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Extrasynaptic Glutamate Spillover in the Hippocampus: Dependence on Temperature and the Role of Active Glutamate Uptake

Fredrik Asztely, Gül Erdemli,* and Dimitri M. Kullmann
Department of Clinical Neurology
Institute of Neurology
Queen Square
London WC1N 3BG
United Kingdom

Summary

At excitatory synapses on CA1 pyramidal cells of the hippocampus, a larger quantal content is sensed by *N*-methyl-D-aspartic acid receptors (NMDARs) than by α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs). A novel explanation for this discrepancy is that glutamate released from terminals presynaptic to one cell can diffuse to and activate NMDARs, but not AMPARs, on a neighboring cell. If this occurs in the living brain, it could invalidate the view that glutamatergic synapses function as private communication channels between neurons. Here, we show that the discrepancy in quantal content mediated by the two receptors is greatly decreased at physiological temperature, compared with conventional recording conditions. This effect of temperature is not due to changes in release probability or uncovering of latent AMPARs. It is, however, partially reversed by the glutamate uptake inhibitor dihydrokainate. The results suggest that glutamate transporters play a critical role in limiting the extrasynaptic diffusion of glutamate, thereby minimizing cross-talk between neighboring excitatory synapses.

Introduction

At Schaffer collateral-CA1 pyramidal cell synapses studied in vitro, the average number of quanta of transmitter (the quantal content) sensed by *N*-methyl-D-aspartate receptors (NMDARs) is consistently larger than that sensed by α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs). This was first suggested by a large discrepancy between the coefficients of variation (CV) of the NMDAR- and AMPAR-mediated components of excitatory postsynaptic currents (EPSCs) (Kullmann, 1994; Selig et al., 1995). In keeping with this, very weak presynaptic stimuli can sometimes give rise to signals mediated by NMDARs but not AMPARs (Isaac et al., 1995; Liao et al., 1995; Durand et al., 1996). Moreover, when the stimulus intensity is adjusted to give intermittent failures of transmission, the failure rate is generally higher for the AMPAR- than for the NMDAR-mediated components of the postsynaptic signal (Liao et al., 1995). Although these observations were originally interpreted as reflecting absence of functional AMPAR clusters at a proportion of 'postsynaptically silent' synapses, an alternative explanation is that NMDARs sense glutamate released not only from directly apposed presynaptic terminals but also spilling over from terminals presynaptic to neighboring cells (Kullmann and Siegelbaum, 1995; Kullmann et al., 1996). AMPARs, on the other hand, only respond to glutamate released from the immediately apposed presynaptic terminals because they have a much lower affinity for glutamate than do NMDARs (Patneau and Mayer, 1990). According to this 'spillover' model, NMDARs sense transmitter released stochastically from a larger number of release sites than AMPARs, explaining the discrepancy in quantal content. The observation that minimal stimulation can elicit pure NMDAR-mediated EPSCs is moreover explained by postulating that none of the active synapses are in direct contact with the postsynaptic cell, and that the NMDAR-mediated signals in fact originate from glutamate released from terminals that are presynaptic to neighboring cells.

We have recently obtained circumstantial evidence for glutamate spillover by comparing long-term potentiation (LTP) of the AMPAR- and NMDAR-mediated components of synaptic signals in CA1 pyramidal cells (Kullmann et al, 1996): pairing-induced LTP of the NMDAR-mediated component is smaller than LTP of the AMPAR-mediated component and is associated with an apparently smaller fractional increase in quantal content, as expected if the major locus of expression were presynaptic and if much of the NMDAR-mediated component were caused by glutamate spilling over from neighboring unpotentiated terminals. In addition, preventing tetanic LTP of the AMPAR-mediated component of the postsynaptic signal in one cell does not abolish LTP of the NMDAR-mediated component, suggesting that NMDARs can sense potentiated glutamate release from synapses on neighboring cells.

Extrasynaptic glutamate spillover could have major repercussions for information processing if it occurs in the living brain, since it implies significant cross-talk between neighboring synapses. The studies referred to above were, however, carried out at room temperature, and since diffusion, receptor kinetics, and active neurotransmitter transport are all temperature dependent, it is important to reexamine the evidence for glutamate spillover under more physiological conditions. Here, we show that the discrepancy in quantal content, although still present, is greatly reduced at physiological temperatures. We provide evidence that active glutamate uptake plays a critical role in limiting the extent of extrasynaptic activation of NMDARs.

Results

Quantal Content of AMPAR- and NMDAR-Mediated Signals at Different Temperatures

If the discrepancy in quantal content mediated by NMDARs and AMPARs (m_{NMDA} and m_{AMPA}, respectively) reflects extrasynaptic glutamate spillover, then differences in the extent of the spillover phenomenon should be reflected in the ratio m_{NMDA}/m_{AMPA}. We applied two

*Present address: Department of Physiology, University of Wales College of Cardiff, Cardiff CF1 1SS, United Kingdom.

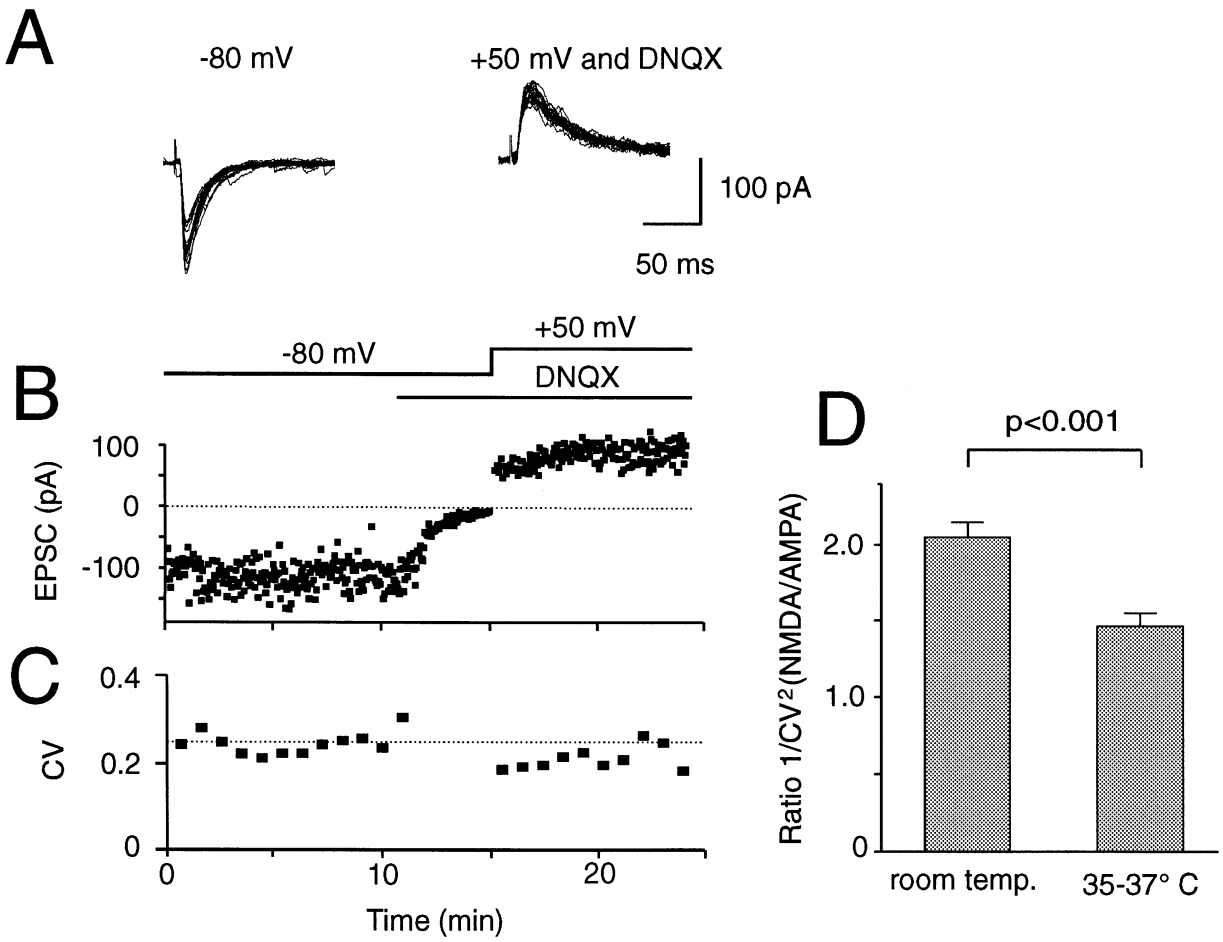


Figure 1. Comparison of EPSC Variance Mediated by AMPARs and NMDARs

(A) Sample EPSCs recorded in one cell at 35°C at −80 (left) and +50 mV in the presence of 10 μM DNQX (right). Twenty successive trials are plotted in each case.

