

Activation of AMPA, Kainate, and Metabotropic Receptors at Hippocampal Mossy Fiber Synapses: Role of Glutamate Diffusion

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Summary

Glutamatergic transmission at mossy fiber (MF) synapses on CA3 pyramidal neurons in the hippocampus is mediated by AMPA, kainate, and NMDA receptors and undergoes presynaptic modulation by metabotropic glutamate receptors. The recruitment of different receptors has thus far been studied by altering presynaptic stimulation to modulate glutamate release and interfering pharmacologically with receptors and transporters. Here, we introduce two novel experimental manipulations that alter the fate of glutamate molecules following release. First, an enzymatic glutamate scavenger reduces the postsynaptic response as well as presynaptic modulation by metabotropic receptors. At physiological temperature, however, the scavenger is effective only when glutamate uptake is blocked, revealing a role of active transport in both synaptic and extrasynaptic communication. Second, AMPA and kainate receptor-mediated postsynaptic signals are enhanced when extracellular diffusion is retarded by adding dextran to the perfusion solution, as is feedback modulation by metabotropic receptors, suggesting that the receptors are not saturated under baseline conditions. These results show that manipulating the spatiotemporal profile of glutamate following exocytosis can alter the involvement of different receptors in synaptic transmission.

Introduction

Several features distinguish mossy fiber (MF) synapses on CA3 neurons from other glutamatergic synapses in the brain. First, excitatory postsynaptic potentials or currents (EPSPs or EPSCs, respectively) undergo profound facilitation with relatively modest increases in presynaptic action potential frequency (Griffith, 1990; Regehr et al., 1994). Second, although under baseline conditions transmission is predominantly mediated by α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, a prominent kainate receptor-mediated component emerges when transmitter release

is enhanced by increasing the presynaptic action potential frequency (Castillo et al., 1997; Vignes and Collingridge, 1997). Third, group 2 presynaptic metabotropic receptors exert a strong inhibitory influence on transmitter release, providing a feedback mechanism to modulate frequency-dependent facilitation (Scanziani et al., 1997). MF synapses are complex and are composed of large presynaptic boutons that may enclose several branched dendritic spines with multiple postsynaptic densities (Chicurel and Harris, 1992). Since mGluR2 receptors are situated in the preterminal region of the membrane (Yokoi et al., 1996), they may be activated by glutamate escaping from the synaptic cleft.

The involvement of each of the different classes of receptors in synaptic transmission is thought to depend on the spatial and temporal extent of extracellular glutamate diffusion following release. For instance, an increase in glutamate concentration within the synaptic cleft could account for the appearance of kainate receptor-mediated signals with brief high frequency trains of stimuli. Similarly, extrasynaptic accumulation of glutamate could explain the contribution of presynaptic metabotropic receptors to feedback regulation of transmitter release. The evidence for this account is, however, indirect, because the available tools to probe synaptic transmission in the brain are restricted to pharmacological agents acting at pre- or postsynaptic receptors, uptake blockers, and manipulations of stimulus protocols designed to alter transmitter release. A more direct test would be to examine the effects of altering glutamate diffusion following release: does increasing or decreasing the rate of glutamate clearance have the expected effect on EPSP amplitude and on presynaptic modulation of transmission by metabotropic receptors?

Here, we apply two novel approaches to alter the spatiotemporal glutamate profile without interfering either with transmitter release or with glutamate receptors and uptake mechanisms. We show that enhancing the clearance of glutamate with an enzymatic glutamate scavenger both reduces the amplitude of the postsynaptic EPSP and interferes with the presynaptic regulation of transmission by metabotropic receptors. We observe evidence for a temperature-dependent role of glutamate uptake. Finally, we show that retarding glutamate diffusion by increasing the viscosity of the extracellular medium enhances transmission mediated by AMPA and kainate receptors as well as feedback regulation by metabotropic receptors. These results argue for a critical role of glutamate diffusion in determining the balance of transmission via postsynaptic AMPA and kainate receptors and in the presynaptic modulation of glutamate release.

Results

We tested two predictions: first, that enhancing the clearance of glutamate from the extracellular space should reduce postsynaptic signaling and presynaptic modulation of transmitter release, and second, that retarding the clearance of glutamate should have the converse effect.

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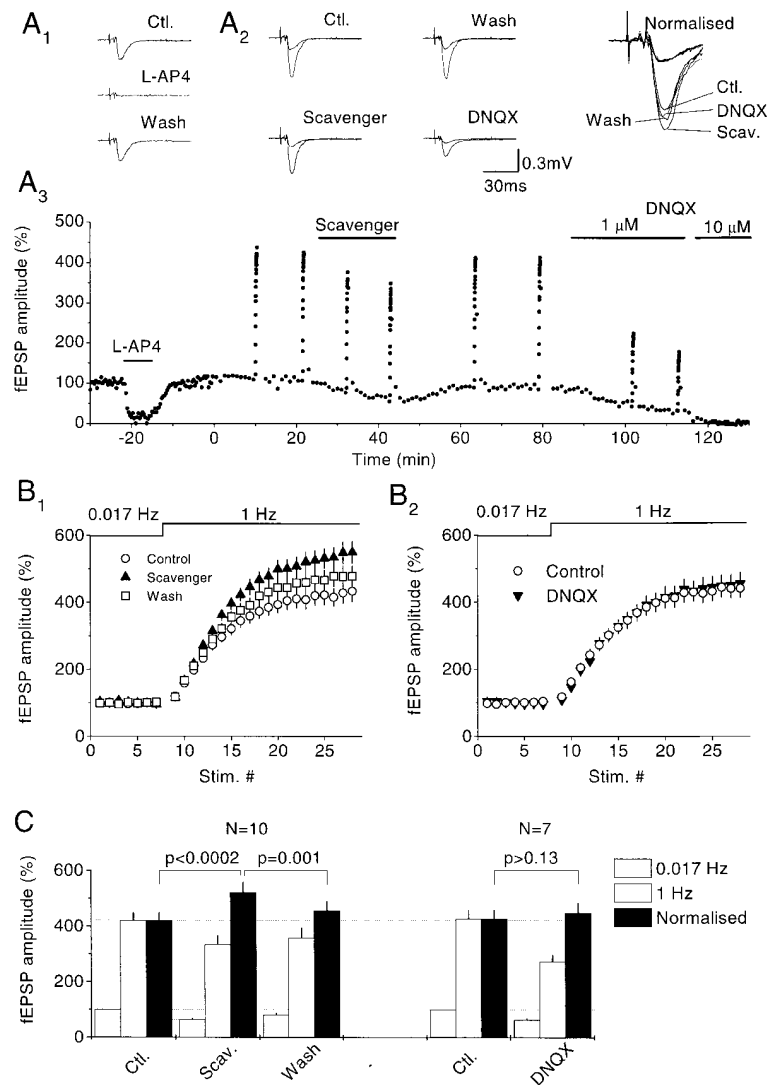


Figure 1. A Glutamate Scavenger Reduces MF fEPSP Amplitude and Enhances Frequency Facilitation at Room Temperature

(A) Results from one experiment.

