EXCITATORY SYNAPTIC TRANSMISSION IN HIPPOCAMPAL NEURONS

By

Gang Tong

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Craig E. Jahr, Ph.D., Mentor

John Resko, Ph.D., Chairman, Graduate Council

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MANUSCRIPTS

To my parents

and to Li-Ping and Fonda

without their love and support, none of this would have been possible

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ABSTRACT

Synaptic transmission involves the regulated release of neurotransmitter in response to neural activity and interaction of the transmitter with postsynaptic receptors to transduce the physiological signals. Most excitatory synapses in the mammalian central nervous system use L-glutamate as the neurotransmitter. Although numerous studies have addressed the physiological, pharmacological, biophysical, and molecular biological characteristics of glutamate receptor channels, less is known about the basic neurotransmission processes, such as transmitter release, transmitter clearance, and transmitter-receptor interactions. The lack of knowledge of the micro physiology of neurotransmission has hindered advances in understanding of such fascinating phenomena as synaptic plasticity, including long-term potentiation and depression. The purpose of this study was to examine such fundamental issues as the number of vesicles released from individual synaptic sites, the postsynaptic glutamate receptor occupancy, the mechanisms of glutamate clearance from the synaptic cleft, and the time course of glutamate concentration in the synaptic cleft. Whole cell and outside-out patch recording techniques were used on primary cultured rat hippocampal neurons.

In the first set of experiments, we found that more than one quantum of glutamate can be released from single synaptic contact sites after each action potential and can interact with the same population of postsynaptic N-methyl-D-aspartate (NMDA) receptors under conditions

which increase the probability of transmitter release. Increasing release probability also results in proportional increases in both AMPA (α-amino-3-hydroxy-5-methy-4-isoxazole propionic acid) and NMDA receptor components of the excitatory postsynaptic current (EPSC). These results suggest that the fraction of AMPA and NMDA receptors occupied by transmitter following the release of a single quantum is similar. Based on AMPA and NMDA receptor responses of outside-out patches to short applications of glutamate, we suggest that both receptor types may be saturated normally by synaptic release at room temperature.

In the second set of experiments, we provide evidence supporting the hypothesis that the glutamate transporter contributes to the clearance of transmitter from the synaptic cleft. The transporter blockers THA (DL-threo-β-hydroxyaspartic acid) and lithium increase the amplitude, but not the decay time, of miniature and evoked AMPA receptor EPSCs recorded at 34 °C but not at 24 °C. The amplitude increase is due to increasing the glutamate concentration in the cleft, suggesting that the glutamate transporter contributes to the clearance of glutamate from the synaptic cleft on the 100 μs time scale.

In the third set of experiments, we have observed that repetitive stimulation of cultured hippocampal neurons loaded with a low affinity NMDA receptor agonist D-glutamate caused a dramatic shortening of both the rising and decaying phases of NMDA receptor EPSCs evoked by autaptic stimulation. The EPSC time course was mimicked by NMDA receptor currents evoked in outside-out patches by brief application of D-glutamate. These data suggest that both the rise and decay times of the

NMDA receptor EPSCs are normally controlled by the slow unbinding rate of the natural neurotransmitter and that the free transmitter is present in the cleft for only a few milliseconds after release.

INTRODUCTION

Glutamate receptors mediate fast excitatory synaptic transmission in many areas of the mammalian central nervous system (CNS). Most neurons in the CNS express two distinct subtypes of glutamate receptors that are directly linked to ion channels. One receptor class, selective for AMPA (also known as the non-NMDA receptor), mediates the current flow of Na^+ and K^+ ions, and is primed for 'normal' fast cell-to-cell signaling. The other receptor type, selective for NMDA, controls the activity of ion channels permeable to both Ca^{2+} and monovalent cations that appear to play an important role in several forms of long-term changes of synaptic activity.

This introduction provides an overview of current ideas about central excitatory neurotransmission in the hippocampus including the physiology and structure of excitatory synapses, the storage of transmitter in the synaptic vesicle, the release of transmitter, and finally the mechanisms that are involved in the clearance of transmitter from the synaptic cleft.

PHYSIOLOGY OF EXCITATORY SYNAPTIC TRANSMISSION

Synaptically released glutamate can bind to at least three subtypes of receptor channels, two of which are ligand-gated ion channels (Collingridge and Lester, 1989; Mayer and Westbrook, 1987). An additional class of glutamate receptor, metabotropic receptors, are G-protein coupled (Nakanishi, 1992; Schoepp and Conn, 1993). The

ionophore-associated receptors are defined by the selective agonists which activate each type, NMDA and AMPA. Following synaptic release, glutamate binds to both NMDA and AMPA receptor channels which are colocalized in the same postsynaptic sites (Bekkers and Stevens, 1989; Hestrin et al., 1990; McBain and Dingledine, 1992), resulting in an EPSC with two distinct components: a fast component induced by AMPA receptor activation and a much slower component caused by the activation of NMDA receptors (Collingridge et al., 1988; Forsythe and Westbrook, 1988; Lester et al., 1990; Hestrin et al., 1990; Robinson et al, 1991; Silver et al, 1992).

Physiology of NMDA receptors.

The NMDA receptor plays important roles in many functions of glutamate in the CNS. It is essential for regulating neuronal excitability, inducing neurotoxicity, and inducing several forms of synaptic plasticity including long-term potentiation (LTP), long-term depression (LTD) and synaptogenesis (Madison et al., 1991; Bliss and Collingridge, 1993). Calcium entry through NMDA receptors is thought to contribute to both neuronal plasticity and neurotoxicity. The NMDA receptor has at least six pharmacologically distinct sites (e.g. Collingridge and Lester, 1989; Mayer and Westbrook, 1987; McBain and Mayer, 1994). These include a glutamate-binding site, a glycine-binding regulatory site (Johnson and Ascher, 1987), a site in the channel that binds channel blockers such as phencyclidine and related compounds, a voltage-dependent Mg^{2+}-binding site (Nowak et al., 1984; Mayer et al., 1984), a Zn^{2+} binding site,

and a fatty-acid binding domain (Petrou et al., 1993).

The NMDA receptor is subject to various regulations. Subunits of the NMDA receptor contain several consensus sites for phosphorylation by protein kinase A, protein kinase C, Ca^{2+}/calmodulin kinase II (CaMKII) and tyrosine kinases (Moriyoshi et al., 1991; Nakanishi, 1992; Kutsuwada et al., 1992). In neurons and oocytes expressing recombinant NMDA receptors, activation of protein kinase C enhances NMDA receptor responses (Chen and Huang, 1991; 1992; Kelso et al., 1992; Yamazake et al., 1992; Kutsuwada et al., 1992). Activity of the NMDA receptor is also regulated by phosphatases in outside-out patches (Tong and Jahr, 1994a). Arachidonic acid and docosahexaenoic acid which can be released by NMDA receptor-mediated activity of phospholipase A_2, enhance the NMDA receptor response (Miller et al., 1992; Nishikawa et al., 1994) by directly binding to the fatty acid-binding domain of the NMDA receptor (Petrou et al., 1993). The state of the actin cytoskeleton is also important for the function of NMDA receptors (Rosenmund and Westbrook, 1993). In addition, NMDA receptors can be modulated by polyamines (McGurk et al., 1990; Rock and MacDonald, 1992; Benveniste and Mayer, 1993), histamine (Bekkers, 1993; Vorobjev et al., 1993), physiological concentrations of hydrogen ions (Traynelis and Cull-Candy, 1990; 1991; Tang et al., 1990; Vyklicky et al., 1990), neurosteroids (Wu et al., 1991; Irwin et al., 1992), and the oxidation/reduction of the NMDA receptor (Aizenman et al., 1989).

Time Course of NMDA and AMPA Receptor EPSCs

The synaptic release of glutamate activates two types of currents with distinct time courses. The synaptic current through AMPA receptor channels activates rapidly with a time to peak of 600 μs or less and decays rapidly with a time constant of a few milliseconds; whereas that through NMDA receptor channels peaks in about 10 ms and does not terminate for hundreds of milliseconds (Bekkers and Steven, 1989; Hestrin et al., 1990; Lester et al., 1990; Clements et al., 1992; Silver et al., 1992; Hestrin, 1992). Several lines of evidence suggest that the slow time course of the NMDA receptor EPSC is caused by prolonged binding of glutamate to the receptors causing repetitive reopening of the bound channels. There appears to be no rebinding of glutamate to the NMDA receptor during synaptic transmission (Hestrin et al., 1990; Lester et al., 1990; Gibb and Colquhoun, 1992; Lester et al., 1994). This notion is confirmed by the finding that NMDA receptor currents activated in outside-out patches by low affinity agonists decay much more quickly than those evoked by high affinity agonists (Lester and Jahr, 1992; Sather et al., 1992). In addition, partial agonists of the glycine site that decrease the affinity of NMDA receptors for glutamate (Kemp and Priestley, 1991) accelerate the decay of NMDA receptor EPSCs (Lester et al., 1993). Therefore, the slow time course of the NMDA receptor EPSC is controlled by the slow unbinding rate of the natural transmitter. We have tested this hypothesis by directly changing the nature of the transmitter present in synaptic terminal (see manuscript III). In addition to the affinity of the agonists, the intrinsic gating properties of the NMDA receptor including complex bursts, clusters, superclusters of opening and desensitization also contribute to

the time course of the NMDA EPSC (Gibb and Colquhoun, 1991).