(B) EPSC peak amplitudes plotted against time, with holding potential and application of DNQX indicated schematically.

(C) CV of successive groups of 20 trials plotted against time. The dotted line shows the average CV for the trials recorded at −80 mV.

(D) $1/CV^2$ for the NMDAR-mediated component, expressed as a ratio of $1/CV^2$ for the AMPAR-mediated component. The averages and SEM are plotted for 50 cells at room temperature (22°–25°C) and 31 cells at physiological temperatures (35°–37°C).

approaches to compare m_{NMDA}/m_{AMPA} at room temperature (22°–26°C) and at near physiological temperature (35°–37°C). First, we measured the trial-to-trial variability of population EPSCs elicited in CA1 cells by repeated stimulation in stratum radiatum under two conditions (Kullmann, 1994): initially the postsynaptic cell was kept hyperpolarized at −80 mV to prevent opening of NMDAR-linked ionophores, so that the EPSCs were mediated by AMPARs; subsequently, the AMPARs were blocked by the selective non-NMDAR antagonist 6,7-dinitroquinoxaline-2,3-dione (DNQX), and the cell was held at +40 to +50 mV to relieve the voltage-dependent block of the NMDARs. Figures 1A–1C show results obtained in one cell in which this measurement was carried out at 35°C. The CV of the EPSC amplitudes was measured for successive groups of 20 trials. CV was 14% smaller for the NMDAR- than for the AMPAR-mediated component ($p < 0.01$; Student's t test). According to the binomial description of transmitter release, CV varies as a decreasing function of m but is independent of mean quantal amplitude. The discrepancy in CV thus implies a larger quantal content for the NMDAR- than the AMPAR-mediated component (Kullmann, 1994). The Poisson limit of the binomial model further predicts that the inverse of CV squared ($1/CV^2 = \text{mean}^2/\text{variance}$) varies linearly with quantal content m. The results in Figure 1C therefore imply that $m_{NMDA}/m_{AMPA} = 1.35$. When this measurement was repeated in 31 cells at 35°–37°C, the ratio of $1/CV^2$ for the NMDAR- and AMPAR-mediated components was 1.47 ± 0.08 (SEM).

Although the difference between $1/CV^2$ for the two components was highly significant, it was smaller than that previously reported from identical experiments carried out at room temperature by Kullmann et al. (1996; see also Selig et al., 1995). We confirmed this in a further eight cells recorded at 22°–25°C: the average ratio of $1/CV^2$ for the NMDAR- and AMPAR-mediated components was 2.0, similar to the value of 2.1 reported by Kullmann et al. (1996). Averaging these together, we obtained a ratio of $1/CV^2$ (NMDA/AMPA) of 2.06 ± 0.10

at room temperature ($n = 50$). This is significantly larger than the average ratio of $1/CV^2$ at 35°–37°C ($p < 0.001$; Student's *t* test). According to the Poisson model, therefore, $m_{NMDA}/m_{AMPA} = 2.06$ at room temperature, but $m_{NMDA}/m_{AMPA} = 1.47$ at physiological temperature. Since, in the more general binomial model, *m* is the product of the number of release sites and the average release probability, these ratios are compatible with the view that NMDARs, on average, sense glutamate released from 106% and 47% more release sites than do AMPARs at the low and high recording temperatures, respectively.

Although the ratio of $1/CV^2$ was smaller at 35°–37°C, it was still >1. Does this imply that the quantal content is still greater for NMDARs than for AMPARs at physiological temperatures? $1/CV^2$ is not exclusively determined by quantal content because it also reflects variability in the quantal amplitude. Although Kullmann (1994) argued that it was not possible to account entirely for the discrepancy between the variability of AMPAR- and NMDAR-mediated signals at room temperature on the basis of differences in quantal variability, this cannot be excluded at physiological temperature where the discrepancy is much smaller. We therefore applied a second approach to look for a discrepancy in quantal content: following Liao et al. (1995), we elicited small EPSCs with low intensity stimulation and compared the proportion of failures of transmission when the postsynaptic cell was hyperpolarized, when the signals are exclusively AMPAR mediated, and at a positive potential, when NMDARs also contribute to the EPSCs.

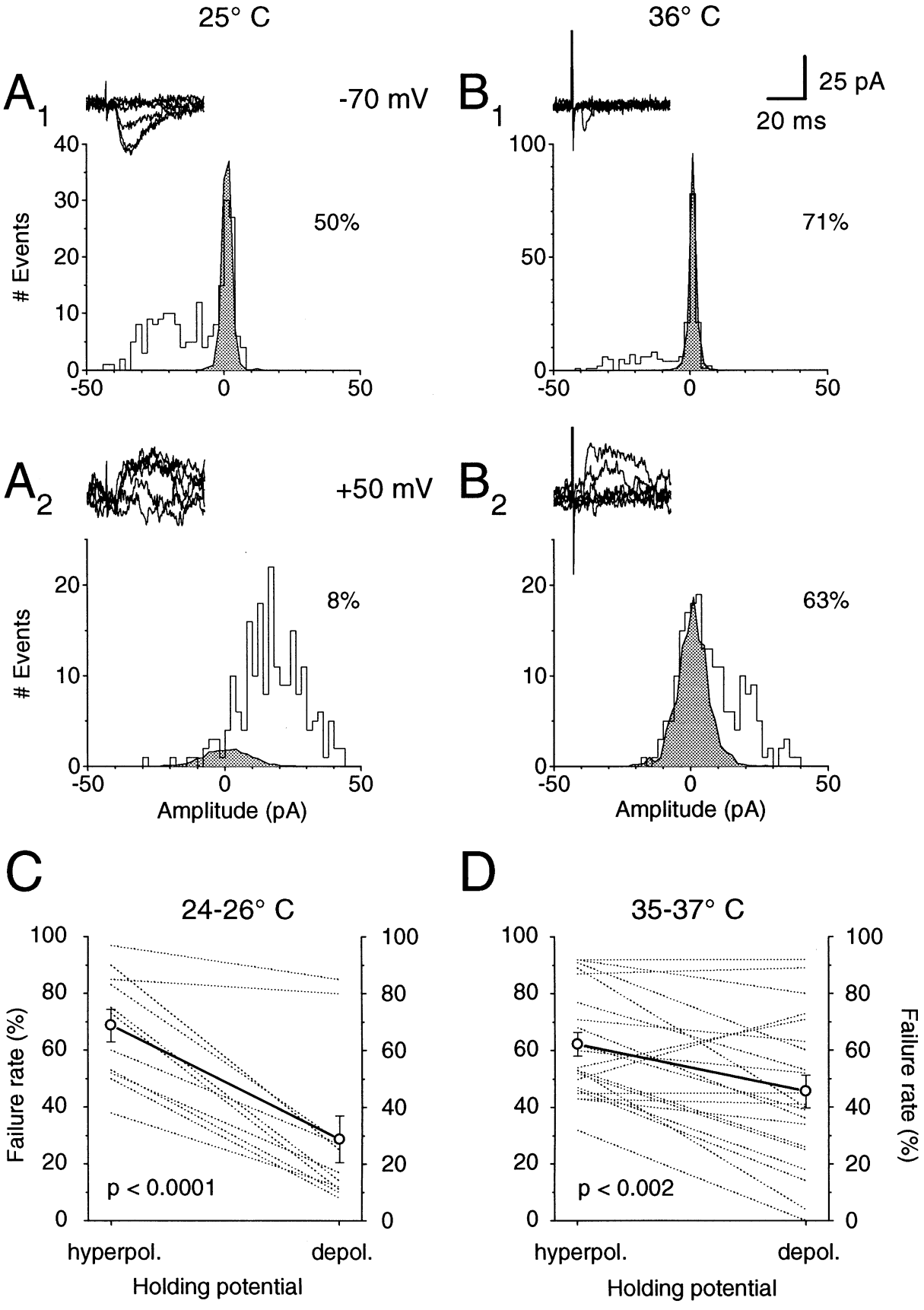
Figures 2A and 2B show a comparison of failure rates in two cells recorded at 25° and 36°C, respectively. A limitation of this approach is that the noise level is consistently larger at positive than at negative potentials, partially because of tonic activation of NMDARs (Sah et al., 1989). This makes it difficult to measure the failure rate at positive potentials. Events whose amplitude was <0 cannot be assumed all to originate from failures of transmission (Liao et al., 1995), since some very small successes may have been biased to negative amplitudes by noise transients. Instead, we treated the overall EPSC amplitude distribution as a mixture of populations or 'components', each of which has a density function equal to that of the noise, and applied a maximum likelihood optimization (Kullmann, 1989) to estimate the probability of a component at 0 pA. If no other component occurred in the range −3 to +3 pA, we considered the probability of the 0 pA component to be an accurate reflection of the failure rate. Figures 2A and 2B show the results of applying the maximum likelihood optimization, and the relative failure rates at negative and positive potentials, in two cells recorded at 25° and 36°C. At 25°C, the failure rate was 50% at −70 mV, dropping to 8% at +50 mV, implying that NMDARs sensed more release sites than AMPARs. At 36°C, the failure rate was more similar: 71% at −70 mV and 63% at +50 mV. When repeated in 11 pathways studied in six cells at 24°–26°C, the average failure rate at positive potentials was 38% ± 8% of the failure rate at negative potentials ($p < 0.0001$; paired Student's *t* test; Figure 2C), in general agreement with Liao et al. (1995). In contrast, in 21 pathways recorded in 15 cells at 35°–37°C, the failure rate at positive potentials was 71% ± 8% of the rate at negative potentials (Figure 2D). This decrease was still highly significant ($p < 0.002$) but was smaller than at room temperature ($p < 0.02$; unpaired Student's *t* test).