(A₁) MF EPSPs were reversibly depressed by applying the metabotropic receptor agonist L-AP4.

(A₂) Sample traces obtained at 0.017 Hz (average of last ten trials, smaller fEPSPs of each pair) and at 1 Hz (average of last seven trials, larger fEPSPs). The traces are taken from a control period (Ctl.), during application of GPT and pyruvate (Scavenger), following washout (Wash), and in the presence of 1 μM DNQX (DNQX). At right, the traces have been normalized by the low frequency fEPSP amplitudes, to show that the frequency facilitation is enhanced in the presence of the scavenger.

(A₃) fEPSP amplitude plotted against time for the same experiment. The fEPSP amplitude increased when the stimulus frequency was intermittently switched from 0.017 Hz to 1 Hz. DNQX (10 μM) was added at the end of the experiment to record the residual fiber volley, which was then subtracted from all the preceding traces.

(B₁) Averaged results from ten experiments (± SEM), showing the fEPSP amplitude plotted against stimulus number. The stimulation frequency was switched from 0.017 Hz to 1 Hz, as indicated at top, and the results were normalized by the amplitude at 0.017 Hz.

(B₂) Summary of the seven experiments where a low concentration of DNQX was added, showing no effect on frequency facilitation.

(C) Histogram showing mean fEPSP amplitude at 0.017 Hz (average of last seven points before switching frequency) and at 1 Hz (average of last ten points). The facilitation ratio is plotted as the black bars. In the presence of the scavenger, the low frequency fEPSP amplitude decreased, and the facilitation ratio increased. A low concentration of DNQX (right) produced a similar decrease in fEPSP amplitude at 0.017 Hz but had no effect on the facilitation ratio.

A Glutamate Scavenger Reduces MF EPSPs and Potentiates Frequency Facilitation

In order to enhance the clearance of glutamate, we applied the enzymatic scavenger system used by O'Brien and Fischbach (1986) in neuronal cultures and extended to brain slices by Rossi and Slater (1993; see also Overstreet et al., 1997). Glutamic-pyruvic transaminase (GPT, alanine transaminase, EC 2.6.1.2) catalyzes the conversion of glutamate and pyruvate to α-ketoglutarate and alanine. We examined MF-CA3 neuron signaling in hippocampal slices at room temperature, to determine whether the scavenger interferes either with anterograde glutamatergic signaling via AMPA receptors or with feedback signaling via metabotropic receptors. We monitored transmission at two stimulus frequencies: at a baseline of 0.017 Hz and during a temporary increase to 1 Hz, designed to elevate the transmitter release probability (Scanziani et al., 1997). In preliminary experiments (data not shown), we confirmed that the enhanced transmission seen with the higher stimulus frequency is further potentiated by the metabotropic glutamate receptor

antagonists (+)α-methyl-4-carboxyphenylglycine (MCPG, 500 μM) and (2S,3S,4S)-methyl-2-(carboxycyclopropyl)glycine (MCCG, 500 μM). This confirms the results of Scanziani et al. (1997) and argues for a feedback regulation of glutamate release at higher stimulus frequencies, mediated by group 2 metabotropic receptors.

Figure 1A shows the results from one experiment where we tested the effect of the scavenger. GPT (5 U/ml) applied together with pyruvate (2 mM) attenuated the MF field EPSP (fEPSP) amplitude. This was accompanied by an increase in the facilitation seen with 1 Hz stimulation. Following washout of the scavenger, there was a partial recovery in both EPSP amplitude and frequency facilitation toward baseline levels. Although these results are consistent with a reversible reduction in presynaptic feedback, the measured fEPSP amplitudes may not faithfully reflect the receptor open probability if the driving force for synaptic currents varies. We therefore routinely added a low concentration of the AMPA receptor blocker 6,7-dinitroquinoxaline-2,3-dione (DNQX, ~0.5 μM) at the end of the experiment, in order to reduce

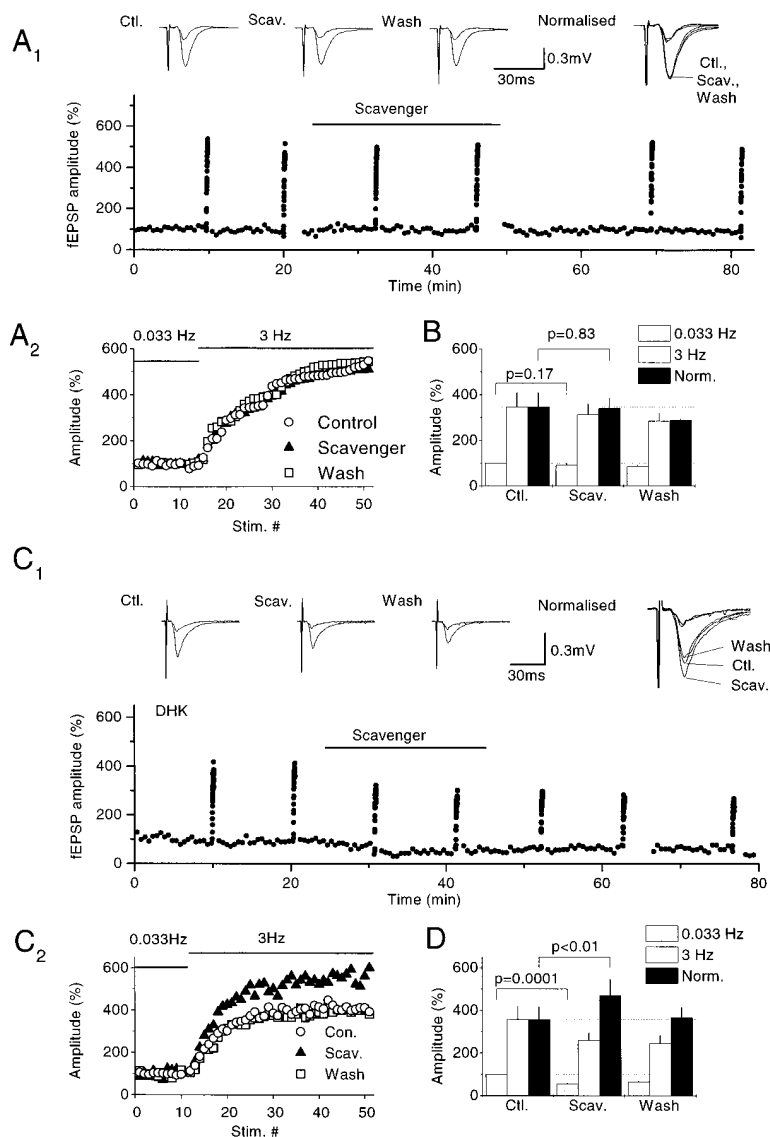


Figure 2. The Scavenger Only Affects fEPSP Amplitude and Facilitation at Physiological Temperature if Glutamate Transport Is Inhibited