The time course of the AMPA receptor EPSC is dependent on either receptor desensitization (Trussell and Fischbach, 1989; Trussell et al., 1993) or the unbinding rate of glutamate from the AMPA receptor (Hestrin, 1992; Colquhoun et al., 1992; Jonas et al., 1993). In chick spinal and cochlear neurons, the onset of desensitization in response to long step application of glutamate exhibits a rapid component of decay, which is similar to the brief application response and the time course of miniature EPSCs (mEPSCs) (Trussell and Fischbach, 1989; Trussell et al., 1993). These data suggest that the decay of AMPA receptor EPSCs is mainly controlled by the desensitization of AMPA receptors. However, in hippocampal and visual cortical neurons (Colquhoun et al., 1992; Hestrin, 1992) the desensitization of AMPA receptors to step application of glutamate is much slower than the decay in response to a brief application, suggesting that the decay of a brief activation is not dominated by desensitization but rather by the faster unbinding rate of glutamate. Because only the response to a brief application of glutamate has a time course similar to mEPSCs, it is reasonable to assume that in hippocampus and cortex, the time course of AMPA receptor EPSCs is controlled by a fast unbinding rate rather than desensitization. The regional variations in gating kinetic properties may be due to different combinations of receptor subunits (Wisden and Seeburg, 1993; Seeburg, 1993).

Colocalization of NMDA and AMPA Receptors at Postsynaptic

Sites.

Colocalization of NMDA and AMPA receptors at postsynaptic sites was first suggested by radioligand binding studies on isolated postsynaptic densities (Fagg and Matus, 1984). Although so far there are no electron microscopic immunochemical studies to test this idea directly, functional studies have strongly supported the colocalization of these receptors. By recording mEPSCs, several groups have found that, like evoked EPSCs, mEPSCs are also composed of two components mediated by NMDA and AMPA receptors (Bekkers and Stevens, 1989; Robinson et al., 1991; Silver et al., 1992; McBain and Dingledine, 1992). Since mEPSCs are presumably caused by the exocytosis of single vesicles, these data suggest that both receptors are colocalized at individual release sites. In addition, by combining iontophoretic mapping of the distribution of NMDA and AMPA receptors along dendrites with subsequent immunohistochemical localization of synapses, NMDA and AMPA responses were shown to be colocalized at "hot spots" that coincided with synaptic connections (Jones and Baughman,1991). It is not clear, however, whether both receptors are evenly distributed in the postsynaptic density, and whether these two types of receptors are mixed or segregated at individual synaptic sites. Since the size of the postsynaptic density can vary 70-fold (0.008 to 0.54 μm^2) (Harris and Stevens, 1989), the receptor distribution may have a significant impact on the saturation and activation of glutamate receptors.

In addition to their synaptic location, both NMDA and AMPA receptors are also located at extrasynaptic sites, although their

physiological function is unknown. These receptors may be tonically activated by ambient glutamate present in the extracellular space (Sah et al., 1989; Blanton et al., 1990; Blanton and Kriegstein, 1992), although the tonic NMDA and AMPA receptor activity could include extrasynaptic and synaptic receptors. The tonic activation of the NMDA receptor imparts a regenerative electrical property to hippocampal neurons that facilitates the coupling between dendritic excitatory synaptic input and somatic action potential discharge (Sah et al., 1989).

Probability of Opening of NMDA and AMPA Receptors.

The probability of opening of NMDA receptors in steady-state whole-cell recording conditions has been estimated to be about 0.002 (Huetter and Bean, 1988). Using a open channel blocker of the NMDA receptor, MK-801, the probability that a liganded channel would open by short but saturating applications of glutamate in outside-out patches of cultured hippocampal neurons is estimated about 0.30 (Jahr, 1992); which is 150 times higher than that in the steady-state condition. By using the same channel blocker method, the opening probability of synaptic NMDA receptors has been estimated recently in hippocampal slices and in cultured hippocampal neurons (Hessler et al., 1993; Rosenmund et al., 1993). The opening probability of synaptic NMDA receptor in hippocampal slice is about 0.3 (Hessler et al., 1993), close to the value in outside-out patches (Jahr, 1992); however, the opening probability in cultured hippocampal neurons is about six-fold lower (Rosenmund et al., 1993). Although these differences may be real, differences in methodology

and analyses could also account for the disparate estimates.

By using variance analysis (Sigworth, 1980), the opening probability of AMPA receptors to glutamate applications was first estimated to be about 0.5 in whole-cell recording in dorsal root ganglion neurons treated with concanavalin A to reduce desensitization (Huettner, 1990). Using the same method, the maximal probability of AMPA receptor opening to saturating concentrations of glutamate is about 0.7 in outside-out patches of cortex and CA3 neurons (Hestrin, 1992; Jonas et al., 1993). The open probability of synaptic AMPA receptor channels has not been estimated yet, although outside-out patches may contain synaptic and extrasynaptic glutamate receptors. In addition, in several brain regions synaptic and extrasynaptic AMPA receptors have the same kinetic properties (Trussell and Fishbach, 1989; Hestrin, 1992; Livsey et al., 1993).

Number of Glutamate Receptors in the Postsynaptic Membrane.

The number of activated postsynaptic glutamate receptors at an individual synaptic site can be estimated from the amplitude of mEPSCs, because mEPSCs are presumably due to exocytosis of single vesicles. Unlike miniature endplate currents at the neuromuscular junction that on average are caused by the opening of about 1500-2000 nicotinic ACh receptors (Gage and Armstrong, 1968; Katz and Miledi, 1972), the mEPSCs in central excitatory synapses require opening of very few NMDA receptors (probably less than 10) and , at most, only 10 times as more as AMPA receptors (Bekkers and Stevens, 1989; Robinson et al., 1991; Hestrin, 1992; Silver et al., 1992; Jonas et al., 1993). If both receptor types

are normally saturated by synaptic release of glutamate, the total number of activated channels would be less than a few hundred at a single synaptic site; this number depends on the probability of opening at the peak of the EPSC. If the probability of opening of synaptic NMDA receptors at peak EPSC is 0.3 (Jahr, 1992; Hessel et al., 1993), the number of NMDA receptors would be about 30 at a single synaptic contact. In the extreme, if the peak opening probability is as low as 0.04 (Rosenmund et al., 1993), the number of NMDA receptors would still be less than 250. The estimates of the number of AMPA receptor channels open at the peak of mEPSCs range from 10 to 100 (Bekkers and Stevens, 1989; Hestrin, 1992; Jonas et al., 1993). If the probability of opening of synaptic AMPA receptors at the peak of the EPSC is 0.7 (Hestrin, 1992; Jonas, 1993), less than 150 AMPA receptors would be present at a single synaptic contact.

Occupancy of Synaptic Glutamate Receptors.

It is generally accepted that the postsynaptic NMDA receptors are saturated (100% receptor occupancy) or nearly saturated by glutamate during normal transmission because of its high affinity (EC_{50} = 3 μM) and the high concentration of glutamate (~1 mM) in the synaptic cleft (Patneau and Mayer, 1990; Larkman et al., 1991; Clements et al., 1992; Perkel and Nicoll, 1993). The occupancy of the AMPA receptors, however, may not be maximal because of its very low affinity (greater than 100-fold lower than NMDA receptor; Kishin et al., 1986; Patneau and Mayer, 1990; Trussell and Fischbach, 1989) implies a very fast unbinding rate (Clements et al., 1992; Perkel and Nicoll, 1993). If the opening rate from the bound but

closed state is similar to or slower than the unbinding rate, significant unbinding may occur before channel opening. However, by using a more complex model, Jonas et al. (1993) estimated that more than 80% of the AMPA receptor binding sites are occupied at the peak of an EPSC at room temperature. Thus, both NMDA and AMPA receptors might be nearly saturated by synaptic release at room temperature. We have examined this issue using outside-out patches as 'artificial' synapses (see manuscript I, II).

Heterogeneity of Postsynaptic Response.

Amplitude histograms of mEPSCs indicate large variability and usually are significantly skewed to the right (Bekkers and Stevens, 1990; Bekkers et al., 1990; Manabe et al., 1992). The mechanisms that underlie this heterogeneity are not clear. Since the size of the postsynaptic density varies greatly (Harris and Landis, 1986; Harris and Stevens, 1989), it is likely that the number of postsynaptic glutamate receptors varies from synapse to synapse. The stochastic properties of receptor-transmitter interactions may also contribute to the variability (Faber et al., 1992), because similar distributions are seen when mEPSCs are obtained by application of hypertonic solution at a fixed dendritic location comprising only one or two synapses (Bekkers and Stevens, 1990). In addition, the simultaneous release of more than one vesicle of transmitter from each synaptic sites could further increase the variability of mEPSC amplitudes. Nonuniformity in the transmitter content of synaptic vesicle and the effects of dendritic cable filtering may also contribute to the broad

distributions.

STRUCTURE OF THE CENTRAL EXCITATORY SYNAPSE

Synapses are specialized sites of contact between neurons that transmit electrical signals from presynaptic cells to postsynaptic cells. A central synapse consists of three distinct structures: a presynaptic terminal containing synaptic vesicles and voltage-gated calcium channels, a postsynaptic membrane containing ligand-gated receptor channels, and a synaptic cleft that physically separates the presynaptic and postsynaptic membranes.

Classically, central synapses are classified into two types (Gray, 1959): Gray type I (or asymmetric) and Gray type II (or symmetric). In the type I synapse, the cleft is slightly widened to approximately 20-30 nm, the active zone is larger, presynaptic dense projections are prominent, and the vesicles tend to be round. In addition, there is an extensive postsynaptic density (PSD), and amorphous dense material appears in the synaptic cleft. Type I synapses are usually excitatory. In the type II synapse, the cleft is 20 nm across. The active zone is smaller, the presynaptic dense projections and the postsynaptic density are modest, and there is no material in the cleft. The postsynaptic thickening often can not be distinguished from a simple thickening of the membrane bilayer. Characteristically, the vesicles of type II synapses are flattened. Type II synapses are usually inhibitory.