Assuming that transmitter release is well described by Poisson statistics, the quantal content should be given by the natural logarithm of the inverse of the failure rate. The failure rate at negative potentials F_- allows an estimate of m_{AMPA}, and, assuming that AMPAR clusters do not occur without NMDARs, the failure rate at positive potentials F_+ allows an estimate of m_{NMDA}. The ratio m_{NMDA}/m_{AMPA} is therefore given by $\ln(F_+)/\ln(F_-)$. This was 3.32 at 22°–25°C and 1.66 at 35°–37°C. Although these estimates are greater than those given by the comparison of $1/CV^2$ (see Discussion), the two approaches yield the same general conclusion: the discrepancy in quantal content mediated by NMDARs and AMPARs is much smaller at physiological temperature than at room temperature.

Are Latent AMPAR Clusters Activated at Physiological Temperatures?

Although the simplest interpretation for the reduced discrepancy in quantal content at physiological temperature is that synaptic cross-talk is greatly attenuated, as a result of decreased glutamate spillover, an alternative explanation is that latent AMPAR clusters are somehow uncovered in a temperature-dependent manner, for instance through a change in the relative activity of kinases and phosphatases. If functional AMPARs were absent from a proportion of synapses at low temperatures, then fewer such 'AMPAR-silent' synapses might be seen at higher temperatures. We tested this hypothesis by measuring the effect of changing the recording temperature on membrane currents evoked by applying AMPA and NMDA directly to the cells. Since these agonists are not transported, and since the putative effect of temperature on receptor clusters should be widely distributed across the synaptic population, the hypothesis predicts that the AMPA current should increase more steeply with temperature than the NMDA current. Figures 3A and 3B show membrane currents recorded in two cells in response to NMDA and AMPA, respectively, pressure applied in s. radiatum. The peak amplitudes of the membrane current increased by similar fractions for a 10°C increase in recording temperature. Figures 3C–3E show that when this measurement was repeated in 8 and 10 cells for NMDA and AMPA, respectively, there was no significant difference in the ratio of currents. In two of the cells studied with AMPA, we began the recording at a high temperature and then decreased the temperature to around 22°C to check for the possibility that the putative differential uncovering of AMPARs could be precluded by 'washout' of a necessary intracellular component, akin to the induction of LTP (Malinow and Tsien, 1990; Kato et al., 1993). We found no difference between these cells and the overall sample.

The present results thus lend no support to the hypothesis that clusters of receptors are differentially regulated by temperature, although complex actions on the kinetics of AMPARs and NMDARs could yet conceal such a phenomenon (see Discussion). These results cannot, however, exclude an alternative interpretation of the 'latent AMPAR cluster' hypothesis: if AMPARs


25° C
36° C
A1
-70 mV
B1
25 pA
20 ms
Events
50%
71%
A2
+50 mV
B2
8%
63%
Amplitude (pA)
C
24-26° C
D
35-37° C
Failure rate (%)
p < 0.0001
p < 0.002
hyperpol.
depol.
Holding potential

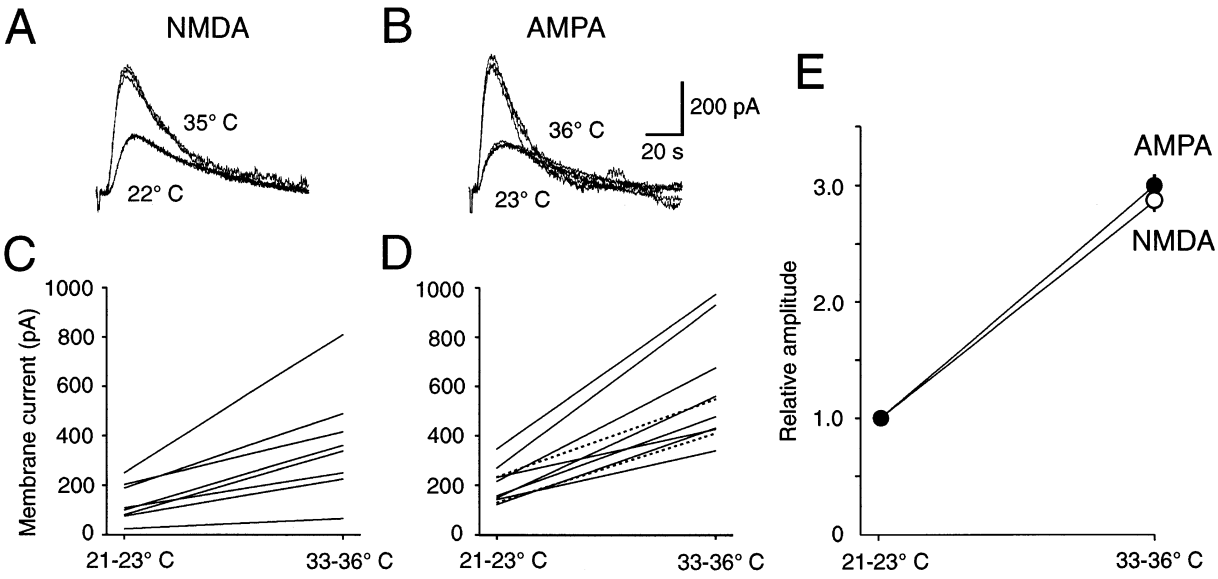


Figure 3. Temperature Dependence of Membrane Currents Elicited by Pressure Application of AMPA and NMDA

(A) Successive membrane currents recorded at 22° and 35°C in response to NMDA application (three trials at each temperature). The deflection before the NMDA current is the response to a voltage pulse to monitor series and input resistance.