(A₁) Results from one experiment. The insets show fEPSPs elicited at 0.033 and 3 Hz and (right) the superimposed traces normalized by the 0.033 Hz response. In all cases, the traces were taken immediately before switching the stimulation frequency.

(A₂) fEPSP amplitude plotted against stimulus number; the scavenger had no effect on frequency facilitation.

(B) Summary of five experiments, showing no significant effect on fEPSP amplitude at either 0.033 or 3 Hz. At physiological temperature, there was a slow rundown in fEPSP amplitude, as witnessed by the washout results. (C₁) Results obtained in one experiment with 50 μM DHK present throughout to inhibit glutamate uptake. The scavenger reduced fEPSP amplitudes at 0.033 Hz and enhanced frequency facilitation, as witnessed by the superimposed traces, normalized by the 0.033 Hz responses (inset at right).

(C₂) Reversible enhancement of frequency facilitation by the scavenger, shown for the same experiment.

(D) Summary of results (n = 6). Following washout, recovery of the fEPSP amplitude at 0.033 Hz was incomplete in some experiments.

the fEPSP to a similar degree as the scavenger. This had no discernible effect on the degree of frequency facilitation, implying that the change in frequency facilitation seen with the scavenger genuinely reflects reduced modulation of transmitter release.

Similar results were obtained in 10 slices (Figure 1B). In three of these experiments, pyruvate (2 mM) was present throughout the experiment, with GPT added subsequently. These experiments gave identical results (see also two-pathway experiments, below). We also verified that GPT applied in the absence of pyruvate (5–10 U/ml, n = 2) was without effect (<10% change in fEPSP), as were the products of the catalyzed reaction, alanine (0.5 mM) and α-ketoglutarate (0.5 mM) applied together (n = 3). On average, the scavenger attenuated the baseline fEPSP amplitude to 64% ± 4% and potentiated frequency facilitation to 124% ± 3% of control. The effect on frequency facilitation was comparable to that of the metabotropic receptor antagonists (128% ± 11%; see above). The results can be explained by an acceleration of the clearance of glutamate from the extracellular space, with two effects. First, it reduces the

activation of postsynaptic AMPA receptors, and second, it interferes with the frequency-dependent modulation of transmission by feedback signaling at metabotropic receptors.

The Scavenger Affects Transmission at Physiological Temperature if Uptake Is Blocked

Although the results illustrated in Figure 1 argue for a role of feedback signaling at room temperature, it is important to determine whether this persists under more physiological recording conditions. We therefore repeated the experiments at 36°C–38°C. Since transmitter release is very temperature sensitive (van der Kloot and Molgo, 1994), we used a higher baseline stimulus frequency (0.033 Hz versus 0.017 Hz at room temperature) and intermittently increased the frequency to 3 Hz instead of 1 Hz. This gave a similar degree of frequency facilitation as in the experiments at room temperature. Figures 2A and 2B show that the scavenger was without significant effect on either the baseline EPSP amplitude or the degree of frequency facilitation (n = 5). A possible

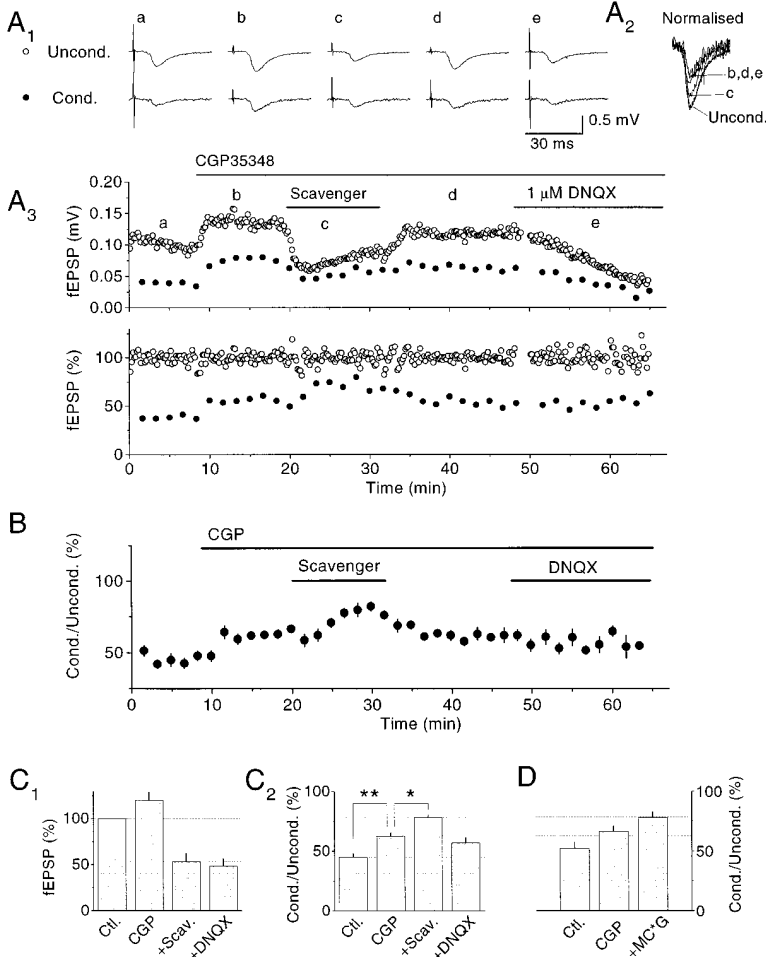


Figure 3. The Scavenger Attenuates Heterosynaptic Depression Mediated by Metabotropic Receptors

(A) Results obtained in one experiment, where two MF inputs were studied.