Presynaptic specializations. Synapses on neurons in the CNS are

usually made by presynaptic terminal swellings, called boutons. Electron microscopic studies have shown that most central synapses have prominent specializations at the presynaptic active zone, termed the 'synaptic grid' (Vrensen et al., 1980; Vrensen and Cardozo, 1981). From the grid emerges 'dense projections' of the presynaptic plasma membrane that may be involved in vesicle docking and release. The dense projections are arranged in more or less trigonal arrays, each of which extends from the presynaptic membrane into the cytoplasm for some distance and is typically surrounded by synaptic vesicles. However, it is uncertain whether these discrete structures exist as such in the unfixed neuron or are formed from cytoskeletal elements during fixation. Adjacent dense projections are just far enough apart that the synaptic vesicle can nestle between them and thus reach the presynaptic membrane. Regions with no presynaptic specialization in the center, known as perforations, are also seen in the presynaptic grid, and these perforations are exactly in register with those in the postsynaptic density (PSD).

Postsynaptic specializations. Like the presynaptic terminal, the postsynaptic membrane also has specializations, the PSD (Siekevitz, 1985; Kennedy, 1993). The term PSD was first used to describe a proteinaceous disc-shaped structure visible on the cytoplasmic face of the postsynaptic membrane of central synapses (Palay, 1956). It represents a fibrous specialization of the submembrane cytoskeleton that attaches to the postsynaptic membrane at sites of close apposition to the presynaptic active zone. Early biochemical studies have shown that the PSD fraction derived from synaptosomes extracted with triton X-100 contains about 10-

15 major protein bands and 10 minor bands (Cohen et al., 1977a; b). The cytoskeletal proteins tubulin, actin, and fodrin are among the most prominent proteins in PSD fractions (Cotman et al., 1974; Cohen et al., 1977 a; b). A wide variety of regulatory enzymes have also been found in the PSD fraction, including calcium-calmodulin kinase II (CaMKII), cAMP-dependent protein kinase, and pertussis toxin-sensitive G-proteins (Kennedy, 1993). The α-subunit of CaMKII comprises about 20 - 40% of the total protein (Miller and Kennedy, 1985), and is five to 10 times more concentrated in PSDs than in the cytosol. In addition, both NMDA and AMPA receptors are in PSDs (Fagg and Matus, 1984; Foster and Fagg, 1987; Rogers et al., 1991; Blackstone et al., 1992). Numerous studies suggest that the PSD plays a critical role in synaptic communication and plasticity (Siekevitz, 1985). It is also suggested that PSDs are involved in control of transmitter receptor clustering, and post-translational modifications that may affect receptor function.

In about 19 to 35% of central synapses, PSDs are often seen to have a perforation, a central region with low density (Jones et al., 1991; Harris et al., 1992). Serial electron microscopic reconstructions show the perforations are not artifacts of the sectioning angle. A perforation can also be seen in the array of freeze-fracture particles in the postsynaptic membrane (Lisman and Harris, 1993). Perforated PSDs take many different forms, which have been classified as 'fenestrated' (containing one or more holes); horse-shoe shaped; or 'segmented' (fragmented into two or more discrete patches that remain within a single spine and associate with a single presynaptic terminal (Jones et al., 1991). The function of

perforations is not clear.

Dendritic spines. Most excitatory synapses of pyramidal and stellate neurons in the hippocampus and cortex are typical spiny synapses, in which a single axon terminal makes synaptic contact with a dendritic spine head. Dendritic spines are tiny, specialized protoplasmic protuberances roughly one micrometer in diameter (Harris and Stevens, 1989). As many as 15,000 spines, at a density of two spines per micrometer of dendritic length, cover the surface of a hippocampal pyramidal neuron. Although spines can have numerous shapes, in general, they can be described as having a "neck" that emerges from the dendrite and ends with a head. In hippocampal CA1 pyramidal neurons, the dimensions of spines are quite variable. The volume of the spine ranges from 0.004 to 0.56 μm^3. Necks range in length from 0.08 to 1.58 μm and in diameter from 0.04 to 0.46 μm. Large spine heads are usually associated with larger synapses, as measured by the size of the associated PSD, and by the number of vesicles in the presynaptic terminal. Because excitatory synapses of the hippocampal and cortical pyramidal neurons rarely occur directly onto a dendritic shaft, virtually all of the excitatory input arrives through the spine synapses.

Because dendritic spines are so closely associated with excitatory synaptic transmission, they constitute the modulation sites for information processing in neurons. At least two functions have been proposed for spines, modulating the membrane conductance in response to synaptic input and the dynamics of intracellular second messengers such as calcium (Koch and Zador, 1993). Early modeling studies

suggested that the spine neck could offer a significant resistance to the electrical charge flowing from the synapse on the head to the dendrite and the soma. Therefore, a changes in neck diameter would lead to significant changes in the somatic EPSC, providing a possible anatomical mechanism for long-term synaptic plasticity (Rall and Segev, 1988). However, recent studies show that at synapses of the Schaffer collateral input to CA1 pyramidal neurons the spine neck conductance is too large relative to the synaptic conductance change to provide effective modulation of the amplitude of the synaptic current generated at the spine head (Koch and Zador, 1993). This suggests that the primary function of the spine is compartmentation of second messengers (Koch and Zador, 1993).

It is generally accepted that in the CA1 region of hippocampus the induction of long-term potentiation (LTP), a leading model for the mechanism of memory, requires a postsynaptic increase in intracellular calcium mediated by calcium influx through the NMDA receptor channel (Bliss and Collingridge, 1993). Thus, detailed studies have focused on the role of spines in modulating calcium dynamics. Because of the small and highly restricted volume of the spine, a small calcium influx during synaptic transmission can cause a large transient increase of intracellular Ca^{2+} into the micromolar range. The elevation of calcium in the spine is isolated from the rest of the dendrite because of the geometry of the neck (Harris and Landis, 1986; Landis, 1988; Muller and Connor, 1991; Guthrie et al., 1991). In addition, the spine head can be protected from the high dendritic calcium concentration by Na^{+}/Ca^{++} exchanges and calcium

pumps in the plasma membrane of the neck if dendritic calcium concentration is increased to the micromolar range (Zador et al., 1990). The specificity of LTP (Nicoll et al., 1988) and the difficulty in inducing LTP-like phenomena by activating voltage-dependent calcium channels (Grover and Teyler, 1990; Aniksztejn and Ben-Ari, 1991; Kullmann et al., 1992; Huang and Malenka, 1993; Wyllie et al., 1994) can be partially explained by the compartmentation of calcium in the spine.

STORAGE OF NEUROTRANSMITTER IN SYNAPTIC VESICLES

Neurotransmitters are stored in synaptic vesicles whose fusion with the plasma membrane can be regulated by neural activity (vesicle hypothesis). This implies that synaptic vesicles are able to accumulate transmitter molecules and store them at a high concentration within the nerve terminal. Classical transmitters, including glutamate, acetylcholine, and γ-aminobutyric acid (GABA) are synthesized in the cytoplasm of the nerve terminal, and vesicular storage requires specific transport activity. We found that the optical isomer of L-glutamate, D-glutamate, can be packaged and released as a false transmitter which activates NMDA receptor EPSCs (see manuscript III). Thus, the following section will review the storage of neurotransmitter in synaptic vesicles.

Identity of Excitatory Neurotransmitters

Although there are several endogenous compounds that can activate glutamate receptor channels in the CNS (Curtis and Watkins,

1960; Curtis et al., 1960; Westbrook and Jahr, 1989; Jahr and Lester, 1992), only L-glutamate satisfies the classical criteria as a neurotransmitter. L-Glutamate is normally present in all neurons for metabolic purposes, but it is concentrated to about 100 mM in synaptic vesicles (Burger et al., 1989) by a low affinity vesicular glutamate transporter (Naito and Ueda, 1985; Edwards, 1992). This proton-driven transporter is extremely selective for L-glutamate, virtually excluding other endogenous acidic amino acid transmitter candidates such as L-aspartate and L-homocysteate (Naito and Ueda, 1985). L-Glutamate is also released in a calcium-dependent manner by electrical or chemical depolarization of neurons in various brain regions, although it is usually co-released with L-aspartate and other amino acids (Fonnum, 1984; Benveniste, 1989; Nicholls, 1989; Nicholls and Attwell, 1990). L-glutamate is the only known endogenous compound that can activate both NMDA and AMPA receptors in concentrations that are expected to be reached in the synaptic cleft (Patneau and Mayer, 1990; Clements et al., 1992). The decay time course of both NMDA and AMPA receptor responses to short applications of L-glutamate mimics closely the decay time course of evoked NMDA and AMPA receptor EPSCs (Lester et al., 1990; Lester and Jahr, 1992; Hestrin, 1992, 1993). In addition, the exogenous action of L-glutamate can be pharmacologically antagonized by many of the same compounds that block transmission at excitatory synapses (Mayer and Westbrook, 1987; Monaghan et al., 1987; Collingridge and Lester, 1989). Finally, removal of glutamate from the synaptic cleft is probably affected both by diffusion (Eccles and Jaeger, 1958) and high-affinity uptake systems (Nicholls and

Attwell, 1990; Amara and Arriza, 1993), although there is until recently no direct evidence that glutamate transporters are involved in rapid clearance of glutamate from the synaptic cleft (Sarantis et al., 1993; Isaacson and Nicoll, 1993; but see Mennerick and Zorumski, 1994; Kovalchuk and Attwell, 1994).

Uptake of Neurotransmitter into Synaptic Vesicles.