(B) Currents in response to application of AMPA in another cell at 23° and 36°C.

(C) Summary of results obtained with NMDA application.

(D) Results obtained with AMPA application. The dotted lines show two cells in which the temperature was decreased.

(E) Data in (C) and (D) normalized by the amplitude of the response at room temperature (±SEM). There was no significant difference in the temperature dependence of the currents evoked by the two agonists.

were translocated from an extrasynaptic site to a synaptic location with increases in temperature, then the total number of functional AMPARs on the surface of the cells may remain constant (explaining the results in Figure 3), although a larger fraction of them would respond to synaptic glutamate release (explaining the smaller discrepancy in quantal content mediated by AMPARs and NMDARs). An indirect argument against this hypothesis comes from examining the expression of LTP: according to the latent AMPAR cluster hypothesis, LTP is associated with an increase in quantal content because AMPAR clusters are uncovered at synapses where they were previously nonfunctional or absent (Kullmann, 1994; Isaac et al., 1995; Liao et al., 1995). If, however, at physiological temperatures, there were fewer synapses devoid of functional AMPAR clusters, then there should be less room for the quantal content to increase. Clearly, LTP can be elicited at physiological temperatures (Bliss and Lømo, 1973), but is it still associated with a large increase in quantal content? We elicited LTP in seven cells at 35°–37°C by pairing low frequency presynaptic stimulation with postsynaptic depolarization and monitored the trial-to-trial variability of the EPSCs with the statistic $1/CV^2$. Figures 4A and 4B show that the magnitude of LTP was comparable to that observed with identical methods at room temperature and associated with a similar increase in $1/CV^2$ (Figure 2 in Kullmann et al., 1996). When the fractional increase in $1/CV^2$ was compared to the fractional increase in amplitude, the average point fell close to the line of identity (Figure 4C), implying an increase in quantal content at least as large as at room temperature. We therefore consider it unlikely that AMPAR clusters are uncovered or translocated as the temperature increases in a manner that shares mechanisms with LTP.

Could Differences in Release Probability Explain the Effect of Temperature?

Another possible explanation for the reduced discrepancy in quantal content at 35°–37°C is that distinct subpopulations of synapses with different release probabilities may have different proportions of functional AMPAR

Figure 2. Comparison of Failure Rates at Negative and Positive Holding Potentials

(A) Results from one cell recorded at 25°C. The histograms show the EPSC amplitude on each of 200 trials while holding the cell at −70 mV (A_1) or at +50 mV (A_2). The shaded histogram is the noise amplitude distribution, scaled by the maximum likelihood estimate of the failure probability. The failure rate fell from 50% at −70 mV to 8% at +50 mV. The traces show six sample records obtained at each voltage.

(B) Results obtained from another cell recorded at 36°C, held at −70 mV (B_1) or at +50 mV (B_2). The failure rate was more similar at the two holding potentials, falling from 71% to 63%.

(C) Failure rates estimated in 11 pathways recorded in six cells at 24°–26°C (dotted lines). The open circles show the average failure rates (±SEM) at −60 to −80 mV (hyperpol.) and at +40 to +50 mV (depol.).

(D) Failure rates estimated in 21 pathways in 15 cells at 35°–37°C. Although the failure rate was again significantly smaller at positive than at negative membrane potentials, the difference was less than at 24°–26°C.

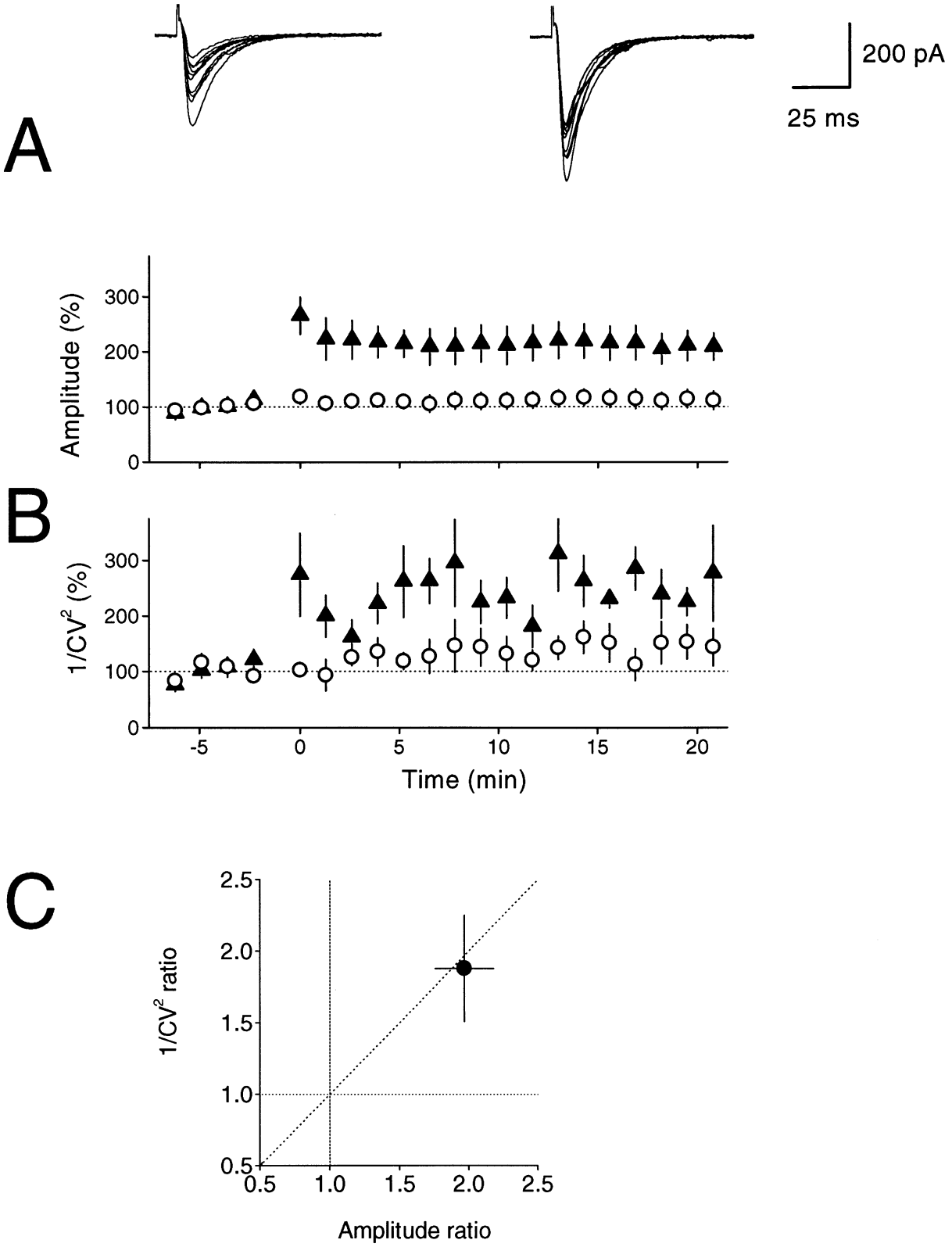


Figure 4. LTP at Physiological Temperatures Is Associated with a Large Increase in $1/CV^2$

(A) Average EPSC amplitude plotted against time and normalized by the average baseline amplitude (n = 7). LTP was induced by pairing postsynaptic depolarization with low frequency stimulation at t = 0 (closed triangles). Open symbols show EPSC amplitude in a control pathway that was not stimulated during the pairing. The sample traces before and after LTP induction are taken from one experiment (10 successive trials in each case).