(A₁) Sample traces obtained at the times indicated in A₃ (top row, fEPSPs in test pathway without preceding train in conditioning pathway; bottom row, fEPSPs elicited in test pathway, 200 ms following the end of a 20 pulse, 20 Hz train in the conditioning pathway).

(A₂) Traces in (A₁) normalized by the amplitude of the unconditioned fEPSPs and superimposed, to show the reduced heterosynaptic depression in the presence of the scavenger (trace c).

(A₃) fEPSP amplitude plotted against time for the same experiment (top) and normalized to set successive groups of 10 unconditioned trials equal to 100% (bottom). Open symbols, unconditioned fEPSPs. Closed symbols, conditioned fEPSPs. The unconditioned fEPSP amplitude increased, and the heterosynaptic depression decreased, following addition of the GABA_B antagonist CGP35348. The scavenger reversibly decreased the fEPSP amplitude and further attenuated heterosynaptic depression. A low concentration of DNQX produced no change in heterosynaptic depression, in spite of a progressive depression of fEPSP amplitude.

(B) Summary of results (n = 7) showing the conditioned fEPSP, normalized by the unconditioned fEPSP amplitude.

(C₁) Mean unconditioned fEPSP amplitude, normalized by the control period (Ctl.), in the presence of CGP35348 (CGP), in the presence of CGP and scavenger (+Scav.), and in the presence of CGP and DNQX (+DNQX). (C₂) Conditioned fEPSP amplitude, as a fraction of the unconditioned amplitude (*p = 0.002; **p < 0.001).

(D) Summary of six experiments where the metabotropic antagonists MCCG (n = 3) or MCPG (n = 3) were added instead of the scavenger. MCCG or MCPG added in the presence of CGP35348 (+MC*G) produced a similar reduction in heterosynaptic depression as the scavenger (horizontal dotted lines).

explanation for the negative result is that glutamate uptake is enhanced at physiological temperature (Wadiche et al., 1995). This may limit the spatiotemporal glutamate profile to the extent that the scavenger has no incremental effect on the occupancy of pre- and postsynaptic receptors. We tested this hypothesis by applying the scavenger in the presence of the uptake blocker dihydrokainate (50 or 100 μM). Figures 2C and 2D show that, under these conditions, the scavenger again significantly reduced baseline transmission and potentiated frequency facilitation, to a similar degree as at room temperature. These results imply that glutamate uptake plays an important temperature-dependent role in limiting both the activation of postsynaptic receptors and the extent of extrasynaptic transmitter diffusion (Asztely et al., 1997; Diamond and Jahr, 1997).

Heterosynaptic Modulation of Transmitter Release

Although the above results, and those of Scanziani et al. (1997), support the view that glutamate escapes the synaptic cleft to activate preterminal metabotropic receptors, they do not exclude an action of glutamate

at as yet unidentified presynaptic receptors within the synaptic cleft where it is released. However, brief trains of stimuli in one MF pathway have recently been reported to depress transmission in another pathway (Vogt et al., 1997, Soc. Neurosci., abstract), prompting the suggestion that glutamate can diffuse between distinct MF synapses. We asked whether the glutamate scavenger could interrupt this form of heterosynaptic modulation at room temperature. We positioned two stimulating electrodes in the dentate gyrus to elicit MF fEPSPs in stratum lucidum. We delivered single stimuli to one pathway at 0.1 Hz, and we preceded every tenth stimulus with a train of 20 action potentials in the other pathway (20 Hz). In agreement with Vogt et al. (1997, Soc. Neurosci., abstract), the conditioned fEPSPs were depressed, relative to the unconditioned fEPSPs (Figure 3A). The heterosynaptic depression was partly reversed by adding the GABA_B antagonist CGP35348 (500 μM), implying that GABA mediates a component of the presynaptic modulation of transmitter release. This effect of CGP35348 was associated with an increase in the amplitude of the unconditioned fEPSP, implying that

GABA_B receptors are tonically activated in the absence of evoked action potentials in the conditioning pathway. In the continued presence of CGP35348, we applied the glutamate scavenger (15 U/ml GPT, with 2 mM pyruvate present throughout the experiment). This led to a reversible decrease in fEPSP amplitude, which was associated with a further decrease in heterosynaptic depression. Similar results were obtained in seven experiments (Figures 3B and 3C). These observations lend further support to the view that glutamate released from one population of MF terminals can reduce transmitter release at other terminals.

We compared the effect of the scavenger to that of blocking metabotropic receptors (Vogt et al., 1997, Soc. Neurosci., abstract). In a separate series of experiments, addition of 1.5 mM MCCG (n = 3) or MCPG (n = 3) in the continued presence of CGP35348 produced a similar decrease in heterosynaptic depression (Figure 3D). The similarity of the effects of the glutamate scavenger with those of MCCG or MCPG application supports the proposal that glutamate, and not an unidentified endogenous agonist at metabotropic receptors, mediates heterosynaptic depression. The scavenger and metabotropic receptor antagonists, however, did not abolish the depression completely (Figure 3D). Whether the residual depression is also mediated by metabotropic receptor activation cannot be determined from these results.

Slowing Glutamate Diffusion Enhances Receptor Activation

A numerical simulation of glutamate diffusion in the perisynaptic space has recently suggested that, for a given amount of glutamate released, AMPA and NMDA occupancy is enhanced by reducing the diffusion coefficient (Rusakov and Kullmann, 1998a). This principle applies not only to receptors located within the synaptic cleft but also, surprisingly, to receptors positioned at a distance from the release site. Although glutamate reaches these receptors more slowly when the diffusion coefficient is low, it subsequently persists for longer and is therefore more likely to activate them. Although less is known about the kinetics of metabotropic receptors, their affinity for glutamate is similar to that of NMDA receptors (Hayashi et al., 1993; Pin and Duvoisin, 1995). Kainate receptors, moreover, have similar kinetic properties to AMPA receptors (Paternain et al., 1995; Lerma, 1997). It is thus likely that retarding diffusion should enhance the activation of all these receptor classes, unless they are already saturated under baseline conditions. Thus far, there has been no experimental method to alter the diffusion coefficient for glutamate. We have, however, argued recently that extracellular macromolecules exert a major influence on the diffusion of small molecules in the extracellular space (Rusakov and Kullmann, 1998b). Macromolecules act as obstacles to the movement of diffusing molecules such as glutamate, resulting in an increase in the effective viscosity of the medium and therefore in the time required for movement away from a release site. This principle is supported by experimental evidence that large molecular weight dextrans, when added to the perfusion solution, retard the movement of the small inorganic ion tetramethylammonium (Prokopová et al., 1996, Physiol. Res., abstract). We therefore

examined the effect of adding 5% dextran (40 kDa molecular weight) to the perfusion solution (rendered isoosmotic by adding water). Our control measurements confirmed that this procedure increases the viscosity of the perfusate from ~1.05 to ~2.45 mPas.