Source of transmitter glutamate. Although glutamate is normally present for metabolic purposes, there are at least two mechanisms by which the presynaptic cytoplasmic glutamate concentration is maintained in the 10 mM range (Nicholls and Attwell, 1990). Neuronal glutamate transporters take up glutamate from the cleft directly into the presynaptic terminal. In addition, glutamate may be synthesized from glutamine by mitochondrial glutaminase. Glutamine is present at about 0.5 mM in the extracellular space and enters the terminal by a low-affinity Na^+-independent pathway. Glia play an important role in maintaining the concentrations of glutamine. High-affinity glial glutamate transporters reuptake extracellular glutamate into glia. In glia, glutamate is converted to glutamine by glial glutamine synthetase. Glutamine then is released into the extracellular space.

Storage of glutamate in the synaptic vesicle. The cytoplasmic glutamate concentration in synaptic vesicles is about 100 mM and is maintained by a low-affinity (K_m ~ 0.3 - 2.0 mM) Na^+-independent vesicular glutamate transporter (Naito and Ueda, 1985; Burger et al., 1989; Nicholls and Attwell, 1990; Maycox et al., 1990; Amara and Arriza, 1993). Assuming

average diameter of 40 nm (Harris and Landis, 1986; Bekkers et al., 1990), an excitatory synaptic vesicle contains about 2000 molecules of glutamate. The driving force for vesicular glutamate transport depends on both electrochemical and proton gradients which are generated by a vesicular ATPase that pumps protons into the vesicle. Proton pumping by the vacuolar class of proton pumps, the V-ATPases, is electrogenic (Rudnick, 1986). In the presence of the permeate anion, chloride (Cl^-), the charge of the proton is balanced, resulting in a net accumulation of HCl and therefore in generation of a proton gradient (Maycox et al., 1988; Salama et al., 1980). In the absence of a permeate anion, a large membrane potential (the electrochemical gradient) builds up after the translocation of only a few protons (Salama et al., 1980; Carlson et al., 1989; Maycox et al., 1988; Hell et al., 1990; Tabb and Ueda, 1991). High concentrations of extracellular chloride (150 mM) inhibit glutamate transport, suggesting a dependence of transport activity on an electrochemical gradient (Carlson et al., 1989; Maycox et al., 1988). The absence of chloride also inhibits glutamate transport, even though under these conditions the electrochemical gradient is maximal (Hell et al., 1990), indicating that Cl^- may be directly involved in the uptake mechanism. Since in the nerve terminal, the physiological concentration of Cl^- is about 4 mM, both gradients (electrochemical gradient and proton gradient) may contribute to the driving force, with a greater effect from the electrochemical gradient.

The vesicular transporter is very selective for L-glutamate; other excitatory amino acids, including L-aspartate, D-aspartate, and L-cysteate, do not transport into the vesicle (Naito and Ueda, 1985;

Villanueva et al., 1990; Tabb and Ueda, 1991; Fykse et al., 1992). The optical isomer of L-glutamate, D-glutamate; α-methyl glutamate; and γ-methylene glutamate inhibit vesicular uptake of [^{3}H]L-glutamate (Naito and Ueda, 1985; Maycox et al., 1988; Tabb and Ueda, 1991; Fykse et al., 1992), suggesting that they may be taken up into synaptic vesicles. Since D-glutamate also excites CNS neurons (Curtis and Watkins, 1960; Grimwood et al., 1991) and binds to rat brain NMDA receptors with an affinity 36 - 74 times lower than L-glutamate (Managhan and Cotman, 1986; Olverman et al., 1988; Grimwood et al., 1991), it can be used as a 'false' transmitter (Colquhoun et al., 1977; Large and Rang, 1978a, b) at glutamatergic synapses (see manuscript III).

RELEASE OF NEUROTRANSMITTER

Chemically mediated synaptic transmission results from fusion of synaptic vesicles with the presynaptic plasma membrane (exocytosis, Almers and Tse, 1990) and subsequent release of the vesicular contents into the cleft (Katz, 1969; Heuser et al., 1979). Of the synaptic vesicles clustered at sites of release, a subset are docked at the active zone, the region of plasma membrane across the cleft from the postsynaptic density. Because release is so fast, only the docked synaptic vesicles are thought to mediate the earliest release of neurotransmitter. The empty sites are then refilled from the reservoir of clustered vesicles. Synaptic vesicles are reformed by endocytosis at the nerve terminal and refilled with neurotransmitter by an active transport process. In the following section, I

will review some basic processes and recent advances in understanding of neurotransmitter release.

Physiology of Neurotransmitter Release

Release is a calcium dependent process. Calcium is required in almost all steps of the transmitter release process (Augustine et al., 1987). Calcium is involved not only in the translocation and docking of synaptic vesicles to the presynaptic plasma membrane, it is also required for synaptic vesicle fusion with the plasma membrane and the endocytosis of the vesicles after release (Augustine et al., 1987). When a nerve impulse arrives and produces a membrane depolarization, calcium influx through voltage-dependent Ca^{2+} channels acts within 100-200 μs to trigger transmitter release suggesting that the channels are located very close to transmitter release sites (Augustine et al., 1987; Adler et al., 1991; Llinas et al., 1992b). The duration of the action potential is an important determinant of the amount of Ca^{2+} that flows into the terminal. If the action potential is prolonged, more Ca^{2+} enters, and therefore more terminals release transmitter causing a larger postsynaptic response.

Multiple Ca^{2+} channel types have been described in mammalian central neuron somata, and several types can be defined based on their sensitivity to specific antagonists (Hille, 1992). More than one type of voltage-gated Ca^{2+} channel is involved in the release of transmitter from central synapses (Augustine et al., 1987; Turner et al., 1992; 1993; Luebke et al., 1993; Yawo and Chuhma, 1993; Takahashi and Momiyama, 1993; Wheeler et al., 1994). ω-conotoxin GVIA (ω-CgTx), which was originally

identified as an irreversible blocker of transmitter release at the neuromuscular junction, has been shown to specifically block N-type Ca^{2+} channels (Plummer et al., 1989). Subsequent studies showed that transmitter release from peripheral neurons and brain synaptosomes is partially blocked by ω-CgTx but not by 1,4-dihydropyridine antagonists which are specific for L-type Ca^{2+} channels (Tsien et al., 1988). ω-CgTx can also block synaptic transmission in brain slices; however the block is incomplete (Takahashi and Momiyama, 1993). At central synapses, another antagonist, ω-Aga-IVA, which specifically blocks P type calcium channel at nM concentrations (Llinas et al., 1992a), has been shown to block glutamate release from synaptosomes and at certain synapses. In the hippocampal slice, Schaffer collateral-CA1 EPSCs are markedly suppressed by ω-Aga-IVA and to a lesser extent by ω-CgTx (Takahashi and Momiyama, 1993; but see Wheeler et al., 1994). Combination of ω-Aga-IVA and ω-CgTx totally blocks the synaptic transmission (Luebke et al., 1993; Yawo and Chuhma, 1993; Takahashi and Momiyama, 1993; but see Wheeler et al., 1994). These data suggest that in the hippocampus, both P- and N-types calcium channel mediate synaptic transmission. In addition to N and P-type calcium channels, a newly identified calcium channel, the Q-type channel, which can be blocked by ω-CTx-MVIIC, may also be involved in hippocampal synaptic transmission (Wheeler et al., 1994) .

Release is in quanta. The quantal hypothesis of transmitter release states that transmitter is probabilistically released in multimolecular packets (quanta), and that normal release consists of an integral number

of these units. The first experimental evidence for quantal release came from studies of postsynaptic responses at the neuromuscular junction (Katz, 1969). Subsequently, quantal analysis has been applied at many synapses including the mammalian central synapse (Redman, 1990). In every case, quanta are thought to be produced by the release of transmitter from synaptic vesicles by exocytosis. However, recent studies show that in certain conditions, transmitter release can be a calcium-independent, nonquantal process (Adam-Vizi et al., 1992; Levi and Raiteri, 1993; Attwell et al., 1993; see below).

Release is not uniform at all synaptic sites. Many studies indicate that release probability is nonuniform at both the neuromuscular junction and at central synapses by using quantal analysis (see Redman, 1990), or more recently by using an open channel blocker of the NMDA receptor channel MK-801 (Rosenmund et al., 1993; Hessler et al., 1993) or dendritic calcium imaging (Murphy et al., 1994). By using MK-801 (Jahr, 1992), two groups (Rosenmund et al., 1993; Hessler et al., 1993) found that release probability can vary widely among central synapses. Two classes of synapses with a six-fold difference in probability of release were observed. These two classes of synapses have different sensitivity to drugs that change transmitter release. By using calcium imaging to visualize the activation of individual postsynaptic elements by mEPSCs, it has been shown that the probability of spontaneous activity differs among synapses on the same dendrite (Murphy et al., 1994). In addition, the release probability in a given synapse could be modified by application of glutamate or phorbol ester.

Mechanisms of Neurotransmitter Release.

Although it is generally accepted that transmitter is released in quanta, it is controversial whether one or more than one can be released from a synaptic contact by each action potential (Redman, 1990; Korn and Faber, 1991). The one vesicle hypothesis states that either zero or one vesicle is released after an impulse, with a given probability from each synaptic contact. Multivesicular release implies that after each action potential, more than one vesicle of transmitter is released simultaneously from a single synaptic contact site, and thus, transmitter from more than one vesicle can act concurrently at the same population of postsynaptic receptors.

The physiological implications for the two mechanisms of release are different. If individual synaptic contacts release only one vesicle, the modulation of synaptic strength at each synapse can only be achieved by changing the postsynaptic sensitivity or, possibly by changing the transmitter clearance rate. However, if more than one vesicle of transmitter is released at single synaptic contacts, the synaptic strength of a single synapse can theoretically be modulated by both pre- and postsynaptic mechanisms.