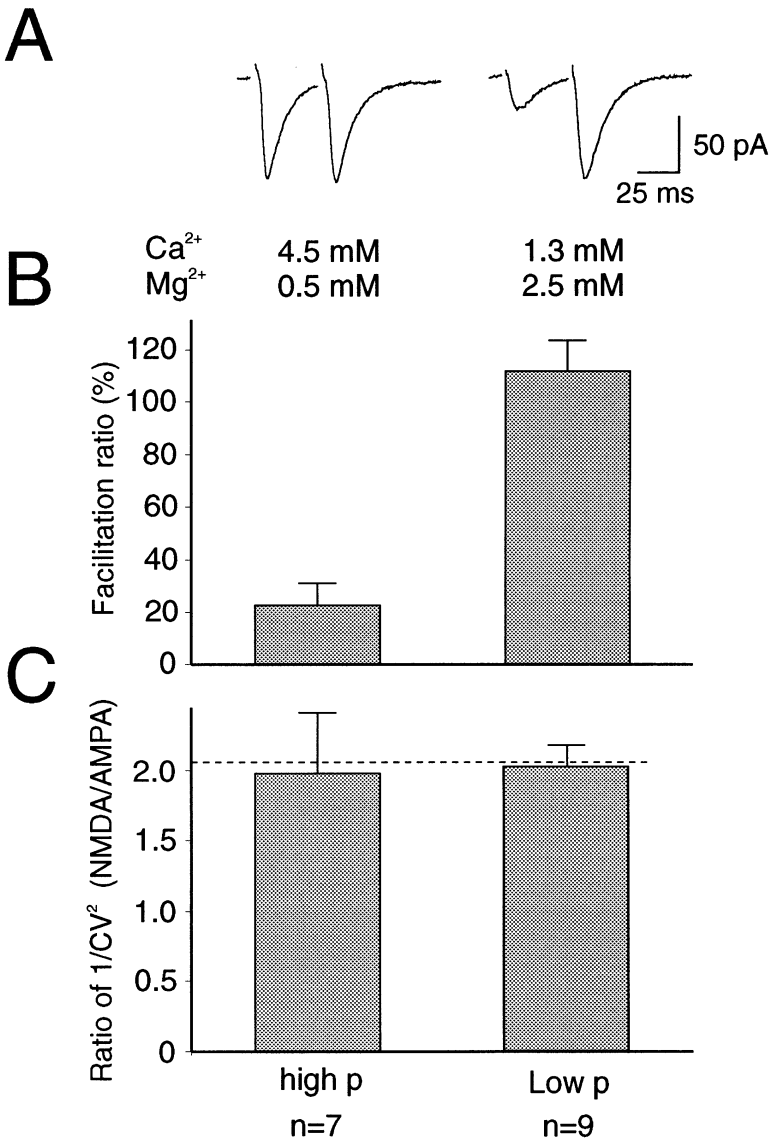


Figure 5. Altering Release Probability with Changes in Ca^{2+}/Mg^{2+} Ratio Has No Effect on the Ratio of $1/CV^2$

(A) Averaged EPSCs recorded in two cells in response to two stimuli delivered with a 50 ms interval, in a perfusion solution containing high Ca^{2+}/low Mg^{2+} (left) or low Ca^{2+}/high Mg^{2+} (right).

(B) Average paired-pulse facilitation ratios measured under the two conditions. The ratio was calculated as $(EPSC_2 - EPSC_1)/EPSC_1 \times 100\%$.

(C) Ratio of $1/CV^2$ (NMDA/AMPA) subsequently measured in the same cells with single pulses. The horizontal dashed line shows the average ratio in the solution used elsewhere in this study.

and NMDAR clusters. Since transmitter release has a high Q_{10} (Van der Kloot and Molgo, 1994), an increase in temperature could alter the relative weighting of synapses with and without AMPARs, possibly increasing the overall proportion of release events witnessed by AMPARs. We tested this hypothesis by repeating, at room temperature, the comparison of $1/CV^2$ mediated by AMPARs and NMDARs, under conditions designed to increase or decrease the transmitter release probability. Nine cells were recorded in a perfusion solution containing 1.3 mM Ca^{2+} and 2.5 mM Mg^{2+} ('low p'), and a further seven cells were recorded in the presence of 4 mM Ca^{2+} and 0.5 mM Mg^{2+} ('high p'). Paired-pulse facilitation ratios were measured to verify that these manipulations designed to alter the release probability were indeed associated with changes in short-term facilitation (Figures 5A and 5B). Since 5 or 10 mM 1,2-bis(2-aminophenoxy)-ethane-N,N,N′,N′-tetraacetic acid (BAPTA) was included in the pipette solution, the effects of the perfusion solution on transmitter release probability were unlikely to be compounded by postsynaptic Ca^{2+}-sensitive processes that have been reported to influence paired-pulse facilitation (Wang and Kelly, 1996). In spite of large differences in paired-pulse facilitation ratio, the ratio of $1/CV^2$ for the NMDAR- and AMPAR-mediated components was very similar under both conditions (Figure 5C): 2.03 ± 0.16 and 1.98 ± 0.44 in 'low p' and 'high p' conditions, respectively, neither of which differed significantly from the ratio measured in 2.5 mM Ca^{2+} and 1.3 mM Mg^{2+} used elsewhere in

(B) $1/CV^2$ calculated for successive groups of 20 trials.

(C) Average increase in $1/CV^2$ (test/control) plotted against average increase in mean amplitude. The Poisson model predicts that the point should fall on the line of identity if LTP is expressed through a pure increase in quantal content.

this study. We were thus unable to detect any effect of changing release probability on the discrepancy in quantal contents mediated by AMPARs and NMDARs.

Role of Glutamate Uptake

The effect of raising the recording temperature is difficult to explain in the context of the latent AMPAR cluster hypothesis but is compatible with the hypothesis that extrasynaptic glutamate spillover is responsible for the discrepancy in quantal content sampled by AMPARs and NMDARs. The simplest explanation is that active glutamate uptake is enhanced, reducing the extent to which glutamate can diffuse to neighboring synapses and reach a sufficient concentration to activate NMDARs. The corollary of this hypothesis is that reducing glutamate uptake by blocking transporters should selectively increase the size of the NMDAR-mediated component of the synaptic signal, with no effect on the AMPAR-mediated component. This has indeed been reported in 7 of 12 cells exposed to the uptake blocker dihydrokainate (DHK, 200 μM) at 32°C (Hestrin et al. 1990). We attempted to repeat these experiments by examining the effect of 100 or 200 μM DHK on isolated NMDAR-mediated EPSCs recorded in the presence of DNQX at 32°–34°C. However, we observed a small depression ($-21\% \pm 9\%$; $n = 4$; $p < 0.05$; data not shown), which was associated with a small increase in $1/CV^2$ ($17\% \pm 8\%$; $p < 0.05$) and a large increase in the holding current. When we examined the effect of DHK on AMPAR-mediated EPSCs, we found that it frequently caused very large spontaneous inward currents: this was seen in 12 of 12 cells at 22°–25°C but only very infrequently at temperatures >35°C. We tentatively interpret these currents as epileptiform discharges in the CA1 region of the slice, which preclude interpretation of the effects of DHK on the amplitude and variability of AMPAR-mediated EPSCs at low temperatures. At 35°–37°C, DHK (50 or 100 μM) caused a small but significant decrease in the average size of AMPAR-mediated EPSCs ($-21\% \pm 9\%$; $n = 8$; $p < 0.05$; data not shown), which was accompanied by an increase in paired-pulse facilitation ratio ($12\% \pm 4\%$; $p < 0.05$) and a nonsignificant decrease in $1/CV^2$ ($-18\% \pm 16\%$).