Figure 4A shows the effect of dextran perfusion on MF fEPSPs elicited at 0.033 and 3 Hz at room temperature. The fEPSP amplitude elicited at 0.033 Hz increased, and this was accompanied by a significant and reversible reduction in frequency facilitation (Figures 4A₂ and 4B). These results are consistent with enhanced activation both of postsynaptic AMPA receptors (see also Figure 5A) and of presynaptic metabotropic receptors. In order to test this hypothesis further, in a separate series of experiments we applied dextran together with the presynaptic metabotropic antagonist MCPG (500 μM). Although dextran perfusion again led to a significant increase in the postsynaptic signal, frequency facilitation in this situation was unaffected (Figures 4C and 4D). The results imply that the effect of increased viscosity on the activation of postsynaptic AMPA receptors can be dissociated from its effect on presynaptic modulation by metabotropic receptors.

Dextran Perfusion Enhances Kainate Receptor-Mediated Signals

High frequency bursts of action potentials greatly enhance the kainate receptor-mediated component of MF EPSPs/EPSCs (Vignes and Collingridge, 1997; Castillo et al., 1997), which can be explained by postulating that a higher glutamate concentration is achieved within the synaptic cleft, leading to greater kainate receptor occupancy. This proposal leads to the prediction that retarding the diffusion of glutamate should also enhance the amplitude of the kainate receptor-mediated signal.

We isolated a kainate receptor-mediated synaptic current in whole-cell recordings from CA3 neurons, by blocking AMPA receptors with the selective antagonist GYKI52466 (100 μM; Paternain et al., 1995) and delivering brief trains of stimuli at 100–200 Hz (Castillo et al., 1997; Vignes and Collingridge, 1997). The residual EPSC was slow, reversed at ~0 mV, and was blocked by adding the nonselective non-NMDA receptor antagonist DNQX, confirming that it was kainate receptor mediated. Figure 5B shows that dextran perfusion reversibly increased the amplitude of the kainate receptor-mediated EPSC. This result implies that modulation of kainate receptor-mediated EPSCs can be achieved by altering glutamate diffusion and does not depend on a change in transmitter release probability. The occupancy of kainate receptors thus varies according to the spatiotemporal profile of glutamate diffusion. Whether the kainate receptors are situated close to the release sites, with a relatively low affinity for glutamate, or are located more remotely, requiring glutamate to diffuse relatively further (Lerma, 1997; Mayer, 1997), cannot be determined on the basis of these results.

Dextran Perfusion Enhances AMPA and NMDA Receptor-Mediated Signals in CA1

The large potentiation of AMPA and kainate receptor-mediated MF synaptic signals can be explained by low

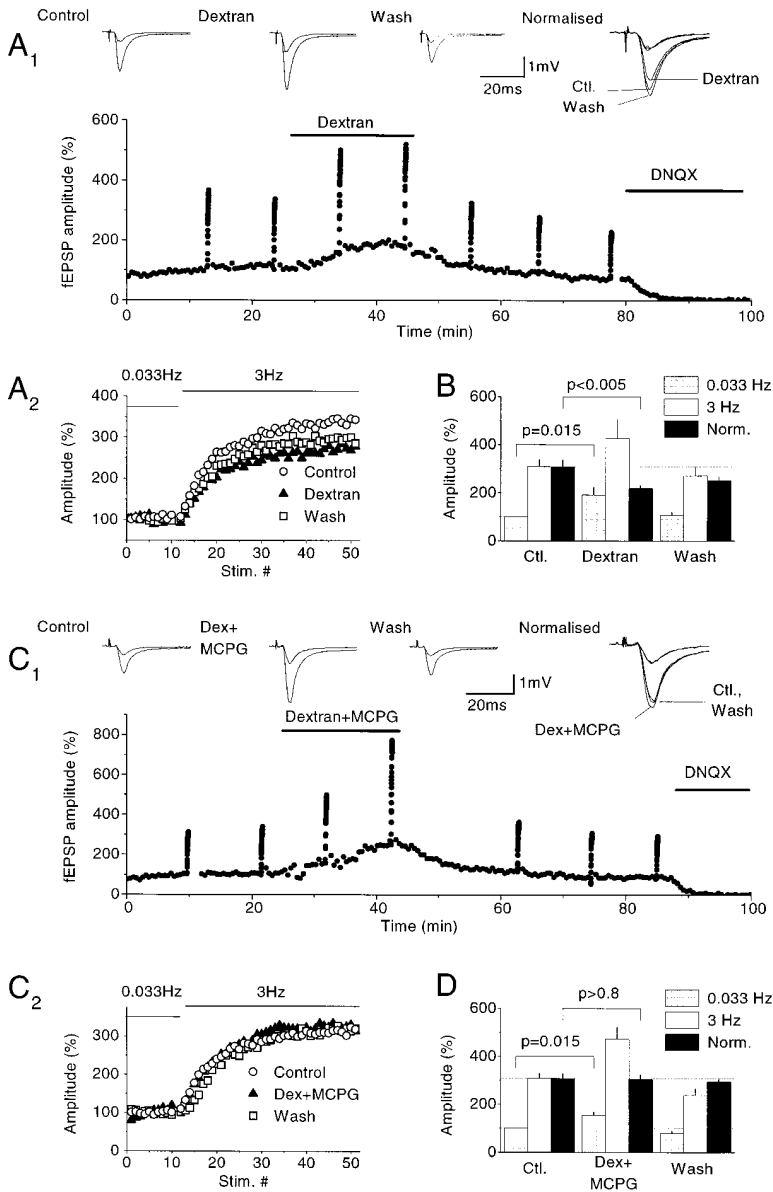


Figure 4. Increasing the Extracellular Viscosity with High Molecular Weight Dextran Enhances fEPSPs and Attenuates Frequency Facilitation

(A₁) Results from one experiment, showing a reversible increase in fEPSP amplitude. The superimposed traces, normalized by fEPSPs at 0.033 Hz (right inset) show a reversible decrease in facilitation.

(A₂) fEPSP amplitudes, normalized by the average amplitude at 0.033 Hz, plotted against stimulus number. Dextran perfusion produced a decrease in frequency facilitation. DNQX (10 μM) was added at the end of the experiment.

