diamond *et al.* 1998; Luscher *et al.* 1998). Another method to detect changes in transmitter release by monitoring presynaptic vesicle cycling also failed to reveal an alteration following LTP induction by conventional stimuli (Zakharenko *et al.* 2001). Surprisingly, an increase was seen after a more intense induction protocol, implying that recruitment of vesicles and/or an increase in release probability at releasing synapses may occur under some special circumstances.

Overall, thus, the evidence that unsilencing normally arises from a presynaptic increase in release probability is not favoured by the balance of evidence. What other explanations remain? One possibility is that the mode of release changes from slow escape of glutamate from a fusion pore to rapid all-or-none release (figure 3*c*). This could change the shape of the glutamate transient within the synaptic cleft, and therefore increase the activation of AMPA receptors, without altering significantly either the activation of NMDA receptors or the total amount of glutamate released into the extracellular space. Some indirect evidence for such a phenomenon has been reported (Choi *et al.* 2000).

Another model that has, thus far, been relatively overlooked, is that LTP is accompanied by a rapid structural modification of the spine and/or presynaptic varicosity that changes the time course of the glutamate transient

sensed by the postsynaptic NMDA receptors (figure 3*e*). This could occur, for instance, by bringing the pre- and postsynaptic parts of the synapse closer together, or by reducing the escape of glutamate from the synaptic cleft. Growth of a spine or filopodial extension expressing receptors towards an existing release site would give a similar result. A cluster of receptors at such a nascent synapse could, under appropriate conditions initially detect glutamate release exclusively via NMDA receptors, and would therefore behave as a silent synapse. As the distance between the postsynaptic process and release site decreased, the AMPA receptors would begin to respond to glutamate. This model does not require changes in either the amount or mode or presynaptic transmitter release, neither does it depend on alterations in AMPA receptor expression. Interestingly, it could account for the observation that LTP is not exclusively expressed by AMPA receptors: a consistent, albeit smaller, increase in the NMDA receptor-mediated component has been reported to occur with either tetanus- or pairing-induced LTP (Asztely *et al.* 1992; Kullmann *et al.* 1996). LTP of isolated NMDA receptor-mediated EPSCs has, moreover, been reported by several groups (Tsien & Malinow 1990; Bashir *et al.* 1991). A simple explanation of this is that NMDA receptors are brought physically closer to the site of release by the structural changes. However, alternative possibilities such as changes in their phosphorylation state or insertion into postsynaptic densities cannot be ruled out.

Although there is evidence for structural rearrangements accompanying LTP, these have generally been detected over tens of minutes (Hosokawa *et al.* 1995; Engert & Bonhoeffer 1999; Toni *et al.* 1999). Nevertheless, this may reflect a trade-off of spatial and temporal resolution: large changes in receptor occupancy occur over sub-micrometre distances in the neuropil, which may fall below the threshold for detection with available methods.

6. CONCLUSION

The silent synapse phenomenon is a major impetus for studying the mechanisms that deliver receptors to synapses. Although the evidence for AMPA receptor insertion during LTP is overwhelming, several steps in this cascade remain to be documented, including the identity and location of the store of reserve receptors and the speed by which this is achieved. Moreover, the absence of AMPA receptors at synapses is not the only possible explanation for silent synapses: the very distinct kinetics of NMDA receptors raise the possibility that glutamate spillover and/or fusion-pore release give rise to selective activation of these receptors at synapses that contain both species. These models also prompt the possibility of some alternative mechanisms for LTP expression, which rely on alterations in the mode of transmitter release or structural changes in the vicinity of release sites, which result in the functional recruitment of AMPA receptors that are insufficiently sensitive to detect glutamate under baseline conditions. Ultimately, silent synapses may result from multiple mechanisms. The relative importance of these mechanisms, and their implications for the earliest steps in LTP expression, remain to be elucidated.

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GLOSSARY

- AMPA: α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
- CV: coefficient of variation
- EPSC: excitatory postsynaptic current
- LTP: long-term potentiation
- NMDA: N-methyl-D-aspartate