We thus failed to reproduce the observations of Hestrin et al. (1990), although the discrepancy between the effect of DHK on $1/CV^2$ for the two components is in keeping with the spillover hypothesis (see below). The effects of DHK on EPSC amplitudes that we observed can to a great extent be explained by an increase in the ambient low micromolar extracellular concentration of glutamate. This could simultaneously activate NMDARs, desensitize AMPARs and NMDARs, and reduce transmitter release through a presynaptic action on metabotropic receptors (Forsythe and Clements, 1990), as has been reported for another glutamate transport inhibitor, L-*trans*-pyrrolidine-2,4-dicarboxylate (Maki et al., 1994). An additional factor that may complicate the interpretation of the results is that DHK has been reported to activate ionotropic glutamate receptors directly (Bridges et al., 1991; Maki et al., 1994). One possible explanation for the difference between the present results and those of Hestrin et al. is that glutamate was cleared from the extracellular space less readily because we used thicker slices (450 versus 200 μm).

Another prediction from the hypothesis that temperature affects clearance of glutamate is that the discrepancy between the quantal content mediated by the two components should be increased by reducing uptake, since NMDARs, but not AMPARs, should sense transmitter released from more distant teminals. This was indeed suggested by the observation that DHK produced an increase in $1/CV^2$ for NMDAR-mediated EPSCs but a tendency to a decrease in $1/CV^2$ for the AMPAR-mediated component. What happens to the ratio of $1/CV^2$ for the NMDAR- and AMPAR-mediated signals measured in the same synaptic pathways? Since presynaptic effects of DHK on release probability should be similarly sensed by AMPARs and NMDARs, this should not invalidate a comparison of $1/CV^2$ for the two components. The quantal contents mediated by AMPARs and NMDARs should also be insensitive to the level of steady-state desensitization since $1/CV^2$ is independent of mean quantal amplitude (Faber and Korn, 1991; Manabe et al., 1993). Figures 6A and 6B show the result of measuring the trial-to-trial variability of the AMPAR- and NMDAR-mediated components at 35°–37°C in the continuous presence of 50–100 μM DHK ($n = 15$). The ratio of $1/CV^2$ (NMDAR/AMPAR) was 1.87 ± 0.12, which was significantly greater than in the absence of DHK ($p < 0.01$). Reducing glutamate uptake thus partially reverses the effect of raising the recording temperature, in agreement with the hypothesis that transporters play a critical role in limiting extrasynaptic spillover.

We further tested the hypothesis that elevating temperature and inhibiting uptake had opposite effects on the discrepancy in quantal contents, by comparing the failure rates at positive and negative potentials in the continuous presence of 100 μM DHK. Figure 6C shows results obtained in 15 pathways from 12 cells recorded at 35°–37°C: the average failure rate at positive potentials was $40\% \pm 8\%$ of the rate at negative potentials. This ratio differed significantly from that observed at 35°–37°C in the absence of DHK ($71\% \pm 8\%$; $p < 0.02$; unpaired Student's *t* test) and was much more similar to that observed at room temperature ($38\% \pm 8\%$; $p = 0.88$; see Figure 2). Assuming a Poisson process, the average failure rates at positive and negative potentials imply a ratio m_{NMDA}/m_{AMPA} of 2.32. Both approaches used to compare the quantal content mediated by AMPARs and NMDARs thus lead to the same conclusion: DHK partially reverses the effect of increasing temperature, consistent with a critical role of glutamate transporters in limiting extrasynaptic spillover.

Discussion

We find that the striking discrepancy between the quantal contents of the AMPAR- and NMDAR-mediated components of the synaptic signal seen at room temperature is greatly reduced at physiological temperature. We interpret this as a reduction in cross-talk between synapses and argue that clearance of glutamate from the extracellular space is more efficient at higher temperatures.

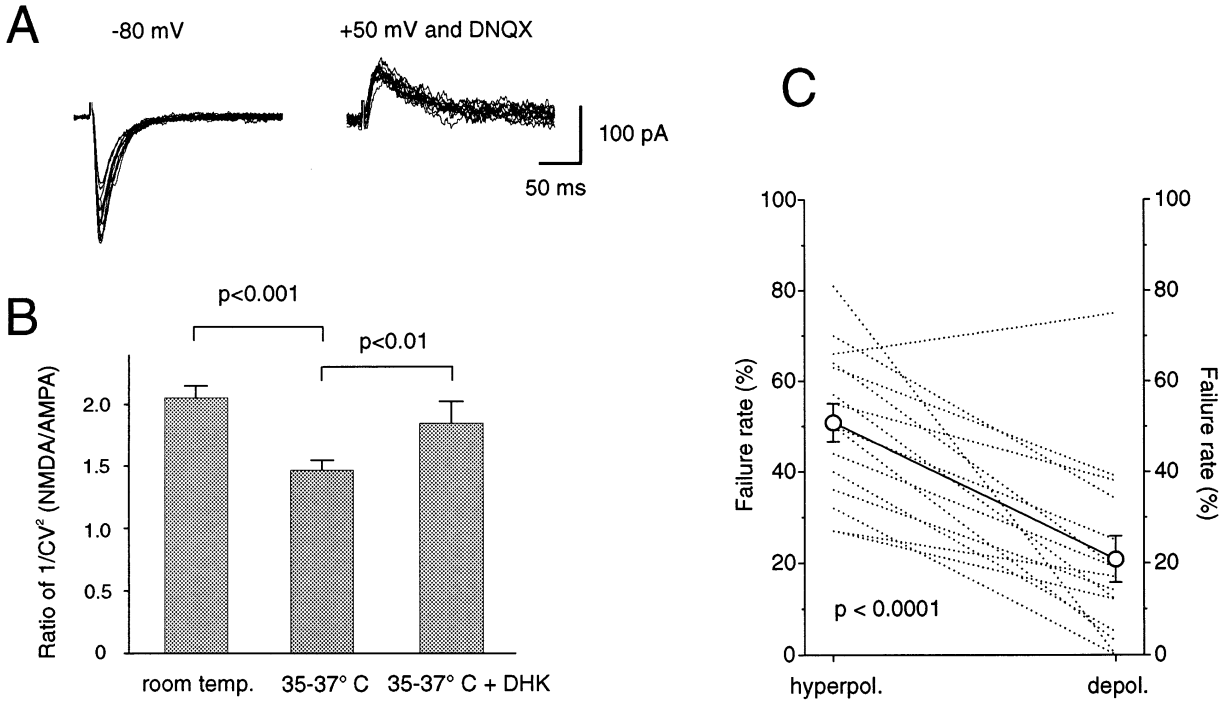


Figure 6. The Glutamate Uptake Inhibitor DHK Partially Reverses the Action of Increasing the Recording Temperature

(A) Sample traces taken from one cell in the presence of 100 μM DHK at 36°C, showing the variability of the AMPAR- and NMDAR-mediated components (15 consecutive trials in each case).

(B) Average ratios of $1/CV^2$ (NMDA/AMPA) recorded at room temperature, 35°–37°C, and at 35°–37°C in the presence of 50–100 μM DHK.

(C) Estimated failure rates in 15 pathways recorded from 12 cells at 35°–37°C in the presence of 100 μM DHK, at −60 to −70 mV (hyperpol.), and at +40 to +50 mV (depol.). The open circles show the average failure rates (±SEM).

What alternative hypotheses could account for the data? It was previously argued by several groups that the discrepancy in quantal contents reflected a proportion of synapses that are normally devoid of functional AMPARs (Kullmann, 1994; Isaac et al., 1995; Liao et al., 1995; Durand et al., 1996). This is, however, difficult to reconcile with several of the present findings. To account for the smaller discrepancy in quantal content at physiological temperature, two possible scenarios can be proposed in the context of the latent AMPAR cluster hypothesis. First, functional AMPARs could appear in a temperature-dependent manner (or clusters of NMDARs could disappear), and second, subsets of synapses with different proportions of latent AMPARs could undergo differential increases in release probability.