One vesicle hypothesis. The one vesicle hypothesis was first suggested by electron microscopic studies which showed that at normal and low release conditions a synaptic vesicle can fuse with the presynaptic plasma membrane, and undergo exocytosis at an active zone of the neuromuscular junction (Heuser and Reese, 1977). The first direct

evidence that supports the one vesicle hypothesis came from studies at inhibitory synapses on the goldfish Mauthner cell (Korn et al., 1981, 1982; Korn and Faber, 1991). By using simultaneous recordings from pairs of histologically identified pre- and postsynaptic neurons, the number of morphologically identified synaptic contacts on the Mauthner cell was equal to the binomial number computed from statistical analysis of inhibitory postsynaptic potential amplitudes. This striking identity implies that each releasing unit functions in an independent, all-or-none manner. Because the size of the derived quantum is relative small, the most likely explanation is that one quantum corresponds to the amount of transmitter released by a given synaptic contact. Similar results have recently been obtained in CA3 hippocampal inhibitory interneurons of guinea-pig (Gulyas et al., 1993). In addition, some studies suggest that when there are several synaptic contacts in a single bouton, they function as independent release sites (Walmsley et al., 1985; Lin and Faber, 1988; but see Trussell et al., 1993).

Multivesicular release. In contrast, several lines of evidence suggest that multivesicular release can occur in both physiological and non-physiological conditions. Early studies showed that 'bursts of activity' of miniature end-plate potentials at insect neuromuscular junctions can be detected with intracellular and focal extracellular recordings, but not using normal extracellular recording or when intracellular recording is made several hundred micrometers away from the original recording site (Rees, 1974). Similar results have been obtained at frog neuromuscular junctions (Cohen et al., 1974) and at guinea-pig hypogastric ganglia

(Bornstein, 1978). These results suggest that bursts of activity occur at individual release sites rather than arising from interactions between different release sites. Thus multiple quanta could be released from each release site.

Using a quick-freezing technique, Heuser showed that, in the presence of 4-aminopyridine, a single stimulus can cause fusion of two or more vesicles with the membrane adjacent to a single active zone in a frog motor nerve terminal (Heuser, 1977; Heuser et al., 1977). Thus, if the contents of a vesicle represent a single quantum of transmitter and an active zone represents a release site (Hubbard and Jones, 1973; Heuser, 1977; Heuser et al., 1977), then single active zones can release more than one quantum. A recent study suggests that at the frog neuromuscular junction, multivesicular release of ACh from an active zone results in repetitive binding of ACh to receptors in the area close to the release site , causing prolongation of endplate currents (Giniatullin et al., 1993). Furthermore, studies of the calyceal synapse of the chick nucleus magnocellularis (Trussell et al., 1993) have shown that simultaneous release of glutamate from many closely spaced sites can interact with the same populations of postsynaptic glutamate receptors, prolonging the time of receptor-transmitter contact and thereby promoting desensitization. We have tested the multivesicular release hypothesis in cultured hippocampal neurons. We found that more than one vesicle of glutamate can be released simultaneously from single synaptic contacts and interact with the same population of postsynaptic glutamate receptors (see manuscript I).

Non-vesicular release. In recent years, it has become clear that in addition to uptake of transmitter from the extracellular space, neurotransmitter transporters can run backward, pumping transmitter out of cells and serving as a calcium-independent, nonvesicular mechanism for transmitter release (Adam-Vizi, 1992; Levi and Raiteri, 1993; Attwell et al., 1993). Although nonvesicular release may play an important role in pathological conditions such as brain anoxia and ischemia (see Attwell et al., 1993; Levi and Raiteri, 1993), the physiological role of this release during normal synaptic transmission is unclear. The concentration of extracellular glutamate obtained by reverse glutamate transport has been estimated to be about 40 μM during an action potential (Szatkowski et al, 1990; Attwell et al., 1993), whereas the concentration of free glutamate probably can rise to 1 mM in the synaptic cleft during calcium-dependent vesicular release (Clements et al., 1992). However, the nonvesicular release of glutamate may affect on desensitization of synaptic NMDA and AMPA receptors and could thereby affect subsequent neurotransmission mediated by calcium-dependent vesicular release.

Non-vesicular release can be evoked in at least three experimental conditions (Levi and Raiteri, 1993). First, non-vesicular release can be induced by increasing intracellular concentrations of free transmitter (Szatkowski et al., 1990). However, in the case of glutamate, the concentration necessary for reverse transport is much higher than physiological levels. Secondly, nonvesicular release of glutamate may be induced by decreasing the Na^+ gradient, since electrogenic glutamate transport is coupled to the transport of Na^+ ions (Szatkowski et al., 1990;

Adam-Vizi, 1992). The Na^+ gradient can be decreased or reversed by manipulating extracellular Na^+, by opening Na^+ channels with veratridine or scorpion venom, by inhibiting the Na^+-K^+ATPase, and by using Na^+ ionophores. Finally, non-vesicular release may also be caused by extracellular transporter substrate analogues that accelerate non-vesicular release by heteroexchange.

Molecular Biology of Neurotransmitter Release

Transmitter release can be considered to involve at least four steps: the translocation (or mobilization) of vesicles from the reserve pool to the releasable pool at the active zones; the docking of vesicles to their release sites at the active zones; the fusion of the synaptic vesicle membrane with the plasma membrane during exocytosis in response to an increase in intracellular Ca^{2+}; and the retrieval and the recycling of the vesicle membrane following exocytosis (Kelly, 1993). In the past few years, major advances have been made in the identification and characterization of several key components of presynaptic terminals and synaptic vesicles.

Vesicle translocation. In general only a small pool of synaptic vesicles participate in exocytosis and recycling, whereas a large pool filled or in the process of being filled with transmitter is kept in reserve within the presynaptic terminal (Kelly, 1993; but see Betz and Bewick, 1992). Electron microscopic studies have demonstrated that synaptic vesicles are suspended in a filamentous network that is believed to be composed mainly of spectrin and actin (Landis et al., 1988; Hirokawa et al., 1989). Translocation of synaptic vesicles may involve release of synaptic vesicles

from this reticulum. In addition to Ca^{2+} (Atlas, 1990; Koenig et al., 1993), the vesicle protein synapsin has emerged as a major component in regulating the translocation of vesicles. The synapsins are represented by a family of four homologous proteins (synapsin Ia, Ib, IIa, IIb) that are derived from two genes by alternative splicing (Sudhof et al., 1989). Synapsin I is bound both to various cytoskeletal proteins, including spectrin and actin, and to synaptic vesicles, apparently involving binding to vesicle-associated CaMKII. The binding of synapsin I to both synaptic vesicles and actin filaments is weakened upon phosphorylation of synapsin I by CaMKII, that is activated by increasing the intracellular calcium concentration (Greengard et al., 1993).

Synaptic vesicle clustering and docking. Docking of synaptic vesicles to the plasma membrane includes recognition and establishment of tight binding with the target membrane (Jessell and Kandel, 1993). This process involves interaction of several vesicular and plasma membrane proteins. Early electron microscopic studies have shown that some special intramembranous particles are located in the vicinity of the release site (Heuser and Reese, 1981). These particles are thought to be calcium channel proteins. Indeed, recent studies show that calcium channels, identified by ω-CgTx binding, line up with the rows of docked synaptic vesicles (Cohen et al., 1991). One possible mechanism of docking is the interaction of synaptic vesicle proteins with the calcium channels. It has been suggested that one of the synaptic vesicle proteins, synaptotagmin, associates with a calcium channel on the plasma membrane (Takahashi et al., 1991; Leveque et al., 1992), apparently together with another integral

plasma membrane protein, syntaxin (Bennett et al., 1992). In this case, the vesicle will be docked near the calcium channel. An alternative docking mechanism is the interaction of vesicle proteins with other plasma membrane proteins. Synaptotagmin can also bind to another presynaptic membrane protein, the α-latrotoxin receptor (Petrenko et al., 1991), and to members of a related family of proteins, the neurexins (Ushkaryov et al., 1992). In addition, it is also proposed that the members of the small G-protein family (Bourne et al., 1990), including rab proteins, play a role in docking of synaptic vesicles (Linstedt and Kelly, 1991; Chavrier et al., 1990; Simons and Zerial, 1993). When rab proteins bind GTP, they tightly associate with a part of a T-shaped vesicle docking apparatus and remain bound until the vesicles reach their target site. The GTP-bound form of rab proteins prevents the association of the docking apparatus with the docking site. When GTP is hydrolyzed to GDP, GDP-bound rab complex is released from the docking apparatus and allows the vesicle to dock. Binding of rab proteins to the docking site is thought to convert the vesicle to a 'fusion-ready' state which enables it to interact with the appropriate target membrane.

Fusion of synaptic vesicle. Exocytosis of neurotransmitter involves the fusion of cytoplasmic membranes including first, a specific interaction of the cytoplasmic leaflets of the vesicular and plasma membranes, and subsequently, a fusion of the two membrane bilayers. Recent studies have shown that synaptobrevin (a vesicle protein), syntaxin (a plasma membrane protein), and SNAP-25 (a synaptic vesicle membrane-associated protein) form an ATP-dependent complex with soluble protein

factors, including NSF (N-ethylmaleimide-sensitive fusion protein) and SNAPs (NSF attachment proteins) (Sollner et al., 1993; Whiteheart et al., 1993). This high molecular weight 'fusion complex' can serve as a membrane receptor, permitting the binding of the SNAPs and NSF fusion proteins to the synaptic vesicle (synaptobrevin) and plasma membrane (syntaxin and SNAP-25) compartments. Synaptobrevin is strongly implicated in exocytosis, since its selective proteolysis by tetanus toxin inhibits neurotransmitter release without apparent affect on other cellular functions, e.g. opening of voltage-sensitive Ca^{2+} channels (Schiavo et al., 1992; Link et al., 1992). In addition to the role of vesicle docking (see above), synaptotagmin, a Ca^{2+}-binding protein, may be also involved in Ca^{2+}-regulated vesicle plasma membrane fusion (Brose et al., 1992; Bommert et al., 1993). Recent electrophysiological studies in chromaffin and mast cells (Almers and Tse, 1990; Spruce et al., 1990; Chow et al., 1992; Alvarez de Toledo et al., 1993) have suggested that exocytosis is preceded by the formation of a pore that has comparable conductance properties to ion channels. The contents of the secretory organelle partially escape at this early step, and the pore can be closed before full vesicle fusion occurs ('kiss-and-run' mechanism, Fesce et al., 1994). Several lines of evidence suggest that quantal release of neurotransmitter from synaptic vesicles may occur by a similar 'kiss-and-run' mechanism (Fesce et al., 1994).

