THE ROYAL SOCIETY

Published online 6 March 2003

Fusion pore modulation as a presynaptic mechanism contributing to expression of long-term potentiation

Sukwoo Choi[1], Jürgen Klingauf[2] and Richard W. Tsien[3*]

[1]*Department of Neuroscience, Ewha Institute for Neuroscience (EIN), School of Medicine, Ewha Womans University, Seoul 110-783, South Korea*

[2]*Max Planck Institute for Biophysical Chemistry, Department of Membrane Biophysics, Am Fassberg 11, D-37077 Göttingen, Germany*

[3]*Department of Molecular and Cellular Physiology, Beckman Center B105, Stanford University School of Medicine, Stanford, CA 94305-5345, USA*

Working on the idea that postsynaptic and presynaptic mechanisms of long-term potentiation (LTP) expression are not inherently mutually exclusive, we have looked for the existence and functionality of presynaptic mechanisms for augmenting transmitter release in hippocampal slices. Specifically, we asked if changes in glutamate release might contribute to the conversion of 'silent synapses' that show *N*-methyl-D-aspartate (NMDA) responses but no detectable α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) responses, to ones that exhibit both. Here, we review experiments where NMDA receptor responses provided a bioassay of cleft glutamate concentration, using opposition between peak $[glu]_{cleft}$ and a rapidly reversible antagonist, L-AP5. We discuss findings of a dramatic increase in peak $[glu]_{cleft}$ upon expression of pairing-induced LTP (Choi). We present simulations with a quantitative model of glutamatergic synaptic transmission that includes modulation of the presynaptic fusion pore, realistic cleft geometry and a distributed array of postsynaptic receptors and glutamate transporters. The modelling supports the idea that changes in the dynamics of glutamate release can contribute to synaptic unsilencing. We review direct evidence from Renger *et al.*, in accord with the modelling, that trading off the strength and duration of the glutamate transient can markedly alter AMPA receptor responses with little effect on NMDA receptor responses. An array of additional findings relevant to fusion pore modulation and its proposed contribution to LTP expression are considered.

Keywords: fusion pore; silent synapse; long-term potentiation; presynaptic mechanism; L-AP5

1. INTRODUCTION

There is wide if not universal consensus that activity-dependent changes in synaptic efficacy such as LTP and LTD are critical for information storage in the brain and the proper development of neural circuitry (McNaughton & Morris 1987; Bliss & Collingridge 1993). However, uncertainty remains about the site or sites of LTP expression. It is our belief that the possible coexistence of postsynaptic and presynaptic expression mechanisms has not been fully explored, owing in part to an investigational desire for conceptual simplicity, and in part to the difficulties of unambiguously distinguishing between various mechanisms.

One important observation, widely replicated at various central synapses (Isaac *et al.* 1995; Liao *et al.* 1995; Durand *et al.* 1996), is that a proportion of glutamatergic synaptic connections exhibit no AMPAR-mediated currents, but show clear NMDAR-mediated currents, particularly upon strong postsynaptic depolarization. These 'silent synapses' can become fully functional upon induction of LTP (Isaac *et al.* 1995; Liao *et al.* 1995; Durand *et al.* 1996). The prevailing hypothesis to account for this conversion invokes a postsynaptic mechanism whereby silent synapses lack functionally active AMPARs but gain them as a result of the insertion of AMPAR-containing vesicles. A proportion of synapses (less than 20%) show no significant labelling by anti-AMPAR antibodies (Nüsser *et al.* 1998) and fluorescently tagged AMPARs can undergo externalization during the course of LTP (e.g. Shi *et al.* 1999, 2001; Hayashi *et al.* 2000).

None of the compelling evidence for postsynaptic changes at silent synapses excludes the possibility of concomitant changes in presynaptic function, as appears to occur in mossy fibre LTP. Indeed, previous work on synapses among cultured dissociated neurons provides unequivocal evidence for the existence of presynaptic mechanisms for expression of NMDAR-induced LTP. This is based on electrophysiological tests with hypertonic solution challenges (Malgaroli & Tsien 1992), the uptake of antibody markers of presynaptic vesicular turnover (Malgaroli *et al.* 1995), and the destaining of synaptic vesicles marked with the fluorescent dye FM1–43 (Ryan *et al.* 1996).

Although experiments in hippocampal cultures have provided proof of principle for the existence of presynaptic mechanisms, their extrapolation to brain tissue faces scepticism. Like others, we continue to explore basic synaptic mechanisms in cultured neurons, but have turned to hip-

*Author for correspondence (rwtsien@stanford.edu).

One contribution of 30 to a Theme Issue 'Long-term potentiation: enhancing neuroscience for 30 years'.

pocampal slices for critical tests of possible LTP expression mechanisms. We have contributed to two new developments: (i) the use of FM dyes to study vesicle turnover in brain slices (Pyle *et al.* 1999), and (ii) the application of NMDAR-based assays of cleft neurotransmitter concentration (Choi *et al.* 2000). In this paper, we summarize our experimental evidence for a presynaptically driven change in cleft neurotransmitter concentration at CA3 to CA1 hippocampal synapses (Choi *et al.* 2000). We present a quantitative framework for exploring the fusion pore hypothesis, of general interest beyond LTP, that models changes in fusion pore properties, the diffusion of glutamate within the synaptic cleft and surrounding tissues, and the interaction of glutamate with postsynaptic receptors and glutamate transporters. We then discuss the relationship between our results and data from others, particularly Renger *et al.* (2001), that supports the involvement of fusion pore modulation in synaptic maturation and plasticity.

2. MATERIAL AND METHODS

(a) *Modelling methods*

The model of buffered diffusion of glutamate inside and outside the synaptic cleft was similar to that of Rusakov & Kullmann (1998). Release of glutamate from a vesicle was simulated by addition of two 'virtual' cylindrical compartments in the middle of the innermost cleft compartment, one representing the vesicle (40 nm in diameter and 40 nm in height), the other constituting the membrane-spanning fusion pore (variable diameter, 10 nm in length). We chose a value $D = 150\ \mu m^2\ s^{-1}$, the highest value that gives results comparable to estimates of the time-course of $[glu]_{cleft}$ (Clements 1996). The diffusion constant of glutamate within the 1–2 nm diameter fusion pore was reduced to $37.5\ \mu m^2\ s^{-1}$ to take account of the fact that dimensions of the diffusing species (0.7–0.8 nm) were close to the initial diameter of the permeation pathway (*ca.* 2 nm) (Spruce *et al.* 1990; Stiles *et al.* 1996). Transporters were incorporated in all compartments outside the cleft, at a concentration of 100 μM, using the kinetic description given by Wadiche & Kavanaugh (1998). To estimate the resulting AMPAR and NMDAR currents we used the kinetic schemes and rate constants published by Jonas *et al.* (1993) and Clements & Westbrook (1991), respectively. Binding of a second antagonist molecule was assumed to show a fourfold positive cooperativity, and the k_{off} of L-AP5 was adjusted in order to simulate the experimentally observed effects of L-AP5.

3. RESULTS

(a) *Experiments with a low-affinity NMDAR antagonist*

To assess the peak $[glu]_{cleft}$ at 'silent synapses' that lacked detectable AMPAR-mediated responses, we employed a well-accepted pharmacological approach that relies on a rapidly unbinding antagonist for NMDARs (Clements 1996). The principle of the method (figure 1*a*) is that the degree of antagonist inhibition will depend upon the peak transmitter concentration during neurotransmission: the higher $[glu]_{cleft}$, the less the inhibition. L-AP5 (K_i *ca.* 40 μM; Olverman *et al.* 1988) was chosen as the low-affinity antagonist because its off-rate is faster than for other available NMDA antagonists. In the presence of this competitive antagonist, NMDAR becomes much more sensitive to *peak concentration* because the race with antagonist rebinding is a matter of on-rate, not equilibrium. The competition between glutamate and a fixed amount of L-AP5 can be likened to finding a seat in a crowded café. Those who are slow or infirm will be at a disadvantage, and may fail to get a seat even after many rounds of customers coming and going, because each competition for a vacancy is a winner-take-all contest. So it is for glutamate binding to the NMDAR in the presence of the competitive antagonist: if a receptor fails to bind neurotransmitter in the first place, it matters not that the ligand would have stayed bound for a long time.