(B) Summary of results (n = 8), showing significant and reversible enhancement in fEPSP amplitude at 0.033 Hz and reduction in frequency facilitation.

(C₁ and C₂) Results from one experiment in which the metabotropic receptor antagonist MCPG was applied together with dextran. This prevented the attenuation in frequency facilitation, without preventing enhancement of transmission.

(D) Summary of results (n = 5), showing that frequency facilitation in the presence of dextran and MCPG was almost identical to the control value (compare also to Ctl. in [B]), in spite of a significant increase in fEPSP amplitude.

mean occupancy of the postsynaptic receptors under baseline conditions. Does this apply to other excitatory synapses in the hippocampus, or is it a property of MF-CA3 transmission—for instance, because several release sites occur within a large synaptic connection (Chicurel and Harris, 1992)? We turned to another well-characterized synapse in the hippocampus: the Schaffer collateral-CA1 synapse. This has a much simpler structure, generally with only a single active zone (Harris and Stevens, 1989; Sorra and Harris, 1993). Figure 5C shows the effect of dextran perfusion on AMPA receptor-mediated synaptic signals elicited in CA1 cells by stratum radiatum stimulation. An enhancement was seen whether the synapses were monitored in whole-cell voltage clamp ($p < 0.1$) or with field EPSP recordings ($p < 0.05$), although it was smaller than that seen at MF-CA3 synapses. Averaging together the results obtained with both recording methods, the potentiation was 16% ±

8%, as compared to 84% ± 32% at MF-CA3 synapses. This result suggests that synapses may respond differently to alterations in glutamate diffusion, depending on their geometries.

We also examined the effect of dextran perfusion on the NMDA receptor-mediated component of Schaffer collateral synaptic signals in CA1, recorded in 10 μM DNQX to block AMPA receptors. We either recorded EPSCs by holding the postsynaptic neuron at a positive voltage or fEPSPs uncovered by lowering the extracellular Mg²⁺ concentration to 0.1 mM. Again, a significant potentiation was obtained with both whole-cell ($p < 0.05$) and field potential recording ($p < 0.05$). Overall, dextran perfusion caused a reversible increase in the EPSC/fEPSP amplitude of 20% ± 6% (Figure 5D). No significant change was seen in the time course of either AMPA or NMDA receptor-mediated EPSCs.

By analogy with the effect of dextran on MF-CA3

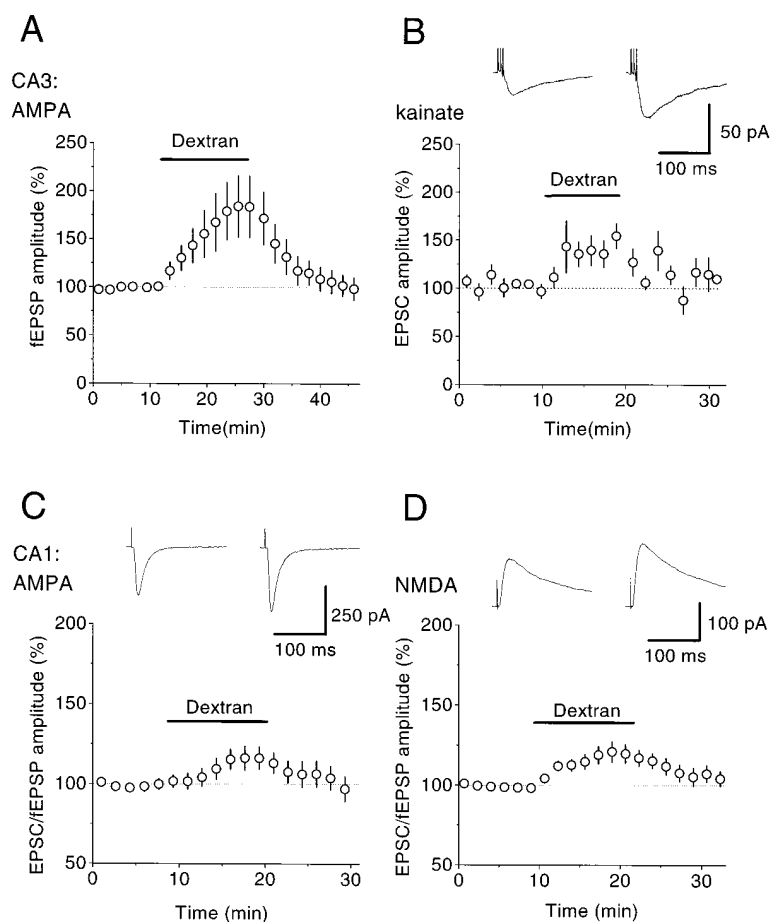


Figure 5. Dextran Perfusion Enhances AMPA and Kainate Receptor-Mediated Transmission at Mossy Fiber Synapses More Than AMPA or NMDA Receptor-Mediated Transmission at Schaffer Collateral Synapses

(A) Effect of dextran perfusion on mossy fiber fEPSP amplitude ($n = 10$), measured at 0.033 Hz.

(B) Dextran perfusion enhanced kainate receptor-mediated EPSCs, isolated by blocking AMPA receptors and delivering brief high frequency trains ($n = 5$).

(Inset) EPSC time course before (left) and during (right) dextran perfusion.

(C) Summary of effect of dextran perfusion on AMPA receptor-mediated Schaffer collateral signals recorded in CA1 pyramidal cells. Similar results were obtained whether extracellular fEPSPs ($n = 12$) or whole-cell EPSCs ($n = 11$, insets) were recorded.

(D) Effect of dextran perfusion on NMDA receptor-mediated fEPSPs ($n = 13$) and EPSCs ($n = 14$, inset).

signaling, these results imply that the occupancy of both AMPA and NMDA receptors following glutamate exocytosis at CA1 synapses is $<100\%$ in the control period. However, given that NMDA receptors have a higher affinity than AMPA receptors (Patneau and Mayer, 1990), their occupancy should be higher, and may even approach saturation. The observation that the potentiation of the NMDA receptor component by dextran is at least as large as that of the AMPA receptor-mediated component is thus unexpected. It can, however, be explained by the proposal that glutamate binds not only to NMDA receptors within the synapses where it is released but also at neighboring synapses (see Discussion).

Discussion

Experimental Manipulation of Extracellular Glutamate Diffusion

We have employed two novel and unique tools to probe glutamatergic transmission. The scavenger catalyzes the conversion of glutamate to α -ketoglutaric acid and reduces its extracellular concentration. Dextran, in contrast, prolongs the presence of glutamate and slows its diffusion away from release sites. The scavenger attenuates anterograde transmission at MF-CA3 synapses, while reducing feedback and heterosynaptic depression of transmitter release via metabotropic receptor activation. Dextran has converse effects and, in

addition, potentiates kainate receptor-mediated transmission. All of these effects can be accounted for by a direct action on the spatiotemporal profile of glutamate diffusion following exocytosis.