Recycling of synaptic vesicles. The final step of release involves endocytosis and refilling of synaptic vesicles (Kelly, 1993). Like other steps of release process, recycling requires interaction of several vesicular

and plasma proteins. One of these protein is dynamin, a GTPase stimulated by microtubules (Chen et al., 1991; Shpetner et al., 1992). Several studies suggest that dynamin, like several other G-proteins, may regulate endocytosis, particularly the step where the vesicle membrane pinches off from the plasma membrane (Hess et al., 1993). One of the other proteins that may involved in recycling is synaptophysin. Synaptophysin is a vesicular transmembrane glycoprotein. However, the role of synaptophysin in endocytosis is still controversial. Furthermore, if release occurs through a fusion pore (see above), then the vesicle membrane does not collapse into and mix with the plasma membrane, and can be retrieved on the spot.

Modulation of Neurotransmitter Release

The release of neurotransmitter from the presynaptic terminals requires activation of voltage-gated Ca^{2+} channels and subsequent entry of Ca^{2+} into the cytoplasm (Katz, 1969; Redman, 1990; Swandulla et al., 1991; Adler et al., 1991; Llinas et al., 1992b). Elevated intraterminal Ca^{2+} then increases the probability that transmitter-containing vesicles fuse with the presynaptic plasma membrane and release their contents into the synaptic cleft. At a given synaptic contact, the probability of release is not constant, but rather is subject to a variety of modulatory influences that can either increase or decrease the probability of release.

Presynaptic $GABA_B$ Receptor. Activation of $GABA_B$ receptors on excitatory axon terminals by exogenous application of baclofen or synaptic release of GABA in the hippocampus causes a decrease in the

amplitude of evoked EPSCs due to a reduction in transmitter release (Dutar and Nicoll, 1988; Thompson et al., 1992; Isaacson et al., 1993). Although the precise mechanisms of presynaptic $GABA_B$ receptors are not clear, activation of postsynaptic $GABA_B$ receptors can increase a K^+ conductance as well as decrease a voltage-dependent Ca^{2+} conductance. Baclofen activates postsynaptic K^+ currents of hippocampal pyramidal and granule cells that are mediated by a pertussis toxin-sensitive G protein (Dutar and Nicoll, 1988; Thompson et al., 1992; Thompson et al., 1993); however, in the same cells, the presynaptic effect of baclofen is mediated by a pertussis toxin-insensitive G-protein.

Presynaptic Adenosine Receptor (A_1 receptor). Like presynaptic $GABA_B$ receptors, presynaptic A_1 receptors can also inhibit excitatory neurotransmission (Fredholm and Dunwiddie, 1988). The mechanism of inhibition by the presynaptic A_1 receptor is almost identical to that of the presynaptic $GABA_B$ receptor (see above; Thompson et al., 1992; Scanziani et al., 1992; Thompson et al., 1993;). However, recent studies suggest that the inhibitory effect of adenosine on release is caused by direct inhibition of ω-CgTx-sensitive presynaptic Ca^{2+} channels rather than activation of K^+ channels (Yawo and Chuhma, 1993).

Opioids. Opioid peptides coexist with classical transmitters in many areas of the central nervous system (Hokfelt, 1991). Opioids can inhibit both inhibitory and excitatory synaptic transmission and block the induction of long-term potentiation in hippocampal neurons by presynaptic inhibition (Cohen et al., 1992; Weisskopf et al., 1993; Wagner et al., 1993). Different presynaptic receptor subtypes are involved in the

inhibition of inhibitory and excitatory synaptic transmission. Inhibition of inhibitory synaptic transmission is mediated by the μ opioid receptor, whereas the κ receptor is involved in the inhibition of excitatory synaptic transmission (Weisskopf et al., 1993; Wagner et al., 1993; Thompson et al., 1993)., Opioids increase a K^+ conductance through a pertussis toxin-sensitive G protein and therefore decrease Ca^{2+} influx during action potentials. The inhibitory effect of opioids can be also blocked by protein kinase C activators.

Presynaptic glutamate receptors. Glutamate released from synaptic vesicles can activate several types of presynaptic metabotropic glutamate receptors (mGluRs) (Forsythe and Clements, 1990; Baskys and Malenka, 1991; Herrero et al., 1992; Schoepp and Conn, 1993; Baude et al., 1993;). The effect of mGluRs on presynaptic release is complex. Activation of presynaptic mGluRs decreases or increases excitatory synaptic transmission (Baskys and Malenka, 1991; Herrero et al., 1992). Two types of mGluRs (mGluR4 and mGluR7; Okamoto et al., 1994; Saugstad et al., 1994) may mediate L-AP4 induced inhibition of synaptic transmission (Forsythe and Clements, 1990). This inhibition is mediated by a G protein coupled process (Trombley and Westbrook, 1992) and may be involved in inhibiting Ca^{2+} channels of the presynaptic terminals (Sladeczek et al., 1988; Schoepp and Conn, 1993). Consistent with this, the pharmacological properties of mGluRs involved in reduction of EPSCs and the inhibition of Ca^{2+} currents are similar. The potentiation effect of mGluRs in glutamate release is also a G protein coupled process, probably mediated by the diacylglycerol-PKC branch of the

phosphoinositide cascade rather than by the generation of inositol triphosphate ($InsP_3$). This potentiation requires the presence of arachidonic acid (Herrero et al., 1992). Since the potentiation can be blocked by protein kinase C inhibitors, or by using an inactive anologue of arachidonic acid, methyl arachidonate, it is possible that this potentiation is mediated by the synergistic activation of PKC by arachidonic acid and by the diacylglycerol generated by metabotropic receptor activation. Thus, the effect of mGluRs is bimodal, causing a depression of synaptic transmission by itself and an increase when arachidonic acid is present. This bimodal effect might partially explain the stimulus dependency of induction of long-term potentiation and long-term depression (Artola and Singer, 1993).

Glutamate may activate another distinct type of presynaptic glutamate receptor (Smirnova et al., 1993a, b; but see Brose, 1993). Although this receptor has similar pharmacological properties to NMDA receptors, its physiological role is unknown. The only evidence that suggests this receptor plays an important role in transmitter release is that induction of LTP induces a transient increase in the amount of mRNA coding for this receptor (Smirnova et al., 1993a, b).

Retrograde messengers. In the past several years, studies have shown that upon activation, postsynaptic cells can produce several diffusible substances that may act as retrograde messages to regulate presynaptic release. This may provide a feedback mechanism regulating the strength of synaptic transmission.

Arachidonic acid (AA). It is generally accepted that

postsynaptic Ca^{2+} entry through the NMDA receptor channel liberates AA from membrane phospholipids apparently via the activation of phospholipase A_2 and phospholipase C (Dumuis et al., 1988). In addition to an increase in the intracellular free concentration of AA, NMDA receptor activation also leads to the release of AA into the extracellular space. The action of AA on synaptic transmission appears to be complicated; it can enhance or inhibit neurotransmitter release. At high concentrations, AA enhances transmitter release from synaptosomes (Freeman et al., 1990; Lynch and Voss, 1990), but it inhibits release at lower concentrations (Herrero et al., 1991; Fraser et al., 1993). However, at low concentrations AA can potentiate glutamate release if metabotropic glutamate receptors are activated (Herrero et al., 1992; see above). Thus, the action of AA on transmitter release depends on not only the intracellular concentration of AA but also the activities of other cellular mechanisms.

Nitric oxide (NO). NO is a gas that is generated by the enzyme NO synthase from the amino acid L-arginine by splitting off stoichiometric amounts of citrulline (Bredt and Snyder, 1992). NO synthase requires Ca^{2+}/calmodulin and the coenzyme NADPH. NO can activate both guanylyl cyclase and adenosine diphosphate (ADP)-ribosyl cyclase. The latter increases the formation of cyclic ADP-ribose that can induce release of calcium from intracellular stores. Early studies showed that NO may be involved in regulating synaptic function since activation of NMDA receptors caused release of NO and subsequent formation of cGMP in the brain (Garthwaite et al., 1988; 1989; East and Garthwaite,

1991). Subsequent studies showed that exogenous application of NO produces a persistent (up to 60 min) increase in the frequency of miniature EPSCs in cultured hippocampal neurons, indicating an increase in presynaptic transmitter release (O'Dell et al., 1991). In addition, NO may also play an important role in the NMDA receptor-mediated neurotransmitter release in the cerebral cortex (Montague et al., 1994). All these findings are consistent with the idea that NO may be a retrograde messenger involved in the induction of long-term potentiation (Schuman and Madison, 1991, 1994; O'Dell et al., 1991; Bohme et al., 1991; Haley et al., 1992; Zhou et al., 1993).