We consider the first alternative, that functional AMPAR clusters appear with increases in temperature, unlikely, since membrane currents elicited by exogenous application of AMPA and NMDA showed the same dependence on temperature. Assuming that the ratio of $1/CV^2$ for the two components gives an unbiased estimate of m_{NMDA}/m_{AMPA} (see below), the proportion of synapses devoid of AMPARs would have to drop from $(2.06 - 1)/2.06 = 51\%$ at 22°–25°C to $(1.47 - 1)/1.47 = 32\%$ at 35°–37°C. This leads to the prediction that the AMPA current should increase with temperature $0.51/0.32 = 1.59$ times as steeply as the NMDA current, which is incompatible with the data in Figure 3. Receptor kinetics, however, depend on the nature of the agonist, and we cannot exclude the possibility that activation of the receptors with glutamate would have shown a steeper increase with temperature for AMPARs than for NMDARs. This cannot be tested in a slice since glutamate is transported in a temperature-dependent manner. Relatively little is known of the temperature dependence of the kinetics of AMPARs and NMDARs studied with glutamate application (Feldmeyer and Cull-Candy, 1993; Chung and Kuyucak, 1995; Silver et al., 1996a, 1996b), but it would clearly be important to determine whether quantitative differences emerge when they are studied in isolated cells. Another weakness of exogenous agonist application is that it does not discriminate between synaptic and extrasynaptic receptors: on the basis of these results, therefore, we cannot exclude the possibility that elevating the temperature causes functional AMPARs to move from the periphery of the synapse, where they are not liganded by synaptically released glutamate, to a position closer to the release site.

An alternative argument against recruitment (and indeed translocation) of AMPAR clusters with temperature comes from the observation that LTP at physiological temperatures is associated with an increase in $1/CV^2$ that is at least as large as at room temperature. This is admittedly indirect, since it presupposes that any temperature-dependent uncovering of AMPARs should occlude that accompanying LTP. The results are nevertheless most simply explained by proposing that the increase in quantal content reflects a genuine presynaptic increase in release probability (Bekkers and Stevens, 1990; Malinow and Tsien, 1990; Kullmann et al., 1996).

The second alternative explanation for the effects of temperature is that different subpopulations of synapses with different ratios of functional AMPARs and NMDARs are sampled because release probability varies steeply with temperature. We consider this unlikely since manipulating the Ca^{2+}/Mg^{2+} ratio at room temperature fails to alter the ratio of $1/CV^2$ for the two components. We witnessed large changes in the paired-pulse facilitation ratio, which, by comparison with Manabe et al. (1993) and Schulz et al. (1995), imply that the average release probability was varied over a 5-fold range. Assuming that this would have differentially affected subpopulations of sites with low and high release probabilities, we would expect to have observed a change in the ratio of $1/CV^2$.

The observation that DHK caused an increase in $1/CV^2$ for NMDAR- but not AMPAR-mediated EPSCs suggests that active uptake normally limits the extent of extrasynaptic spillover. This was further supported by the finding that DHK partially reversed the effect of increasing temperature on the ratio of $1/CV^2$ for AMPAR- and NMDAR-mediated signals. The results were, however, complicated by actions of DHK on the tonic membrane current at positive potentials, probably mediated by an elevation of the ambient glutamate concentration, acting on NMDARs. DHK also appeared to decrease the presynaptic release probability, which may again reflect the effect of an elevated glutamate concentration on presynaptic metabotropic glutamate receptors (Maki et al., 1994; see also Sarantis et al., 1993), although direct actions of DHK on the presynaptic terminals cannot be excluded. At room temperature, DHK always caused large intermittent inward currents through AMPARs. In preliminary experiments, L-*trans*-pyrrolidine-2,4-dicarboxylate (PDC) did not have this effect. Although this difference could reflect the lower selectivity of DHK for transporters over glutamate receptors, the dissociation of transport inhibition from epileptiform activity parallels the observation that down-regulation of neuronal but not glial transporters can cause seizures (Rothstein et al., 1996). Indeed, some evidence exists that DHK inhibits a neuronal transporter (Robinson et al., 1993; although see Arriza et al., 1994). We chose not to use PDC for the rest of the experiments because, unlike DHK, it is transported into the cell, where it can displace cytoplasmic glutamate through heteroexchange (Barbour et al., 1991; Arriza et al., 1994). This could exacerbate the accumulation of extracellular glutamate (Isaacson and Nicoll, 1993), leading to further receptor desensitization. PDC has indeed been reported to decrease NMDAR-mediated EPSCs in hippocampal slices (Sarantis et al., 1993), although additional direct actions on glutamate receptors may also play a role.

The small depression of AMPAR-mediated EPSCs that we and others (Sarantis et al., 1993; Mennerick and Zorumski, 1994; Takahashi et al., 1995) have observed with uptake inhibitors was not reported by several other groups (Isaacson and Nicoll, 1993; Barbour et al., 1994; Tong and Jahr, 1994b). Tong and Jahr (1994b) in fact reported an increase in the amplitude of AMPAR-mediated EPSCs with another uptake blocker, D-L-threo-β-hydroxyaspartic acid, at 34°C but not at 24°C, which was interpreted as a prolongation of the glutamate transient in the synaptic cleft, thereby increasing AMPAR occupancy. Although the much greater role of transporters at high temperatures agrees with our conclusions, the opposite effect of inhibiting uptake on AMPAR-mediated EPSCs may simply reflect differences in the diffusional barriers (Tong and Jahr, 1994b), which determine whether glutamate accumulates sufficiently to desensitize receptors and/or depress the release probability. An additional factor that may influence the effect of transport inhibitors is whether numerous release sites occur within a synapse, since this may allow interactions between release sites to be uncovered even when monitored by AMPARs (Trussell et al., 1993; Tong and Jahr, 1994a; Silver et al., 1996b). This does not appear to be the case for excitatory synapses on CA1 cells (Perkel and Nicoll, 1993).

The simplest explanation for the effect of increasing temperature in the present experiments is that glutamate uptake is enhanced, thereby reducing the degree of intersynaptic cross-talk. This interpretation is, however, not without its difficulties: although the Q_{10} for active glutamate uptake has been estimated between 1.95 (Schwartz and Tachibana, 1990) and 2.5–3 (Wadiche et al., 1995), this refers to complete cycles of the transporters. The turnover time of the $EAAT_2$ transporter has recently been estimated at 70 ms (Wadiche et al., 1995), which is much slower than the glutamate transient in the vicinity of a synapse: following release of 4000 molecules into an infinite disk 15 nm wide representing the synaptic cleft, diffusion kinetics predict that the glutamate concentration should drop to <10 μM within 5 ms. Transporters could still play a major role in limiting extrasynaptic diffusion simply by virtue of the binding sites available to glutamate molecules as they diffuse out of the synaptic cleft (Tong and Jahr, 1994b; Takahashi et al., 1996). For a high affinity site, however, the unbinding reaction is likely to have a higher activation energy and therefore a higher Q_{10} than the binding reaction, so it is not clear that this explains why less extrasynaptic spillover appears to occur at higher temperatures. If, however, a larger fraction of the transporters were positioned with the glutamate-binding site exposed to the extracellular space as temperature increased, then the paradox would be resolved. Among other explanations are that transporters are under temperature-dependent regulatory control (Casado et al., 1993; Dowd and Robinson, 1996) or that some transporters may have a much faster cycling rate than the cloned $EAAT_2$ type (Schwartz and Tachibana, 1990).

Although the simplest unifying explanation for the present results is that glutamate transport plays a critical and temperature-dependent role in limiting spillover, we cannot exclude the possibility that the relative affinities of AMPARs and NMDARs for glutamate depend on the recording temperature: if the affinity of NMDARs were relatively lower at physiological compared with room temperature, then there could be less synaptic cross-talk. Although no evidence for differential temperature sensitivity of AMPAR and NMDAR kinetics was seen with agonist application, it will again be necessary to look at the effects of glutamate application at different temperatures in a reduced system to test this hypothesis directly.