Synaptic transmission was evoked by minimal stimulation of the Schaffer collateral-commissural pathway to hippocampal area CA1 (figures 1*b* and 2*a*). At silent synapses that lacked detectable AMPAR currents but showed clear NMDAR responses, NMDA EPSCs were almost completely inhibited by 250 μM L-AP5 (12 out of 12 cells, mean inhibition = 99.7 ± 1.0%). Pairing of pre- and postsynaptic activity was imposed and was successful in inducing potentiation in 6 out of 12 cells; in these cases, failures of AMPAR responses sharply decreased after pairing as previously reported (Isaac *et al.* 1995; Liao *et al.* 1995; Durand *et al.* 1996). By contrast, no LTP was ever induced when the pairing procedure was carried out in the presence of 50 μM D-AP5 ($n = 8$ out of 8, $p < 0.004$), as expected for NMDAR-dependent plasticity.

We found a marked change when we tested the effects of L-AP5 on NMDAR currents in cases where silent synapses lacking AMPAR currents were converted into fully functional ones. Whereas L-AP5 reduced NMDAR currents by 99.2 ± 3.4% before pairing, the inhibition was much milder after pairing (50.2 ± 3.3%, $n = 6$, $p < 0.001$). The change from full inhibition to half inhibition was all the more convincing because each potentiated slice served as its own control. The reduction in the degree of L-AP5 inhibition indicated that the peak value of $[glu]_{cleft}$ was significantly increased along with the potentiation.

The interpretation of the findings with L-AP5 was clarified by control experiments that addressed several key questions.

Was the difference in antagonist sensitivity specific to the rapidly reversible antagonist? We performed control experiments using the slow unbinding antagonist, R-CPP. In contrast to L-AP5, R-CPP produced a degree of inhibition that did not significantly change during LTP expression. The proportion of current amplitude remaining in the presence of R-CPP was 0.580 ± 0.048 before pairing and 0.542 ± 0.043 after pairing ($p > 0.05$, $n = 4$). The finding that a blockade of NMDA receptor responses was specific to a fast unbinding antagonist but not a slow unbinding antagonist supported the idea that peak $[glu]_{cleft}$ increased during LTP.

(i) *How stable were the recordings?*

No significant changes in series resistance were found during our whole-cell recordings (figure 1*b*).

(ii) *How was it possible to perform the L-AP5 test before washout of the ability to induce LTP?*

The lack of synaptic enhancement in half (6 out of 12) of the recordings can be attributed to washout of LTP under whole-cell recording (Malinow & Tsien 1990). We kept the time before LTP induction short by testing the

antagonist on NMDA EPSCs first, without knowledge of whether the fixed stimulus would yield an all-silent connection at −60 mV. On average, the pairing protocol was applied 13.3 min after the initiation of whole-cell recording. In line with our 50% success in LTP induction around this time, previous work in acute slices has indicated that LTP could be induced with an acceptable rate of success 12–20 min after the initiation of whole-cell recording (Magee & Johnston 1997; Otmakhova *et al*. 2000). By contrast, organotypic slices exhibit more rapid washout, requiring that baseline recordings be restricted to 2–5 min before LTP induction (Hayashi *et al.* 2000; Montgomery *et al.* 2001), leaving insufficient time for a test with L-AP5 (Montgomery *et al.* 2001).

(iii) *Do synapses displaying mixed AMPA/NMDA responses without induction behave similarly to those that have subsequently undergone LTP?*

Even if washout of the ability to induce LTP is a problem, one may test L-AP5 responsiveness at non-silent control synapses. When we studied such cases of mixed transmission (significant AMPA current at −60 mV as well as NMDAR current at +40 mV), we found that blockade of NMDAR currents by L-AP5 (250 μM) was always incomplete, even before any induction protocol (16 out of 16 cells, mean inhibition = 68.9 ± 3.7%). By contrast, at silent synapses, NMDA EPSCs were almost completely inhibited by L-AP5 (250 μM) (12 out of 12 cells, mean inhibition = 99.7 ± 1.0%).

(iv) *Is there a credible postsynaptic explanation for the change in antagonist sensitivity?*

Incorporation of NMDARs is not part of the prevailing hypothesis. In any case, the drug-free NMDA current hardly increases on average, so the change in antagonist sensitivity cannot be attributed to the hypothetical incorporation of L-AP5-insensitive NMDARs.

(v) *Can the competitive antagonist approach provide quantitative information about peak* $[glu]_{cleft}$*?*

A more quantitative estimate of peak $[glu]_{cleft}$ sensed by NMDARs at silent synapses can be obtained on the basis of antagonist characteristics, empirically derived from outside-out patch recordings from hippocampal glutamate receptors (Choi *et al.* 2000). Because complete block of channel opening can only occur if antagonist binding greatly outraces neurotransmitter binding (Clements 1996), we estimated that the peak $[glu]_{cleft}$ at silent synapses was far less than 170 μM. This would be far lower than the *ca*. 2 mM estimated for conventionally active synapses (Clements *et al.* 1992; Diamond & Jahr 1997), and would be expected to produce negligible activation of AMPARs, given their highly cooperative [glu] dependence (Rosenmund *et al.* 1998).

(b) *Modelling transmitter release and postsynaptic action*

(i) *Hypothetical explanations of the increased* $[glu]_{cleft}$

How might the glutamate concentration sensed by NMDA receptors undergo such a sharp increase in association with potentiation? The switch-like nature of LTP expression weighs against cell biological mechanisms that would be expected to develop gradually, such as major changes in cleft width, transport buffer capacity, or alignment between presynaptic release sites and postsynaptic receptor clusters (Renger *et al.* 2001). Setting these aside as highly speculative, several additional hypotheses must be considered. First, the glutamate content of vesicles might increase (Pothos *et al.* 1998). Second, glutamate might 'spill over' to a postsynaptic site from near-neighbour synapses before pairing, only to be supplanted after potentiation by neurotransmitter release from the presynaptic terminal directly apposed to the site (Kullmann & Asztely 1998). Third, changes in $[glu]_{cleft}$ might arise from altered fusion pore dynamics (Choi *et al.* 2000). If this occurred as a result of LTP, vesicular glutamate at silent synapses might trickle out slowly enough to minimally activate AMPA receptors before induction, but produce easily detectable peak concentrations after LTP.

(ii) *Precedents from other systems*

Electrophysiological studies in non-neuronal secretory cells, mostly using capacitance measurements, have demonstrated that fusion pores can show multiple modes of operation (e.g. Spruce *et al.* 1990; Lollike *et al.* 1998). These modes can be distinguished as non-expanding and rapidly expanding (figure 3*a*), corresponding respectively to slow and incomplete secretion or a rapid spike of release as detected by amperometry (Bruns & Jahn 1995). Acceleration of fusion pore expansion has been inferred as a consequence of altered interactions between munc18 and the SNARE protein syntaxin (Fisher *et al.* 2001). The rate of fusion pore expansion can be sharply increased by PKC (Scepek *et al.* 1998; Graham *et al.* 2000), a kinase long ago implicated in LTP (Malinow *et al.* 1989). Our own findings with optical probes in cultured hippocampal synapses suggest the existence of transient fusion pore openings with lifetimes in the millisecond range (N. C. Harata, S. Choi, J. L. Pyle, A. M. Aravanis and R. W. Tsien, unpublished observation). Recent capacitance measurements have revealed the existence of non-expanding fusion pores in microvesicles similar to small synaptic vesicles (Klyachko & Jackson 2002). The conductance of the non-expanding fusion pore is 11 times smaller than that of large dense core vesicles, leaving open the possibility of even slower transmitter release than that which had been supposed. Taken together, these observations lend credence to the possibility that presynaptic fusion pores at hippocampal synapses may be under modulatory control.