Carbon monoxide (CO). CO may also play an important role in synaptic function comes as haem oxygenase-2, which catalyses the production of CO, is widely distributed throughout the CNS (Verma et al., 1993). CO can activate guanylyl cyclase (Marks et al., 1991). There is no direct evidence that CO regulates presynaptic release. However, two recent studies show that CO, as a retrograde messenger, may play an important role in long-term potentiation (Zhou et al., 1993; Stevens and Wang, 1993). Thus, CO may also regulate presynaptic release.

Platelet-activating factor (PAF). PAF is a naturally occurring alkyl ether phospholipid that acts as an extracellular mediator in a wide range of biological processes (Braquet et al., 1987). Like arachidonic acid, PAF is also generated by the hydrolysis of membrane phospholipids by phospholipase A_2 (Bazan, 1989). PAF increases the frequency, but not the amplitude or the time course, of miniature EPSCs in hippocampal neurons (Clark et al., 1992; Kato et al., 1994). PAF may also act as a

retrograde messenger in long-term potentiation (Wieraszko et al., 1993; Kato et al., 1994). The mechanism by which PAF increases presynaptic release is not clear. PAF has been shown to activate phosphoinositide turnover, to increase free fatty acids, to increase the intracellular calcium concentration, and to activate protein kinase C in some cells (Yue et al., 1991), which can lead to increasing presynaptic release.

CLEARANCE OF NEUROTRANSMITTERS FROM THE SYNAPTIC CLEFT

Clearance of transmitter after release is critical to synaptic function, because if transmitter persists in the synaptic cleft for long time, a new signal would not get through. At the neuromuscular junction acetylcholine clearance normally depends on hydrolysis by acetylcholinesterase, the clearance of L-glutamate from central excitatory synapses is not well understood. Several factors may contribute to the clearance of neurotransmitter in the central synapse.

Mechanisms of clearance of neurotransmitters.

Generally, there are three mechanisms by which transmitter is removed from the synaptic cleft: diffusion, enzymatic degradation, and reuptake. Diffusion removes some fraction of all chemical messengers. Enzymatic degradation of transmitter is used primarily by the cholinergic and some peptidergic systems . At the neuromuscular junction, acetylcholine clearance normally depends on hydrolysis by

acetylcholinesterase (AChE). Diffusion of ACh out of the cleft becomes rate limiting only when AChE is blocked (Katz and Miledi, 1973). In the CNS, it is generally accepted that neurotransmitter uptake into neurons and glial cells is the major mechanism of clearance of transmitter from the extracellular space for at least some synapses. Physiological studies have shown that blockade of uptake prolongs and increases the synaptic response mediated by a number of neurotransmitters including GABA (Dingledine and Korn, 1985; Isaacson et al., 1993; Pekling et al., 1990; Thompson and Gahwiler, 1992), norepinephrine (Egan et al., 1983; Suprenant and Williams, 1987), and serotonin (Bobker and Williams (1991).

Clearance of L-glutamate.

Although L-glutamate is not enzymatically inactivated the relative contributions of diffusion and reuptake are unknown (Nicholls and Attwell, 1990). Free diffusion alone would result in a very rapid decline of transmitter concentration at central excitatory synapses (τ = 0.3 ms; Eccles and Jaeger, 1958). However, diffusion will be affected by several parameters, such as the cleft geometry (Eccles and Jaeger, 1958; Wathey et al., 1979; Lisman and Harris, 1993), the density of transmitter-binding proteins such as receptors or transporters (Katz and Miledi, 1973), the tortuosity of diffusion pathways (Lisman and Harris, 1993), and the density of release sites (Trussell et al., 1993). If the free glutamate concentration in the synaptic cleft decays with a time constant of about 1 - 2 ms (Clements et al., 1992), it is possible that diffusional barriers exist

and reuptake could be important. However, the physiological role of glutamate transporters in the clearance of glutamate from the synaptic cleft has been difficult to establish. Blockers of the plasma membrane glutamate transporter, such as acid and L-trans-pyrrolidine-2,4-dicarboxylic acid (L-trans-PDC), prolong the response to exogenous application of glutamate but do not prolong the time course of either the AMPA or NMDA components of the EPSCs at room temperature (Hestrin et al., 1990; Sarantis et al., 1993; Isaacson and Nicoll, 1993; but see Mennerick and Zorumski, 1994; Kovalchuk and Attwell, 1994). Interpretation of these results, however, may be complicated by the imprecise pharmacology of blockers, receptor kinetics, receptor desensitization, receptor saturation, and prolonged receptor binding. Indeed, several recent studies show that blockade of glutamate transporters prolong NMDA receptor EPSCs, and AMPA EPSCs in the presence of the blocker of desensitization, cyclothiazide (Mennerick and Zorumski, 1994; Kovalchuk and Attwell, 1994).

Time Course of ACh at the Neuromuscular Junction.

Inhibition of AChE prolongs ACh in the cleft, causing an increasing of both amplitude and duration of synaptic potentials (Fatt and Katz, 1951). Under such conditions, the action of ACh is terminated solely by diffusion from the cleft because no ACh carriers have been identified. Detailed studies have suggested a simple scheme for the time course of ACh in the synaptic cleft: the saturated disk hypothesis (Hartzell et al., 1975; Salpeter, 1987). After release of 7000 - 12,000 ACh molecules per

quantum, the concentration of ACh increases almost instantaneously across the cleft to a level high enough (~0.5 mM) to saturate both ACh receptors and AChE within an area centered on the release site. The fraction of ACh molecules released that initially bind to postsynaptic ACh receptors is determined by the ratio of receptors to AChE and the distributions of ACh receptors and AChE. Since the density of receptors (10,000 receptors per μm^2) is about 3.8 times of that of AChE (2,600 AChE per μm^2), about 80% of the ACh molecules bind to the receptors and 20% to the AChE. Binding itself causes a rapid fall in ACh concentration. Therefore, at 100 μs after release, the concentration of ACh in the cleft has fallen to levels sufficiently low that the rebinding of ACh to receptors is negligible. The concentration then remains low since AChE can hydrolyze ACh much faster (~10 ms^{-1}) than they dissociate from receptors (τ = 1 ms). This model predicts that inhibition of AChE should have a more pronounced effect on the duration than on the amplitude of synaptic potentials. Consistent with this idea, studies have shown that the duration is increased 3 to 5 times, while the amplitude is increased only 1.5 to 2 times after block of AChE (Fatt and Katz, 1951; Katz and Miledi, 1973).

Time Course of Free Glutamate in the Synaptic Cleft

Central excitatory synapses differ from the neuromuscular junction in that transmitter clearance depends on diffusion and reuptake rather than enzymatic breakdown (see above). Central synapses also differ in their morphology (Eccles and Jaeger, 1958; Harris and Landis, 1986) and

quantal characteristics of transmitter release (Malinow and Tsien, 1990; Redman, 1990). Furthermore, the time course of free glutamate at central excitatory synapses must account for both fast and slow components of EPSCs mediated by colocalized AMPA and NMDA receptors (Bekkers and Stevens, 1990; Hestrin et al., 1990). Nevertheless, recent studies show that like ACh, glutamate released from synaptic vesicle is present in the synaptic cleft for a short time. The competitive NMDA receptor antagonist D-2-amino-5-phosphonovalerate (D-AP5) (Davies et al., 1981), which can only bind to the NMDA receptor when the receptor is free from glutamate, only block the NMDA receptor EPSC only when it is applied prior to the synaptic response (Lester et al., 1990). The lack of effect of AP5 when applied after the start of the EPSC indicates that the transmitter does not rebind during the prolonged decay phase of the NMDA EPSC. Furthermore, brief applications (1 - 5 ms) of glutamate to outside-out patches generate NMDA receptor responses that accurately mimic the time course of the NMDA receptor EPSC. These data imply that transmitter is only present in the synaptic cleft for a short time (Lester et al., 1990).

The time course of glutamate in the synaptic cleft has been estimated by using a low affinity competitive NMDA receptor antagonist, D-α-aminoadipate (D-AA) (Clements et al., 1992). Since D-AA has a very fast unbinding rate, the amount of inhibition of the NMDA receptor EPSC by D-AA is dependent on the time during which an effective glutamate concentration is present in the cleft. The longer glutamate is present in the synaptic cleft, the more the D-AA will dissociate from the NMDA receptor

and be replaced by glutamate, thus reducing the inhibition of NMDA receptor EPSCs by D-AA. The time course of glutamate in the synaptic cleft can be calculated by measuring the effectiveness of D-AA as a synaptic response blocker if one knows the binding and unbinding rates for glutamate and D-AA. Results from these experiments demonstrated that the average free glutamate concentrations at postsynaptic receptors peaks at 1.1 mM and decays exponentially with a time constant of 1.2 ms. Because the unbinding rate of D-AA is not extremely fast (200 s^{-1}), however, the concentration profile of glutamate in the first ~200 μs after release is not measurable by this approach and they could be much higher.

Physiology of Plasma Membrane Glutamate Transporters

Two different glutamate uptake systems are present in neuronal and glial plasma membranes of the CNS (Kanner and Schuldiner, 1987; Nicholls and Attwell, 1990; Bridges et al., 1987): a high affinity sodium-dependent L-glutamate transporter and a lower-affinity, chloride-dependent transporter. The physiological role of the latter system is unclear, but the former is theoretically capable of maintaining an extracellular L-glutamate concentration of close to 100 nM at equilibrium (Nicholls and Attwell, 1990). Pharmacological, kinetic and molecular cloning studies have suggested the existence of multiple glutamate transporter subtypes (Kanner and Schuldiner, 1987; Nicholls and Attwell, 1990; Kanai, et al., 1993; Amara and Arriza, 1993; Chamberlin and Bridges, 1993, Arriza et al., 1994). The high-affinity glutamate transporter

is relatively non-specific: it transports L-and D- aspartate in addition to L-glutamate. However, the optical isomer of L-glutamate, D-glutamate is a poor substrate for this uptake system (Balcar and Johnston, 1972; Kanai and Hediger, 1992; Eliasof and Werblin, 1993).