The estimates of m_{NMDA}/m_{AMPA} given by comparing

$1/CV^2$ for the two components were consistently smaller than those given by comparing failure rates: 2.06 versus 3.32 at 22°–25°C, 1.47 versus 1.66 at 35°–37°C, and 1.87 versus 2.32 at 35°–37°C in the presence of DHK. Both methods rely on assuming that release is governed by a Poisson description, so the discrepancy may reflect deviation from this model, and although it gives a good description of release at another central nervous system synapse (Isaacson and Walmsley, 1995), the higher release probabilities in the present experiments may invalidate this assumption. The two approaches are also sensitive to different sources of bias that may further explain the disagreement. $1/CV^2$ is not entirely determined by the quantal content but also reflects variability in quantal amplitude, both 'intra-' and 'intersynaptic' (Kullmann, 1994). If NMDAR-mediated quanta are more variable than AMPAR-mediated quanta, then the ratio of $1/CV^2$ will underestimate m_{NMDA}/m_{AMPA}. The spillover hypothesis in fact predicts just such a phenomenon, since, in addition to large NMDAR-mediated quanta caused by a saturating concentration of glutamate released from immediately apposed terminals, other much smaller events may arise from a relatively low concentration of transmitter diffusing from more distant release sites. The comparison of failure rates, on the other hand, is sensitive to sampling error, since the data are necessarily biased toward cells with low noise, and where the quantal events can be readily discriminated from failures of transmission. Another assumption inherent in the comparison of failure rates is that synapses with functional AMPAR clusters but no NMDARs do not occur. If this assumption is incorrect, then the failure rate at positive potentials may lead to an overestimate of the quantal content mediated by NMDARs. Although synapses with AMPARs but no NMDARs have been described in cultured cells (Bekkers and Stevens, 1989), it remains to be determined if this occurs in vivo.

Does synaptic cross-talk occur in the living brain? The comparison of $1/CV^2$ and the comparison of failure rates at 35°–37°C gave the same conclusion: the quantal content mediated by NMDARs was between 47% and 66% larger than that mediated by AMPARs. We have argued that extrasynaptic spillover occurs at room temperature and that the effect of changing temperature is to reduce this phenomenon. The simplest explanation for the residual discrepancy in quantal contents at physiological temperature is therefore that it reflects a smaller degree of spillover, although we cannot rule out some noncongruence of AMPAR and NMDAR clusters. There are, however, major limitations in extrapolating this to the living brain, since uptake mechanisms may still be compromised in tissue that has been traumatized by the slice preparation and incubation. If some residual spillover occurs in vivo, then synapses may not act as entirely private communication channels between neurons.

Experimental Procedures

Recordings were obtained from CA1 cells in hippocampal slices (450 μm) from 4- to 5-week-old guinea pigs. Slices were stored at room temperature in an interface chamber containing a solution of NaCl (119), KCl (2.5), $MgCl_2$ (1.3), $CaCl_2$ (2.5), $NaHCO_3$ (26.2), NaH_2PO_4 (1), and glucose (11) (concentrations in mM), which was gassed with 95% O_2–5% CO_2. Slices were transferred as needed into a recording chamber where they were submerged in a perfusion solution with the same composition except for the addition of 100 μM picrotoxin to block $GABA_A$ receptors. The CA3 region was routinely cut away to prevent epileptiform discharges from spreading to CA1. Fibers in s. radiatum were stimulated with bipolar stainless-steel electrodes at 0.1 or 0.33 Hz. A second pair of stimulating electrodes was used to activate another afferent pathway in most experiments.

The temperature of the recording chamber was monitored throughout the experiments and controlled through a Peltier device, which heated or cooled the perfusion solution as it entered the chamber. The temperature could be increased or decreased by 10°C to a stable level within 5 min.

Whole-cell voltage-clamp recordings were made with pipettes containing Cs gluconate (97.5), CsCl (17.5), HEPES (10), BAPTA (5 or 10), NaCl (8), MgATP (2), GTP (0.3), and QX314 Br (5) (pH 7.2, osmolarity 295 mOsm). In the experiments where LTP or transmission failure rates were studied, the pipettes contained Cs gluconate, (117.5), CsCl (17.5), HEPES (10), EGTA (0.2), NaCl (8), MgATP (2), GTP (0.3), and QX314 Br (5). Membrane currents were amplified and filtered at 1 kHz, sampled at 2 or 4 kHz with either an Axopatch 1D or an Axopatch 200 A amplifier (Axon Instruments), and stored on a personal computer for off-line analysis. The series resistance was continuously monitored by delivering a voltage step command at the end of each trace recorded. The holding potential was generally −80 mV to record AMPAR-mediated EPSCs and +40 or +50 mV to record NMDAR-mediated EPSCs. Junctional potentials were not corrected, but the reversal potentials of the EPSCs were routinely measured and fell close to 0 mV.

The EPSC amplitudes were measured by averaging a 5–10 ms period coinciding with the peak of the average time course, subtracted from a baseline of similar duration before the stimulus artefact. For the calculation of the trial-to-trial variance of the EPSC amplitude (Var_{EPSC}), identical measurements were also made from interleaved traces where no stimuli were delivered. The variance of the background noise (Var_{Noise}) was subtracted from the EPSC variance, and $1/CV^2$ was estimated for successive epochs of 20 or 25 trials as the ratio of the mean amplitude squared to the corrected variance:

$$(\overline{EPSC})^2/(Var_{EPSC} - Var_{Noise}).$$

In the minimal-stimulation experiments, the stimulus strength was reduced until clear failures of transmission were seen (stimulation rate 0.33 Hz). A minimum of 200 trials were recorded at a negative membrane potential (−60 to −80 mV) and then again at a positive potential (+40 to +50 mV). Further trials were then recorded at a negative potential to verify that LTP had not been induced and that the failure rate had not drifted. In some experiments, this sequence was repeated more than once, and the electrode position, stimulus strength, and/or recording temperature were adjusted to collect more data. The peak measurement window was positioned to include the peak of the averaged EPSCs measured at both holding potentials. In some of these experiments, a shorter baseline window was placed between the stimulus artefact and the onset of the EPSC. The background noise was oversampled by repeating this measurement at five equally spaced points on each noise sweep. The noise distribution was fitted with a sum of 2 Gaussians with a maximum likelihood estimator (Kullmann, 1989). This was then used to resolve the amplitude distribution into a mixture of discrete components, each of which had the same shape as the noise function, with the following constraints: the sum of the probabilities of all components equalled 1, and the amplitude of the first component was fixed at 0 pA. The number, probabilities, and amplitudes of the components were otherwise allowed to change freely to maximize the likelihood of the solution. If no other components occurred in the range −3 to +3 pA, then the probability of the 0 pA component was taken as an estimate of the failure rate. This was generally unambiguous at negative potentials, but at positive potentials, in about a third of the cases, another component occurred in the range −3 to +3 pA, implying some uncertainty about the failure rate. These data sets were rejected.

For the measurement of the membrane currents elicited by AMPA and NMDA, tetrodotoxin (0.5 or 1 μM) was included in the perfusion

solution, and the drugs were dissolved in 0.9% saline at a concentration of 500 μM (NMDA or AMPA) or 1 mM (AMPA). They were delivered via patch pipettes positioned in s. radiatum, with brief pulses of compressed nitrogen controlled by a Picospritzer II (General Valve Corp.) with 2 min between pulses. The agonists were applied at least five times before changing the temperature, and results were only accepted if the membrane currents were stable.

LTP was elicited in the experiments illustrated in Figure 4 by pairing 120 pulses at 2 Hz with depolarization to 0 mV within 10 min of breaking into whole-cell mode. Paired-pulse facilitation ratios (Figure 5) were measured with an interpulse interval of 50 ms.

Drugs were purchased from Sigma, except for QX314 Br (Alomone Laboratories) and DNQX, AMPA, NMDA, and DHK (Tocris Cookson). DNQX was dissolved in DMSO to a final DMSO concentration of 0.1%.

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