(iii) *Model of quantal responses at a glutamatergic synapse*

To explore the implications of fusion pore modulation, we constructed a detailed model of glutamatergic transmission (figures 3 and 4). Taking a lead from other secretory systems (figure 3*a*), we assumed that the fusion pore can switch between two patterns, a non-expanding fusion pore with a small conductance (mode I) and a rapidly expanding fusion pore (mode II) (figure 3*b*). The expansion rate of 25 nm ms^{-1} was typical of that thought to support rapid exocytosis in other systems (Stiles *et al.* 1996). We followed Rusakov & Kullmann (1998) in modelling the synaptic cleft and the porous extracellular space outside it (figure 4*a*). AMPA and NMDA receptors on the postsynaptic membrane were described by kinetics given respectively by Jonas *et al.* (1993) and Clements &

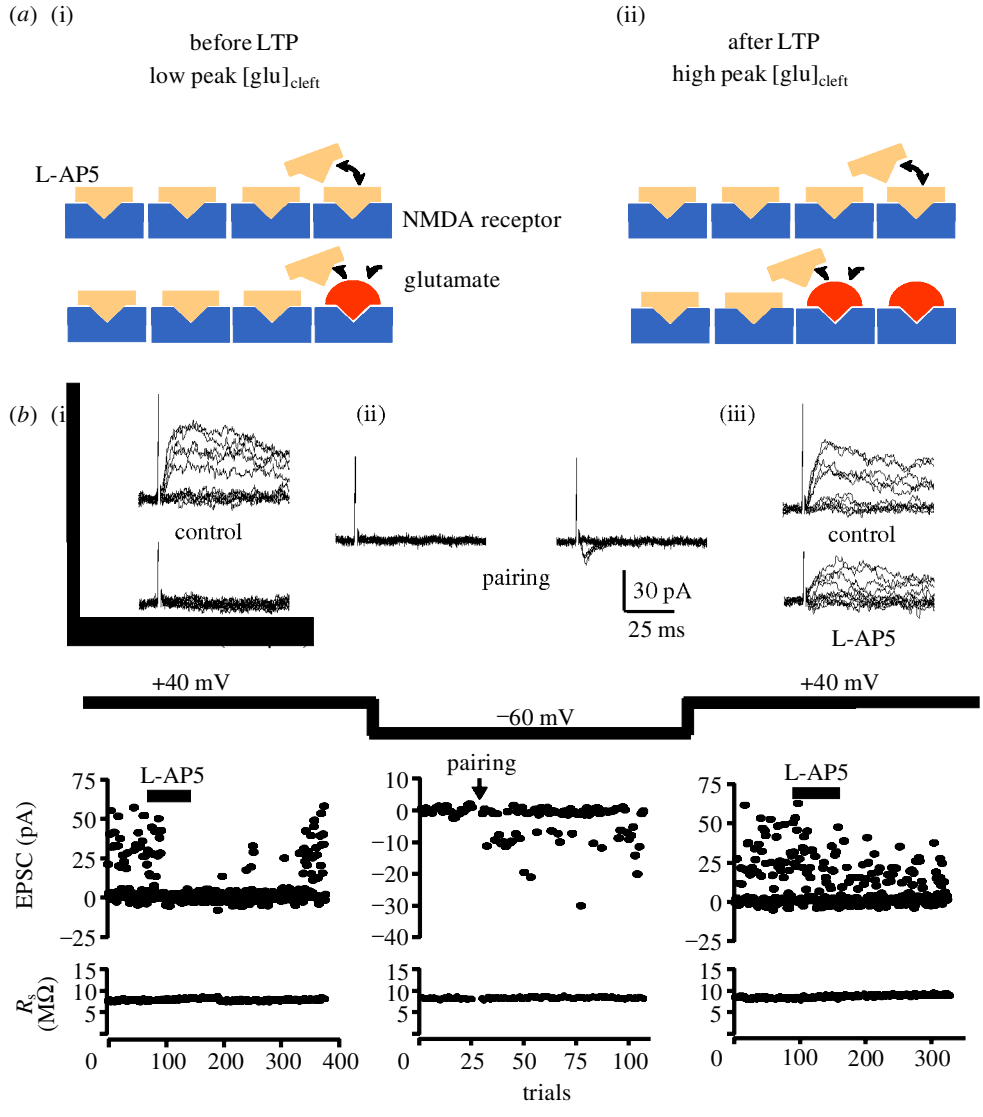

Figure 1. Use of an NMDAR antagonist to probe cleft glutamate concentration. (*a*) Theoretical basis for assessment of $[glu]_{cleft}$ using the fast unbinding antagonist, L-AP5. At a given time, there are a fraction of unoccupied receptors owing to the fast unbinding rate of L-AP5. Then, the probability for glutamate to bind to unoccupied receptors is proportional to the ratio between the concentration of glutamate and L-AP5. The higher the peak glutamate concentration, the less the inhibition by L-AP5. Therefore, if LTP involves increases in peak $[glu]_{cleft}$, one would expect less inhibition by L-AP5 after LTP induction. (After Clements (1996).) (*b*) Representative experiment showing that the degree of inhibition of NMDA EPSCs by L-AP5 decreased during conversion of silent synapses into functional ones. EPSCs were elicited at a frequency of 0.5 Hz. (i) and (iii) Groups of 10 consecutive NMDAR-mediated current records, taken at +40 mV before and after exposure to 250 μM L-AP5. (ii) AMPAR EPSCs, taken at −60 mV, showing conversion from all-silent to non-silent transmission. Note sudden appearance of AMPAR EPSCs and lack of change of series resistance (R_s). (From Choi *et al.* (2000).)

Westbrook (1991), with AMPAR desensitization rates set at a log midpoint between the estimates of Jonas & Sakmann (1992) and Raman & Trussell (1995). Figure 4*b* illustrates the calculated time-course of $[glu]_{cleft}$ for a vesicle containing 6000 transmitter molecules and a fusion pore undergoing the stepwise opening and immediate rapid expansion of mode II. The calculated $[glu]_{cleft}$ reached a peak value in the millimolar range, then decayed rapidly, in conformity with previous findings (Clements *et al.* 1992; Diamond & Jahr 1997; Liu *et al.* 1999). This standard transient may be compared with model predictions for the three scenarios for silent synapses. For a 10-fold reduction in vesicular transmitter contents, the calculated $[glu]_{cleft}$ was in essence a scaled-down version of the standard transient. For spillover, we calculated the glutamate concentration due to a neighbouring synapse positioned an average intersynaptic distance away (460 nm (Rusakov & Kullmann 1998)). The simulated $[glu]_{cleft}$ reached a lower peak, but then closely approximated the standard transient. Finally, for a non-expanding fusion pore (mode I, figure 2*b*), the predicted $[glu]_{cleft}$ also reached a peak near 100 nM, but then showed an extremely slow decay, owing to the extended time required for transmitter to escape the vesicle. The transient illustrated in figure 4*b* was based on the assumption that the fusion pore opened instantaneously to a fixed diameter of 1.8 nm, then closed again after 5 ms. The prolonged waveform of neurotransmitter is reminiscent of behaviour observed with amperometry in secretory cells (Bruns & Jahn 1995; Zhou *et al.* 1996).