Unlike all other available pharmacological tools, the scavenger and dextran appear not to act on presynaptic transmitter release, on postsynaptic receptors, or on glutamate transporters. In the case of the scavenger, GPT on its own is without effect on synaptic transmission, and only in the presence of pyruvate does it alter glutamatergic signaling. Although the simplest explanation for the effect of the scavenger is to bind and catalyze the conversion of synaptically released glutamate to α -ketoglutarate, several other possibilities need to be considered. First, diverting pyruvate away from the tricarboxylic acid cycle could potentially interfere with presynaptic glutamate synthesis. This is, however, very unlikely to explain the results, since both glucose and pyruvate were present in excess. Second, reducing the tonic extracellular glutamate concentration (Rossi and Slater, 1993) could affect the occupancy of receptors, transporters, and other binding sites. Reducing the tonic activation of presynaptic metabotropic receptors would, however, be expected to enhance rather than depress evoked transmitter release. As for reducing the occupancy of AMPA receptors, this too would be expected to enhance transmission, since fewer receptors would be desensitized prior to evoked glutamate release (see

below). One remaining possibility is that other binding sites, either within or without the synaptic cleft (such as transporters and "orphan" receptors), are normally partly occupied by glutamate molecules prior to evoked release. If glutamate molecules are stripped off these sites by the action of the scavenger, then a large number of high affinity binding sites could be exposed, ready to "soak up" part of the vesicle contents released in response to a presynaptic stimulus. Whether this phenomenon contributes to the action of the scavenger remains to be determined.

Dextran, on the other hand, is a biologically inert macromolecule, which is used clinically as a plasma expander because of this property. Since it was applied in an isoosmotic solution, an effect on cell volume is unlikely. Although we cannot exclude an action at as yet unidentified pre- or postsynaptic receptors, its effects on glutamatergic transmission are in exact agreement with the predicted consequences of enhancing receptor occupancy.

The novel approaches to manipulate the glutamate transient employed here underline the importance of diffusion in determining receptor activation. Although the scavenger technique is specific for glutamate, analogous enzymatic methods may be adapted to address other forms of chemical transmission. Manipulation of the extracellular viscosity using high molecular weight dextrans, on the other hand, should be equally effective for all neurotransmitters, since its consequences depend on the fundamental mechanisms of extracellular diffusion.

Increasing Extracellular Viscosity Potentiates Synaptic Receptor Activation

In the experiments at MF synapses at room temperature, we found no evidence for a "safety factor" for either anterograde or feedback signaling. That is, enhancing or retarding the clearance of glutamate had opposite effects, as would be expected if receptor occupancy was normally incomplete and could be increased or decreased along a continuum. Dextran perfusion produced a large potentiation of AMPA ($84\% \pm 32\%$) and kainate ($45\% \pm 14\%$) receptor-mediated EPSPs/EPSCs, implying that these classes of receptors are far from saturated by glutamate released in response to a brief train of action potentials. A possible explanation for the wide range over which EPSPs/EPSCs can be graded by manipulating diffusion is that glutamate can spread from one release site within the large MF synaptic connection to receptor clusters opposite other release sites (Chicurel and Harris, 1992). This proposal leads to the prediction that experimental modulation of the glutamate profile should have a smaller effect at simpler synapses such as those made by Schaffer collaterals on CA1 neurons, which generally have only a single ultrastructurally defined active zone (Harris and Stevens, 1989; Sorra and Harris, 1993). In agreement with this prediction, dextran perfusion had a more modest effect on AMPA and NMDA receptor-mediated EPSCs in this system.

If the AMPA receptors at CA1 synapses have a high occupancy, then an even larger proportion of NMDA

receptors should be bound by glutamate following release, since they have a much greater affinity for glutamate (Patneau and Mayer, 1990). The NMDA receptors should then be almost saturated, and this component of the EPSCs should be unaffected by dextran. The fact that dextran perfusion increased the NMDA component at least as much as the AMPA component is thus unexpected. It can, however, be explained by proposing that glutamate diffuses out of the synaptic cleft and activates high affinity NMDA receptors at neighboring synapses (Kullmann and Asztely, 1998). Dextran perfusion potentiates this "spillover" phenomenon, explaining the increase in the NMDA receptor-mediated component. We have recently simulated the effect of altering the glutamate diffusion coefficient on AMPA and NMDA receptor-mediated EPSCs generated by exocytosis and diffusion within a realistic representation of the CA1 neuropil (Rusakov and Kullmann, 1998a). We have also estimated the relative contribution of geometric obstacles and extracellular macromolecules to the overall tortuosity that determines diffusion in the brain (Rusakov and Kullmann, 1998b). Under baseline conditions, the overall tortuosity factor of the hippocampal and neocortical neuropil (λ), measured with small ions, is generally estimated as ~ 1.6 (Nicholson and Syková, 1998). The diffusion coefficient in a porous medium approximating the neuropil D^* is related to the free diffusion coefficient D by $D^* = D/\lambda^2$. Since D for glutamate in a free medium is $\sim 0.75 \mu\text{m}^2\text{ms}^{-1}$ (Longworth, 1953), this implies that D^* for glutamate is normally $\sim 0.3 \mu\text{m}^2\text{ms}^{-1}$, not taking into account spatial inhomogeneities and interactions with transporters and other binding sites, which could further slow diffusion (Diamond and Jahr, 1997; Rusakov and Kullmann, 1998a). Nicholson and Tao (1993) have shown that high molecular weight dextran penetrates the neuropil, albeit more slowly than small inorganic ions. We have estimated that 2% dextran (40 kDa molecular weight) increases the tortuosity for small diffusing ions to ~ 1.8 (Rusakov and Kullmann, 1998b), and this is confirmed by experimental measurements of the diffusion of tetraethylammonium ions (Prokopová et al., 1996, *Physiol. Res.*, abstract). Assuming a hydrodynamic radius for this dextran molecule of ~ 8 nm (Tao and Nicholson, 1996), the tortuosity factor in 5% dextran can be estimated to approach its theoretical maximum of 2.2 (Rusakov and Kullmann, 1998b). This corresponds to a halving of D^* to $\sim 0.15 \mu\text{m}^2\text{ms}^{-1}$. Given reasonable estimates of the contents of a single vesicle, and of the distance separating neighboring CA1 synapses (Rusakov et al., 1998), our simulations predict that reducing the glutamate diffusion coefficient over this range increases the amplitude of both AMPA and NMDA receptor-mediated components by $\sim 20\%$ – 30% (see Figure 9 in Rusakov and Kullmann, 1998a). The increase in the AMPA component is principally because of enhanced occupancy of receptors within the synapse where glutamate is released. In the case of the NMDA component, the increase is almost entirely due to enhanced opening of receptors positioned at neighboring synapses. These estimates are in reasonable agreement with the effects of dextran perfusion on CA1 EPSPs/EPSCs reported here, although a more accurate simulation would take into account the structural heterogeneity and variability

in receptor density that are seen within the population of synapses (Nusser et al., 1998 [this issue of *Neuron*]).