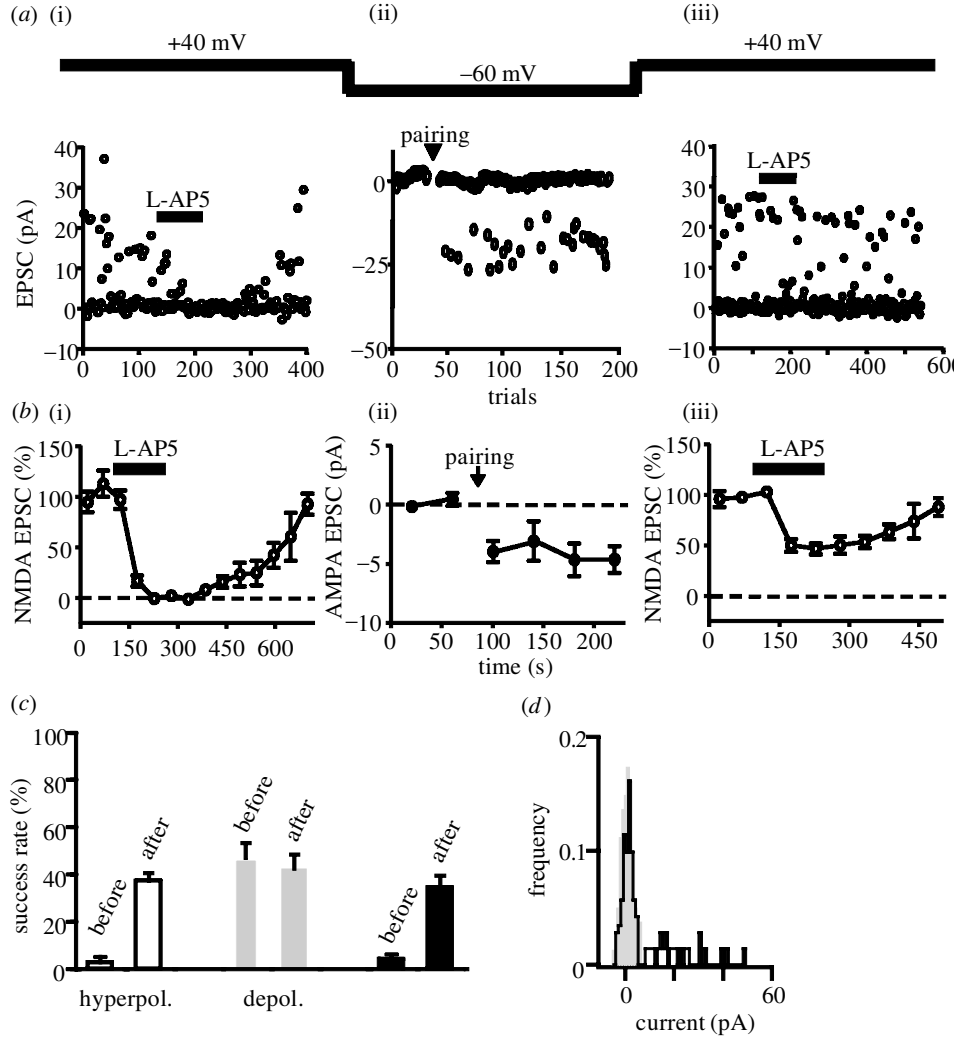

Figure 2. Collected results showing the use of L-AP5 to probe $[glu]_{cleft}$. (*a*) Another representative experiment further illustrating the clearcut difference in L-AP5 responsiveness before and after switch-like expression of LTP. (*b*) Pooled data from silent synapses before pairing, showing that 250 μM L-AP5 completely inhibited NMDAR EPSCs ($n = 12$). L-AP5 was applied for 150 s. Pairing given within 800 s after the start of the whole-cell recording caused an immediate but stable recruitment of AMPAR responses (ii) as seen in data averaged without exclusion of failures. After pairing and successful potentiation, repeated application of L-AP5 (same concentration and duration) inhibited NMDAR EPSCs to a lesser extent ((iii), $n = 6$). (*c*) Summary showing rate of EPSC successes (non-failures) before and after pairing in cells in which pairing induced potentiation ($n = 6$). The proportion of synaptic failures was estimated by doubling the fraction of responses with amplitude less than zero. The success rate of NMDA EPSCs at +40 mV (shaded bars) did not change significantly during LTP ($p > 0.05$). (*d*) Amplitude distribution of NMDAR EPSCs during exposure to 250 μM L-AP5, before pairing (shaded histogram) and after pairing (open histogram). Note that the behaviour of NMDARs treated with 250 μM L-AP5 mimicked that of AMPARs at silent synapses. From Choi *et al.* (2000).

(iv) *Distinguishing among expression scenarios*

The simulated $[glu]_{cleft}$ transients for reduced vesicular content or 'spill-over' each satisfied the criterion of generating nearly undetectable AMPA currents. However, in both of these cases, the predicted NMDA currents were also much smaller than their counterparts during standard transmission (simulations not shown). Only for the non-expanding fusion pore did the transient in $[glu]_{cleft}$ result in relatively large NMDA and relatively tiny AMPA components (figure 4*c*,*d*). Note that the fusion pore modelling provides a presynaptic rationale for the finding that LTP is associated with relatively little increase in NMDA current compared with the large potentiation of the AMPA current. Likewise, with appropriate assumptions about antagonist binding kinetics, it was possible within the non-expanding/expanding pore scenario to simulate the very different effects of 250 μM L-AP5 on silent synapses and standard transmission (figure 4*e*,*f*), whereas this was not feasible for the other cases (simulations not shown).

Another sharp distinction between scenarios hinges on the degree of AMPA receptor desensitization. Little desensitization arises from short-lived transients generated by 'spill-over' or by an expanding fusion pore, regardless of whether vesicular contents are normal or reduced. By contrast, the non-expanding fusion pore produced a prolonged, low-amplitude $[glu]_{cleft}$ transient that quickly drove AMPARs into a desensitized state. Accordingly, removal of desensitization has widely different consequences (figure 4*g*): only a modest (*ca.* 15%) increase of the simulated AMPA current in the case of a brief $[glu]_{cleft}$ transient (mode II), but a more than threefold increase in the ampli-

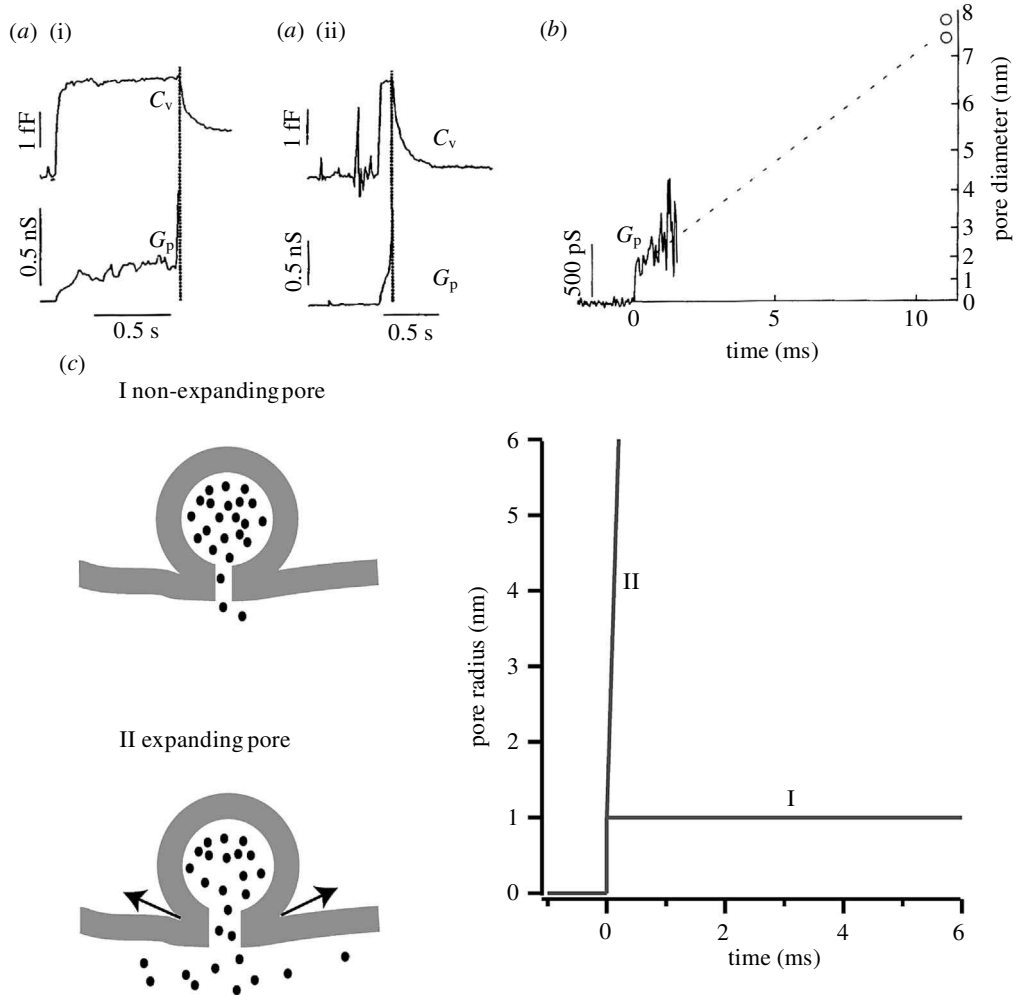

Figure 3. Modes of fusion pore opening studied with capacitance measurements in neutrophils. (*a*) Variety of behaviour, including a long-lived plateau of pore conductance (G_p), followed by rapid expansion (i), or a brief plateau of G_p, followed by rapid expansion (ii) C_v, vesicular capacitance. (From Lollike *et al.* (1998).) (*b*) Capacitance measurement in neutrophil from Spruce *et al.* (1990) showing the genesis of increased pore conductance (alternatively expressed as pore radius, right). (*c*) Proposed fusion modes (see text), opening to a small, fixed level (2 nm pore diameter), labelled mode I; opening to the same level but undergoing immediate rapid expansion at a rate of 25 nm ms^{-1}, typical of that believed to support rapid exocytosis, labelled mode II.

tude of the tiny events associated with a non-expanding pore (mode I). Indeed, the modelling suggested that transmission by AMPARs might become detectable at 'silent synapses' if desensitization of the receptors were pharmacologically inhibited. Events revealed in this way should have a slow rise and a relatively abrupt decay, faster than the decline of 'standard' events. This set of predictions was borne out by our experiments with cyclothiazide in area CA1 (Choi *et al.* 2000; see also Gasparini *et al.* 2000).

4. DISCUSSION

(a) *Changes in glutamate dynamics and pairing-induced LTP at CA3–CA1 synapses*

We have reviewed evidence that pairing-induced LTP in young hippocampal slices is associated with a dramatic increase in cleft neurotransmitter concentration. Our detection method relied on a local sensor, the NMDAR, that by definition must be present at synapses undergoing NMDAR-dependent, associative LTP. The use of a rapidly dissociating antagonist, L-AP5, is a well-accepted biophysical method for assaying peak cleft glutamate concentration (Clements 1996). By testing the method on NMDA receptors in outside-out patches of hippocampal membrane, we were able to set some quantitative limits. Before pairing, peak $[glu]_{cleft}$ at silent synapses was far below 170 μM, low enough to escape clear detection by postsynaptic AMPARs. After potentiation, peak $[glu]_{cleft}$ reached millimolar values, thus supporting NMDAR responses only partially blocked by 250 μM L-AP5, and yielding clearly detectable transmission via AMPARs (Choi *et al.* 2000). The use of NMDARs as reporters leaves open the possibility of concomitant changes in postsynaptic receptor properties, including incorporation of new AMPA receptors, phosphorylation of AMPARs, etc. Kinetic changes in the mode of gating of presynaptic fusion pores represent a precisely targeted action for which there is ample precedent in non-neuronal cells (figure 3*a*). The proposed mechanism offers considerable functional advantages for both synaptic plasticity and development. A basal state of local, NMDAR-only transmission would maximize the input-specificity of Hebb's rule, in contrast to the absence of proximal NMDA transmission envi-

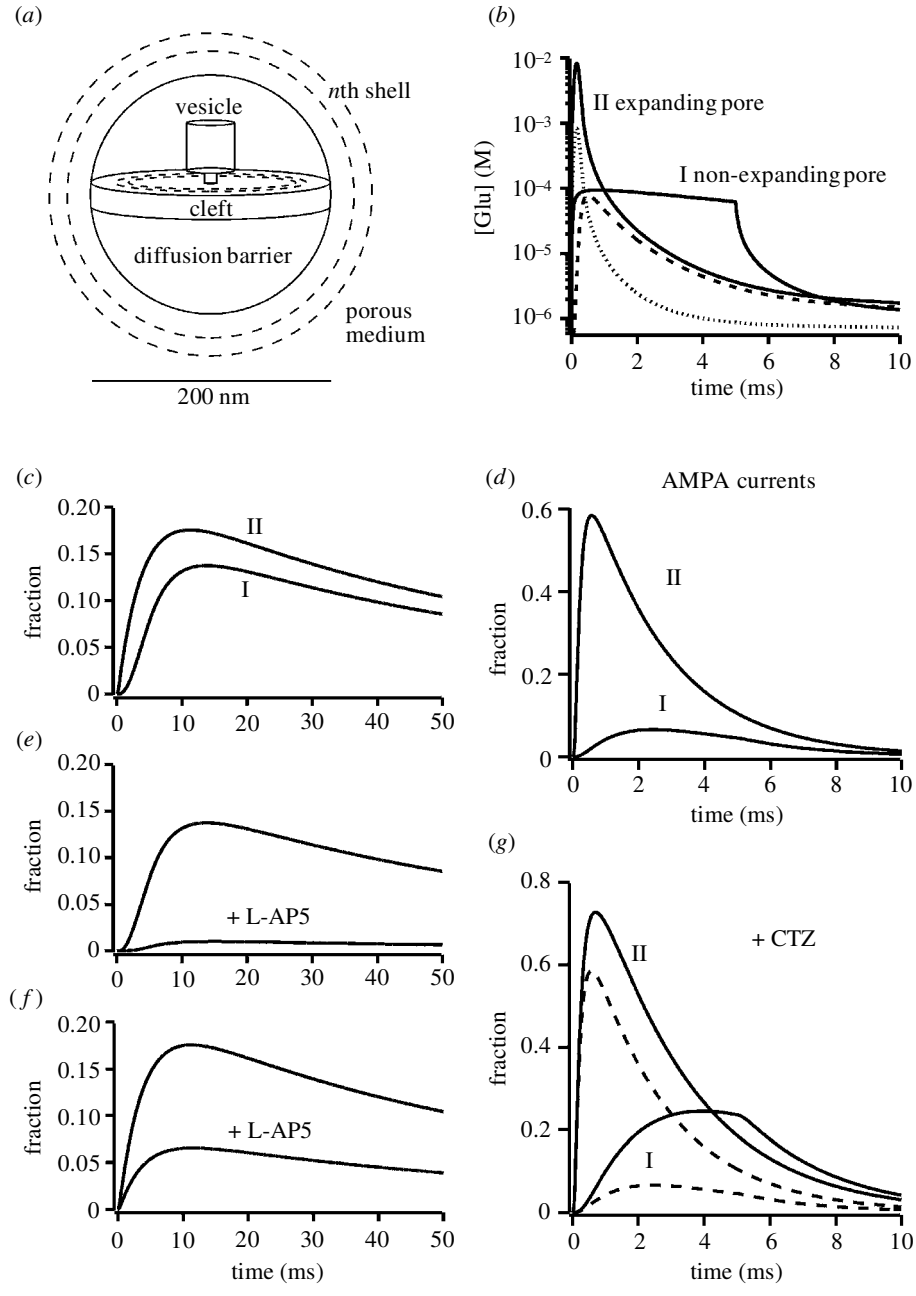

Figure 4. Modelling presynaptically based changes in glutamatergic transmission. (*a*) Cleft model supplemented with simulation of glutamate efflux from presynaptic vesicle (see § 2). A disc-shaped cleft was assigned a width of 20 nm and a radius of 100 nm. The glutamate diffusion coefficient (D_{glu}) was assumed to be 150 $\mu m^2 s^{-1}$. This value was chosen to allow the simulation of $[glu]_{cleft}$ to resemble experimental estimates with respect to the time-course of decay (Clements *et al.* 1992). (*b*) Calculated $[glu]_{cleft}$ for various scenarios: 'standard transmission', a vesicular content of 6000 glutamate molecules, escaping through a rapidly expanding fusion pore (mode II); similar to 'standard transmission' but with only 600 glutamate molecules (dotted line); similar to 'standard transmission' but assessed at a position 465 nm away from the centre of the cleft (dashed line); non-expanding fusion pore (mode I, see text). (*c*) fractional activation of NMDARs driven by $[glu]_{cleft}$ for modes I and II. Note that NMDARs show similar amplitudes because of combined influence of strength and duration of $[glu]_{cleft}$ transient. A small change in the time course of NMDAR activation was predicted but would be difficult to resolve because of stochastic channel gating. (*d*) Fractional activation of AMPARs for modes I and II. Differential between fractional activation of AMPARs would be further increased if AMPAR kinetics took account of binding of four glutamates (Rosenmund *et al.* 1998). In contrast to changes in $[glu]_{cleft}$, increasing the number of AMPARs would not change activation kinetics significantly but would simply increase the amplitude of the current. (*e*), (*f*) Simulated effects of L-AP5 on NMDAR activation with scenarios I, non-expanding pore (*e*) and II, expanding pore (*f*). (*g*) Simulated effects of cyclothiazide on AMPAR activation with scenarios I and II.

sioned in the spillover hypothesis (Kullmann & Asztely 1998). It would also allow for rapid, stepwise increases in AMPAR transmission (e.g. figure 2*a*), difficult to achieve by other cell biological mechanisms (Liao *et al.* 1999).