Although reducing the diffusion coefficient should also affect the time course of EPSCs, our simulations show that the expected changes in rise time are small (<1 ms), possibly explaining why they were not detected in the present study.

Increasing Glutamate Clearance Reduces Synaptic Receptor Activation

One potentially powerful method to resolve the issue of relative AMPA and NMDA receptor occupancy would be to enhance glutamate clearance with the scavenger. The interpretation of such experiments would, however, be difficult, because NMDA receptors are desensitized at low micromolar concentrations (Lester and Jahr, 1992) similar to those that are thought to occur in the extracellular space in the absence of exocytosis (Sah et al., 1989; Bouvier et al., 1992). That is, at the resting level of extracellular glutamate, a large proportion of receptors is unavailable to open in response to exocytosis. Reducing the tonic glutamate concentration with the scavenger (Rossi and Slater, 1993) would then bring some receptors out of the desensitized state and paradoxically enhance the evoked EPSC amplitude, counteracting any direct effect of the scavenger on glutamate diffusing to the receptors. This phenomenon may have a much smaller effect on AMPA receptor-mediated EPSCs, since these receptors desensitize at a higher glutamate concentration (Jonas et al., 1993). Indeed, our finding that the scavenger reduces, rather than enhances, the AMPA component at mossy fiber synapses argues that its effect on glutamate diffusion overrides any decrease in the resting desensitization level of the receptors (Trussell et al., 1993).

At physiological temperature, in contrast to room temperature, the scavenger was without effect on either postsynaptic fEPSP amplitude or on frequency facilitation, unless glutamate uptake was blocked with dihydrokainate. This implies that glutamate uptake plays a major role in determining receptor occupancy for both pre- and postsynaptic receptors in vivo. If binding of glutamate to transporters is enhanced by raising the temperature to a greater extent than binding to (and catalysis by) GPT, then this could explain why the scavenger appears not to affect receptor occupancy at physiological temperature. The conclusion that glutamate uptake plays a greater role at physiological temperature is supported by evidence that intersynaptic glutamate cross-talk, sensed by NMDA receptors, is reduced by raising the recording temperature (Asztely et al., 1997; Min et al., 1998).

Conclusion

The present study shows that the contribution of various receptor types to the function of hippocampal synapses depends on the spatiotemporal profile of neurotransmitter diffusion following exocytosis. This profile is determined not only by the properties of transmitter release, but also by the extracellular environment. Uniform perturbations of the environment may, however, have non-uniform effects on the function of synaptic connections with different architectures.

Experimental Procedures

Male Hartley guinea pigs (4–5 weeks old) were killed by cervical dislocation followed by decapitation. Transverse hippocampal slices (450 μ m thick) were cut on an oscillating tissue slicer (FHC, Bowdoinham, ME) and stored in an interface-type chamber before transfer into a submersion-type recording chamber. The perfusion solution contained (in mM) NaCl (119), KCl (2.5), MgCl₂ (4), CaCl₂ (4), NaHCO₃ (26.2), NaH₂PO₄ (1), glucose (11), and picrotoxin (0.1) and was gassed with 95% O₂/5% CO₂. The divalent cation concentrations were kept high to suppress polysynaptic transmission. Data were rejected if epileptiform bursting was observed. In the experiments on CA1 synapses, the CaCl₂ and MgCl₂ concentrations were reduced to 2.5 and 1.3 mM. The experiments were carried out at room temperature (23°C–25°C), except where indicated. Physiological temperature (36°C–38°C) was achieved by controlling the temperature of the perfusion solution and of the chamber with a Peltier effect device.

Stimuli were delivered via bipolar stainless steel electrodes. For MF experiments, one or two stimulating electrodes were positioned in stratum granulosum of the dentate gyrus. Two tests were routinely applied to verify that the signal recorded in stratum lucidum was a MF fEPSP. First, increasing the stimulation frequency caused pronounced facilitation (>2.5-fold at 1 Hz at room temperature or at 3 Hz at physiological temperature). Second, application of L(+)-2-amino-4-phosphonobutyric acid (L-AP4, 10 μ M) depressed the fEPSP amplitude to <20% of control (Manzoni et al., 1995). At the end of each experiment, DNQX (10 μ M) was added to block AMPA receptors, and the residual fiber volley was subtracted from the fEPSPs. Where two pathways were studied, we verified that the fEPSP elicited in one pathway was not facilitated by a preceding stimulus to the other pathway. For CA1 experiments, either one or two electrodes were positioned in stratum radiatum (stimulus frequency, 0.1–0.3 Hz).

Recordings were made either with extracellular field potential electrodes containing 3 M NaCl or with whole cell voltage-clamp pipettes containing Cs gluconate (117.5), CsCl (17.5), HEPES (10), EGTA (0.2), NaCl (8), Mg-ATP (2), GTP (0.3), and QX314 Br (5) (pH 7.2, 295 mOsm). The access resistance was monitored with a voltage step and was <20 M Ω . GPT (porcine heart, 115 kDa dimer) was dialyzed for at least 3 hr with a 10 kDa cutoff membrane (Slide-A-Lyzer, Pierce Chemical, Rockford, IL) prior to addition to the perfusion solution. Dextran (40 kDa molecular weight) was added to the standard perfusion solution in a concentration of 5% and rendered isoosmotic by diluting the solution with water (~6%). Viscosity measurements were performed using a falling ball viscosimeter (Gilmont, Barrington, IL). Dextran perfusion had no consistent effect on the access or input resistance of the neurons recorded in whole-cell mode.

Drugs were purchased from Sigma, except for QX314 Br (Alomone Laboratories) and DNQX, L-AP4, MCPG, and PDC (Tocris Cookson). CGP35348 was a gift from Ciba-Geigy.

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