(b) *Insights from a model for glutamate dynamics in the synaptic cleft*

We have presented a quantitative model of glutamatergic transmission that adheres closely to published infor-

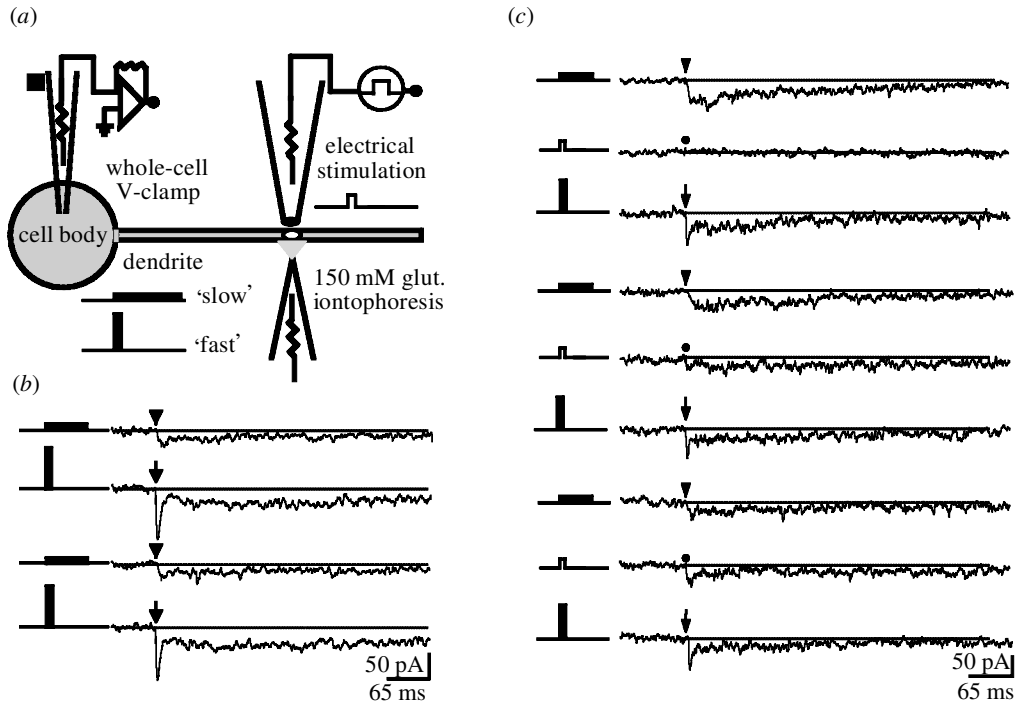

Figure 5. Concentration profile of neurotransmitter delivery determines AMPAR activation. (*a*) Experimental design for alternately stimulating presynaptic release and iontophoretically probing postsynaptic receptors. Iontophoresis and stimulating electrodes are brought to within 1 μm of an isolated synapse. Filled vertical and horizontal bars represent 'fast' (1 ms, 100 nA) and 'slow' (10 ms, 10 nA) iontophoretic application parameters. (*b*) AMPA receptors are not significantly activated by a slow flux of glutamate. Slow pulses elicited NMDAR-only responses, while fast pulses elicited AMPAR and NMDAR responses from the same site. Because AMPAR activation was sensitive to sudden increases in neurotransmitter concentration, AMPA-quiet responses could be generated at synapses with functional AMPARs. (*c*) Silent synapses contain functional AMPARs. $EPSC_{AMPA\text{-}quiet}$ responses, resulting from endogenous transmitter release, were evoked by presynaptic electrical stimulation at a 9 DIV synapse (open vertical bars). Trials with synaptic stimulation were interleaved with iontophoretic applications of neurotransmitter. Presynaptically evoked $EPSC_{AMPA\text{-}quiet}$ responses were scattered among full-fledged AMPAR responses to fast iontophoretic pulses, indicating that AMPARs were functional despite the finding of AMPA-quiet synaptic events ($n = 4$). (From Renger *et al.* (2001), with permission.)

mation on fusion pore, cleft geometry and postsynaptic receptor kinetics. We were able to simulate an increase in $[glu]_{cleft}$ due to the conversion of presynaptic fusion pores from non-expanding to rapidly expanding. Modelling of this kind provides some useful lessons.

(i) It dispels simple-minded calculations which estimate peak $[glu]_{cleft}$ by taking the transmitter content of a vesicle and dispersing it uniformly within the volume of the synaptic cleft, without consideration of rates of diffusion into and out of the cleft.

(ii) It successfully simulates the finding that $[glu]_{cleft}$ decays with multiple exponential components (Clements 1996).

(iii) It provides a theoretical framework for understanding why neither AMPARs nor NMDARs are regularly saturated by quantal release of glutamate (Dube & Liu 1999; Liu *et al.* 1999; McAllister & Stevens 2000; Oertner *et al.* 2002).

(iv) It provides perspective on the hypothesis that NMDA-only transmission reflects failures of release from the immediately apposed presynaptic terminal, amidst spillover of glutamate from neighbouring boutons (Kullmann & Asztely 1998). Simulation of the diffusion of glutamate from nearby synapses did not predict enough spillover to support full-blown NMDAR-only synaptic responses (figure 2); in addition, the incidence of NMDAR successes failed to increase after pairing (figure 1), excluding the predicted increase in the release probability of the immediately presynaptic terminal.

(v) It focuses interest on the concept of 'kiss and run' fusion, and the possibility that a fusion pore might open up to a small, sustained conductance level, possibly shutting even before all the neurotransmitter had escaped.

(c) *Complementary studies in hippocampal cultures and slices from other groups*

The idea that the concentration profile of glutamate delivery governs AMPAR activation was directly tested by the group of Guosong Liu (Renger *et al.* 2001). Using iontophoretic application of various glutamate waveforms (figure 5), they demonstrated that 'fast' (1 ms, 100 nA) application of glutamate elicited both AMPAR and NMDAR currents, whereas 'slow' (10 ms, 10 nA) applications evoked very similar NMDAR currents but no detectable AMPAR response. Both kinds of responses were registered as inward currents near the normal resting potential, the NMDAR distinguished by its slow kinetics. Their results are in excellent accord with our modelling

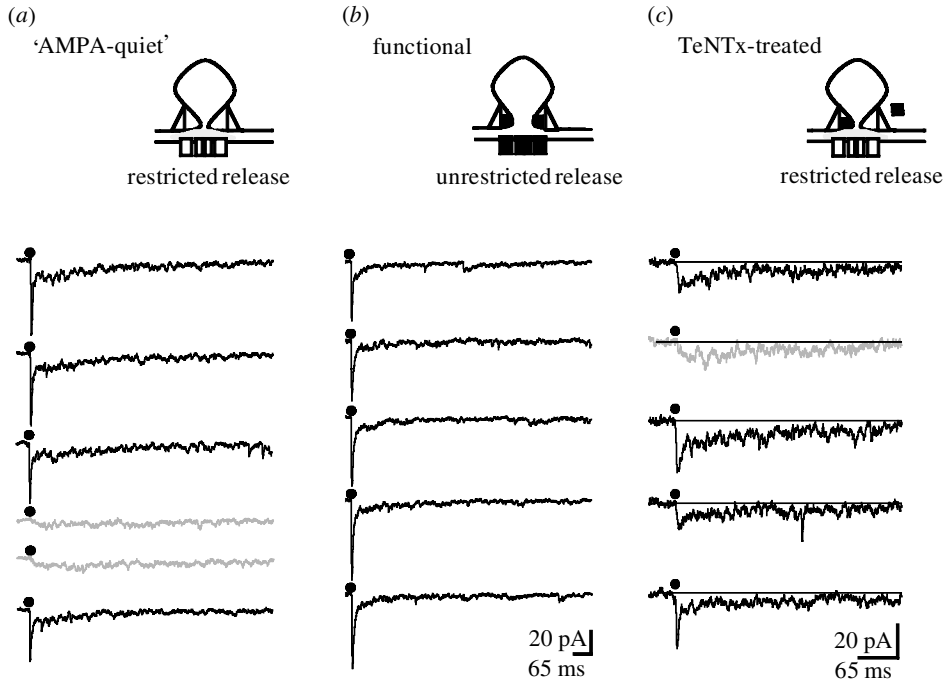

Figure 6. Maturation of glutamate flux affects receptor activation. Neurotransmitter concentration could be altered through controlling the diffusion of glutamate into the synaptic cleft. For example, the synaptic vesicle pore conductance may be larger during a functional than a silent event. (*a*) AMPA-quiet events could be due to slow release through a non-expanding fusion pore. Current traces (bottom) show that series of evoked events varied between $EPSC_{dual}$ (black traces) and $EPSC_{AMPA\text{-}quiet}$ (grey traces) responses at single synapses. (*b*) Functional events could be due to fast release through rapidly expanding fusion pore. As shown in current traces (bottom), a higher proportion of evoked $EPSC_{dual}$ events was observed. (*c*) TeNtx treatment converts functional events to AMPA-quiet ones. Representative series of evoked EPSCs (bottom) from a single synapse (DIV 21) after 1 h TeNTx treatment (0 mM Mg^{2+}). Evoked EPSCs fluctuated between $EPSC_{AMPA\text{-}quiet}$ and $EPSC_{dual}$ among trials, reminiscent of evoked EPSCs from immature synapses. (From Renger *et al.* (2001), with permission.)

of the downstream responses to glutamate release from rapidly expanding and non-expanding fusion pores. Renger *et al.* (2001) went on to stimulate synaptic transmission, and found 'AMPA-quiet' as well as mixed synaptic responses. The 'AMPA-quiet' responses could be interleaved with clearcut AMPAR responses to 'fast' iontophoretic application. Thus, it was concluded that a 'silent synapse' can be generated by a fluctuating presynaptic mechanism, even under conditions where clearcut and steady AMPAR responsiveness already exists.

In another important experiment, Renger *et al.* (2001) showed that AMPA-quiet behaviour gradually disappears with development between 9 and 13 days *in vitro* (figure 6*a*,*b*). However, application of a well-known presynaptic toxin that cleaves SNARE proteins, TeNTx, converted the fully functional synapses back into ones that alternated between silent and non-silent behaviour (figure 6*c*). The effectiveness of TeNTx provides another indication that the presynaptic fusion machinery may be involved causing modulation of $[glu]_{cleft}$.

A change in the mode of opening of presynaptic fusion pores provides one way of explaining previous findings of an increased uptake of presynaptic markers such as the antibody against synaptotagmin I (Malgaroli *et al.* 1995), or accelerated destaining of the fluorescent vesicle marker FM1–43 (Ryan *et al.* 1996). The use of FM1–43 in slices by the Siegelbaum and Stanton groups has supported the idea that presynaptic vesicular dynamics are significantly altered during certain forms of LTP and LTD (Stanton *et al.* 2001; Zakharenko *et al.* 2001, 2002). Finally, imaging of EPSCaTs at single synapses by Fine, Bliss and colleagues (Emptage *et al.* 1999) has demonstrated an increase in the likelihood of these jointly NMDAR-, AMPAR-dependent events. One might interpret their data as a graded increase in the proportion of AMPA-unsilent events, from an initial nonzero value to a higher level after induction (see also Renger *et al.* 2001), thus providing an analogue increase in synaptic strength (Dixon *et al.* 2002). The EPSCaT imaging experiments inherently select for unitary connections that are not entirely AMPA-silent, whereas the experiments in figures 1 and 2 were designed to probe 'all-silent' synapses. Nevertheless, both sets of results are compatible with scenarios wherein presynaptic terminals distribute their time among distinct fusion modes.

(d) *Possible coexistence of postsynaptic and presynaptic mechanisms for unsilencing*

It is important to note that a mechanism of fusion pore modulation and modification of postsynaptic receptor properties are not mutually exclusive. Perhaps both mechanisms coexist but unfold over somewhat different time domains. There is evidence that postsynaptically silent synapses containing only NMDARs might form first during early development, while AMPA receptors are added later by some mechanism akin to LTP (Gomperts *et al.* 1998; Liao *et al.* 1999; Nüsser *et al.* 1998). However, there is disagreement between the relatively low proportion of synapses that are immunochemically identifiable as potentially NMDA receptor-only synapses (17–28%; Nüsser *et*

al. 1998) and the much higher proportion of physiologically silent events. This opens up the possibility that physiologically silent events do not represent one entity, but a mixture of pre- and postsynaptically silent synapses. One might even imagine the simultaneous coexistence of pre- and postsynaptic mechanisms for attenuating synaptic strength, a 'belt and braces' scenario, that would maximize the distinction between dormant and awakened synaptic connnections. Unsilencing might involve coordinated changes in the mode of release and an increase in postsynaptic receptivity. This has some teleological appeal, but it also calls for a case-by-case examination of the specific role of each mechanism in different brain regions at different stages of development. In one particular region of interest, the associational connections among CA3 neurons, Montgomery *et al.* (2001) have presented compelling evidence that postsynaptic unsilencing must occur. It would be interesting to look for presynaptic fusion pore modulation in the same region, under conditions in which the L-AP5 approach is experimentally feasible.

We are grateful to D. Ramot for a careful reading of the manuscript. This work was supported by a Silvio Conte Center for Neuroscience Research (NIMH) to R.W.T. and by Korea Ministry of Science and Technology grant M1-0108-00-0051 under the neurobiology research programme to S.C.

REFERENCES

Bliss, T. V. & Collingridge, G. L. 1993 A synaptic model of memory: long-term potentiation in the hippocampus. *Nature* **361**, 31–39.

Bruns, D. & Jahn, R. 1995 Real-time measurement of transmitter release from single synaptic vesicles. *Nature* **377**, 62–65.

Choi, S., Klingauf, J. & Tsien, R. W. 2000 Postfusional regulation of cleft glutamate concentration during LTP at 'silent synapses'. *Nature Neurosci.* **3**, 330–336.

Clements, J. D. 1996 Transmitter time course in the synaptic cleft: its role in central synaptic function. *Trends Neurosci.* **19**, 163–171.

Clements, J. D. & Westbrook, G. L. 1991 Activation kinetics reveal the number of glutamate and glycine binding sites on the N-methyl-D-aspartate receptor. *Neuron* **7**, 605–613.

Clements, J. D., Lester, R. A., Tong, G., Jahr, C. E. & Westbrook, G. L. 1992 The time course of glutamate in the synaptic cleft. *Science* **258**, 1498–1501.

Diamond, J. S. & Jahr, C. E. 1997 Transporters buffer synaptically released glutamate on a submillisecond time-scale. *J. Neurosci.* **17**, 4672–4687.

Dixon, D. B., Bliss, T. V. P. & Fine, A. 2002 Individual hippocampal synapses express incremental (analog) and bi-directional long-term plasticity. *Soc. Neurosci. Abstr.* **28**, 150.3.

Dube, G. R. & Liu, G. 1999 AMPA and NMDA receptors display similar affinity during rapid synaptic-like glutamate applications. *Soc. Neurosci. Abstr.* **25**, 992.

Durand, G. M., Kovalchuk, Y. & Konnerth, A. 1996 Long-term potentiation and functional synapse induction in developing hippocampus. *Nature* **381**, 71–75.

Emptage, N., Bliss, T. V. P. & Fine, A. 1999 Single synaptic events evoke NMDA receptor mediated release of calcium from internal stores in hippocampal dendritic spines. *Neuron* **22**, 115–124.

Fisher, R. J., Pevsner, J. & Burgoyne, R. D. 2001 Control of fusion pore dynamics during exocytosis by Munc18. *Science* **291**, 875–878.

Gasparini, S., Saviane, C., Voronin, L. L. & Cherubini, E. 2000 Silent synapses in the developing hippocampus: lack of functional AMPA receptors or low probability of glutamate release? *Proc. Natl Acad. Sci. USA* **97**, 9741–9746.

Gomperts, S. N., Rao, A., Craig, A. M., Malenka, R. C. & Nicoll, R. A. 1998 Postsynaptically silent synapses in single neuron cultures. *Neuron* **21**, 1443–1451.

Graham, M. E., Fisher, R. J. & Burgoyne, R. D. 2000 Measurement of exocytosis by amperometry in adrenal chromaffin cells: effects of clostridial neurotoxins and activation of protein kinase C on fusion pore kinetics. *Biochimie* **82**, 469–479.

Hayashi, Y., Shi, S. H., Esteban, J. A., Piccini, A., Poncer, J. C. & Malinow, R. 2000 Driving AMPA receptors into synapses by LTP and CaMKII: requirement for GluR1 and PDZ domain interaction. *Science* **287**, 2262–2267.

Isaac, J. T., Nicoll, R. A. & Malenka, R. C. 1995 Evidence for silent synapses: implications for the expression of LTP. *Neuron* **15**, 427–434.

Jonas, P. & Sakmann, B. 1992 Glutamate receptor channels in isolated patches from CA1 and CA3 pyramidal cells of rat hippocampal slices. *J. Physiol. (Lond.)* **455**, 143–171.

Jonas, P., Major, G. & Sakmann, B. 1993 Quantal components of unitary EPSCs at the mossy fibre synapse on CA3 pyramidal cells of rat hippocampus. *J. Physiol. (Lond.)* **472**, 615–663.

Klyachko, V. A. & Jackson, M. B. 2002 Capacitance steps and fusion pores of small and large-dense-core vesicles in nerve terminals. *Nature* **418**, 89–92.

Kullmann, D. M. & Asztely, F. 1998 Extrasynaptic glutamate spillover in the hippocampus: evidence and implications. *Trends Neurosci.* **21**, 8–14.

Liao, D., Hessler, N. A. & Malinow, R. 1995 Activation of postsynaptically silent synapses during pairing-induced LTP in CA1 region of hippocampal slice. *Nature* **375**, 400–404.

Liao, D., Zhang, X., O'Brien, R., Ehler, M. D. & Huganir, R. L. 1999 Regulation of morphological postsynaptic silent synapses in developing hippocampal neurons. *Nature Neurosci.* **2**, 37–43.

Liu, G., Choi, S. & Tsien, R. W. 1999 Variability of neurotransmitter concentration and nonsaturation of postsynaptic AMPA receptors at synapses in hippocampal cultures and slices. *Neuron* **22**, 395–409.

Lollike, K., Borregaard, N. & Lindau, M. 1998 Capacitance flickers and pseudoflickers of small granules, measured in the cell-attached configuration. *Biophys. J.* **75**, 53–59.

McAllister, A. K. & Stevens, C. F. 2000 Nonsaturation of AMPA and NMDA receptors at hippocampal synapses. *Proc. Natl Acad. Sci. USA* **97**, 6173–6178.

McNaughton, B. L. & Morris, R. G. M. 1987 Hippocampal synaptic enhancement and information storage within a distributed memory system. *Trends Neurosci.* **10**, 408–415.

Magee, J. C. & Johnston, D. 1997 A synaptically controlled, associative signal for Hebbian plasticity in hippocampal neurons. *Science* **275**, 209–213.

Malgaroli, A. & Tsien, R. W. 1992 Glutamate-induced long-term potentiation of the frequency of miniature synaptic currents in cultured hippocampal neurons. *Nature* **357**, 134–139.

Malgaroli, A., Ting, A. E., Wendland, B., Bergamaschi, A., Villa, A., Tsien, R. W. & Scheller, R. H. 1995 Presynaptic component of long-term potentiation visualized at individual hippocampal synapses. *Science* **268**, 1624–1628.

Malinow, R. & Tsien, R. W. 1990 Presynaptic enhancement shown by whole-cell recordings of long-term potentiation in hippocampal slices. *Nature* **346**, 177–180.

Malinow, R., Schulman, H. & Tsien, R. W. 1989 Inhibition of postsynaptic PKC or CaMKII blocks induction but not expression of LTP. *Science* **245**, 862–866.

Montgomery, J. M., Palvlidis, P. & Madison, D. V. 2001 Pair recordings reveal all-silent synaptic connections and the postsynaptic expression of long-term potentiation. *Neuron* **29**, 691–701.

Nüsser, Z., Lujan, R., O'Brien, R. J., Kamboj, S., Ehler, M. D., Rosen, K. R., Fischbach, G. D. & Huganir, R. L. 1998 Cell type and pathway dependence of synaptic AMPA receptor number and variability in the hippocampus. *Neuron* **21**, 545–559.

Oertner, T. G., Sabatini, B. L., Nimchinsky, E. A. & Svoboda, K. 2002 Facilitation at single synapses probed with optical quantal analysis. *Nature Neurosci.* **5**, 657–664.

Olverman, H. J., Jones, A. W., Mewett, K. N. & Watkins, J. C. 1988 Structure/activity relations of N-methyl-D-aspartate receptor ligands as studied by their inhibition of [^{3}H]D-2-amino-5-phosphonopentanoic acid binding in rat brain membranes. *Neuroscience* **26**, 17–31.

Otmakhova, N. A., Otmakhov, N., Mortenson, L. H. & Lisman, J. E. 2000 Inhibition of the cAMP pathway decreases early long-term potentiation at CA1 hippocampal synapses. *J. Neurosci.* **20**, 4446–4451.

Pothos, E. N., Przedborski, S., Davila, V., Schmitz, Y. & Sulzer, D. 1998 D2-Like dopamine autoreceptor activation reduces quantal size in PC12 cells. *J. Neurosci.* **18**, 4106–4118.

Pyle, J. L., Kavalali, E. T., Choi, S. & Tsien, R. W. 1999 Visualization of synaptic activity in hippocampal slices with FM1-43 enabled by fluorescence quenching. *Neuron* **24**, 803–808.

Raman, I. M. & Trussell, L. O. 1995 The mechanism of alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate receptor desensitization after removal of glutamate. *Biophys. J.* **68**, 137–146.

Renger, J. J., Egles, C. & Liu, G. 2001 A developmental switch in neurotransmitter flux enhances synaptic efficacy by affecting AMPA receptor activation. *Neuron* **29**, 469–484.

Rosenmund, C., Stern-Bach, Y. & Stevens, C. F. 1998 The tetrameric structure of a glutamate receptor channel. *Science* **280**, 1596–1599.

Rusakov, D. A. & Kullmann, D. M. 1998 Extrasynaptic glutamate diffusion in the hippocampus: ultrastructural constraints, uptake, and receptor activation. *J. Neurosci.* **18**, 3158–3170.

Ryan, T. A., Ziv, N. E. & Smith, S. J. 1996 Potentiation of evoked vesicle turnover at individually resolved synaptic boutons. *Neuron* **17**, 125–134.

Scepek, S., Coorssen, J. R. & Lindau, M. 1998 Fusion pore expansion in horse eosinophils is modulated by Ca^{2+} and protein kinase C via distinct mechanisms. *EMBO J.* **17**, 4340–4346.

Shi, S. H., Hayashi, Y., Petralia, R. S., Zaman, S. H., Wenthold, R. J., Svoboda, K. & Malinow, R. 1999 Rapid spine delivery and redistribution of AMPA receptors after synaptic NMDA receptor activation. *Science* **284**, 1811–1816.

Shi, S. H., Hayashi, Y., Esteban, J. A. & Malinow, R. 2001 Subunit-specific rules governing AMPA receptor trafficking to synapses in hippocampal pyramidal neurons. *Cell* **105**, 331–343.

Spruce, A. E., Breckenridge, L. J., Lee, A. K. & Almers, W. 1990 Properties of the fusion pore that forms during exocytosis of a mast cell secretory vesicle. *Neuron* **4**, 643–654.

Stanton, P. K., Heinemann, U. & Muller, W. 2001 FM1-43 imaging reveals cGMP-dependent long-term depression of presynaptic transmitter release. *J. Neurosci.* **21**, RC167.

Stiles, J. R., Van Helden, D., Bartol Jr, T. M., Salpeter, E. E. & Salpeter, M. M. 1996 Miniature endplate current rise times less than 100 microseconds from improved dual recordings can be modeled with passive acetylcholine diffusion from a synaptic vesicle. *Proc. Natl Acad. Sci. USA* **93**, 5747–5752.

Wadiche, J. I. & Kavanaugh, M. P. 1998 Macroscopic and microscopic properties of a cloned glutamate transporter/chloride channel. *J. Neurosci.* **18**, 7650–7661.

Zakharenko, S. S., Zablow, L. & Siegelbaum, S. A. 2001 Visualization of changes in presynaptic function during long-term synaptic plasticity. *Nature Neurosci.* **4**, 711–717.

Zakharenko, S. S., Zablow, L. & Siegelbaum, S. A. 2002 Altered presynaptic vesicle release and cycling during mGluR-dependent LTD. *Neuron* **35**, 1099–1110.

Zhou, Z., Misler, S. & Chow, R. H. 1996 Rapid fluctuations in transmitter release from single vesicles in bovine adrenal chromaffin cells. *Biophys. J.* **70**, 1543–1552.

GLOSSARY

AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

AMPAR: AMPA receptor

CA1 and CA3: anatomical regions of the hippocampal formation (CA stands for '*cornu Ammon* or Ammon's horn)

EPSCaT: excitatory postsynaptic Ca^{2+} transient

FM1-43: *N*-(3-triethylammoniumpropyl)-4-(4-(dibutylamino)styryl)pyridinium dibromide, a fluorescent dye used to monitor vesicular traffic

LTD: long-term depression

LTP: long-term potentiation

NMDA: *N*-methyl-D-aspartate

NMDAR: NMDA receptor

PKC: protein kinase C

R-CPP: 3-(*R*)-2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid

SNARE: soluble *N*-ethylmaleimide-sensitive-factor attachment protein

TeNTx: tetanus neurotoxin