Glutamate transport is an electrogenic process. The mechanism of sodium-dependent glutamate transport was studied initially using radiotracing flux studies. These studies showed that the transport of glutamate into cells up its concentration gradient is driven by the movement of sodium down its transmembrane electrochemical gradient and that the process is electrogenic, with the positive charge moving in the direction of the glutamate (Kanner and Sharon., 1978;a,b; Stallcup et al., 1979). These observations indicate that the process of glutamate transport can be studied electrophysiologically. Whole-cell voltage-clamp techniques have been used to measure the robust uptake of glutamate into glial cells, neurons and *Xenopus* oocytes expressing glutamate transporters (Nicholls and Attwell, 1990; Eliasof and Werblin, 1993; Arriza et al., 1994; Kanai and Hediger, 1992; Storck et al., 1992). These studies demonstrate that glutamate transport requires extracellular sodium and intracellular potassium (Barbour et al., 1988). In addition, there is also evidence for a pH dependence of the glutamate transporter (Erecinska et al., 1983). By using pH-sensitive dyes to visualize intracellular pH changes, it has been shown that during each transport cycle one pH-changing anion (OH^- or HCO_3^-) is counter-transported from the cell (Bouvier et al., 1992). Glutamate transport is also dependent on the membrane potential. Membrane depolarization inhibits uptake. A simple scheme has emerged:

for each glutamate anion transported into the cell, three sodium ions are co-transported into the cell, and one potassium ion is transported out of the cell. This stoichiometry suggests that one net positive charge is moving inward per glutamate anion entering the cell. Furthermore, if a hydroxyl per glutamate is countertransported as well (Bouvier et al., 1992), the stoichiometry could be 2 Na^+: 1 K^+: 1 glutamate :1 OH^- (or HCO_3^-), and transport would still be electrogenic. Thus, the glutamate transport process elicits a depolarizing positive inward current accompanied by intracellular acidification and extracellular alkalization (Erecinska et al., 1983).

Molecular Biology of Glutamate Transporters

In the last two years major advances in the cloning of plasma membrane glutamate transporters in mammals have been made (Kanai et al., 1993; Amara and Arriza, 1993; Kanner, 1993). Three high-affinity sodium-dependent glutamate transporters (EAAC1, GLAST1, GLT-1) were cloned independently (Storck et ., 1992; Kanai and Hediger, 1992; Pines, et al., 1992). These three glutamate transporter subtypes have approximately 50% amino acid sequence identity and are likely to have similar transmembrane topologies. The exact transmembrane topology, however, is controversial because the interpretation of hydrophathy analysis has been ambiguous. The rat transporters GLAST-1 (Strock et al., 1992) and GLT-1 (Pine et al., 1992; Danbolt, et al., 1992; Levy, et al., 1993) are reported to be expressed by glial cells. In contrast, EAAC1 mRNA is abundantly expressed in the pyramidal layer of the hippocampus, the

granular layer of the dentate gyrus, the granular cell layer of cerebellum and layers II-VI of cerebral cortex, and thus, may be localized to neurons (Kanai and Hediger, 1992). The membrane localization of these glutamate transporter is unknown. The human counterparts of these transporters have been cloned recently (Arriza et al., 1994).

Modulation of Plasma Membrane Glutamate Transporters.

Although our knowledge of this aspect of neurotransmitter function is rudimentary, some data show that the reuptake process is subject to physiological regulation.

Arachidonic acid. Arachidonic acid, which is released from neurons by a mechanism involving activation of phospholipase A_2 following an increase in intracellular $[Ca^{2+}]$, inhibits several sodium-coupled uptake systems, including the uptake system for glutamate (Chan et al., 1983; Barbour et al, 1989; Volterra et al., 1992; Racagni, 1992). Arachidonic acid can be released from postsynaptic cells when NMDA receptors are activated, or when AMPA and metabotropic receptors are activated simultaneously (Dumuis et al., 1988, 1990). In addition to the effect of arachidonic acid on the NMDA receptor channel (Miller et al., 1992) and on the presynaptic release, the modulation of the glutamate transporter by arachidonic acid may provide another possible positive feedback mechanism for regulating synaptic transmission.

Phosphorylation. Molecular biological studies have shown that glutamate transporters have several consensus sequences for phosphorylation by protein kinase A and protein kinase C (Storck et al.,

1992; Pines et al., 1992; Kanai and Hediger, 1992) indicating that phosphorylation may regulate glutamate transporters. In cultured glial cells, glutamate transporter activity (V_{max} but not K_m) was increased by application of phorbol esters (Casado et al., 1991)and this effect was blocked by protein kinase C inhibitors. Furthermore, in transfected cells expressing glutamate transporters, activators of PKC increase both the activity and the phosphorylation of the glutamate transporter (Casado et al., 1993). Because activation of NMDA receptor channels can increase the intracellular calcium concentration (MacDermott et al., 1986; Jahr and Stevens, 1987; Ascher and Nowak, 1988; Alford et al., 1993; Perkel et al., 1993) and hence stimulate Ca^{2+}-dependent kinase activity, the modulation of glutamate transporters by phosphorylation may provide a negative feedback mechanism for the function of excitatory synaptic transmission.

Others. Glucocorticoids, the adrenal steroid hormones secreted during stress, decrease the affinity but not the V_{max} of glutamate transporters under conditions of reduced glucose availability in cultured hippocampal astrocytes (Virgin et al., 1991). In addition, dopaminergic activity has a modulatory effect on the glutamate transporter (Nieoullon et al., 1983; Kerkerian et al., 1987). Electrical stimulation of the frontal cortical areas increases the high affinity glutamate transport in rat striatum. This increase is due to an increase in affinity of glutamate transporter. A low concentration of dopamine, which has no effects on the basal level of glutamate transport, inhibits this stimulation induced increase. In human astrocytoma cells, substance P, which couples to

phosphoinositide acting via an NK_1 subtype of substance P receptors (Johnson and Johnson, 1992), inhibits glutamate transport (Johnson and Johnson, 1993). Finally, glutamate transport in primary cultures of rat astrocytes is increased by activation of α_1 adrenergic receptors and decreased by activation of β adrenergic receptors (Hansson and Ronnback, 1991).

SUMMARY

The goal of this dissertation is to characterize several fundamental issues of excitatory synaptic transmission in the CNS. Three sets of experiments were performed as presented in the following manuscripts.

First, we tested the hypothesis that more than one quantum of glutamate could be released from single synaptic contact sites by each action potential and could interact with the same population of postsynaptic NMDA receptors. The occupancy of synaptic NMDA and AMPA receptors during synaptic transmission was also examined. Whole-cell recording of autaptic EPSCs and low affinity NMDA receptor antagonist displacement techniques were employed.

Second, we examined the role of glutamate transporters on the strength of excitatory synaptic transmission, by testing the hypothesis that glutamate transporters contribute to the clearance of glutamate during neurotransmission. Using a combination of transporter blockers and near physiological temperatures, we found that glutamate transporters are important in clearance of glutamate from the cleft on the 100 μs time scale. Furthermore, synaptic AMPA receptors may not saturated by synaptically released glutamate at near physiological temperature.

Finally, using the low affinity NMDA receptor agonist D-glutamate as a false transmitter, we tested the hypothesis that both the rise and decay time course of the NMDA receptor EPSC are dependent on the affinity of transmitter normally released, and that the concentration of free transmitter is elevated in the synaptic cleft for only a few milliseconds after release.

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MULTIVESICULAR RELEASE OF NEUROTRANSMITTER FROM EXCITATORY SYNAPSES OF CULTURED HIPPOCAMPAL NEURONS

Running title: Multi-vesicular release

Gang Tong and Craig E. Jahr
The Vollum Institute and the Department of Cell Biology and Anatomy
Oregon Health Sciences University
Portland, OR 97201-3098

Correspondence: Craig E. Jahr
Vollum Institute, L474
OHSU
3181 SW Sam Jackson Park Road
Portland, OR 97201-3098
voice: 503-494-5471
FAX: 503-494-6972
email: jahr@ohsu.edu

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INTRODUCTION

Chemically mediated synaptic transmission results from fusion of synaptic vesicles with the presynaptic plasma membrane and subsequent release of the vesicular contents into the cleft (Katz, 1969; Heuser et al., 1979). The loci of vesicular exocytosis have been defined ultrastructurally and consist of pre- and postsynaptic membrane specializations and numerous vesicles in the presynaptic terminal (DeRobertis, 1967; Palay and Palade, 1955). Studies of the release process at the neuromuscular junction and at central synapses have reported that the number of released quanta by presynaptic stimulation is never larger than, although sometimes equal to, the number of structurally defined sites. This suggests that at each morphologically defined release site a maximum of one vesicle is released by each invading action potential (Redman, 1990; Korn and Faber, 1991). Thus, any change in synaptic efficacy caused by presynaptic modulation of release is due to an alteration of the number of release sites from which a single vesicle is released. This "one site-one vesicle" hypothesis does not explain how each release site controls its coterie of docked vesicles so that only one can release its contents with each stimulus -- this, despite the approximately 50-fold range in area of synaptic specializations from site to site (Harris and Stevens, 1989). If only one vesicle can be released per site per spike, then the limits of transmitter concentration in the synaptic cleft at a given release site will depend only on the variability in the number of transmitter molecules from vesicle to vesicle and will not vary with release probability. If, however, transmitter from more than one vesicle can act concurrently at

the same population of postsynaptic receptors (i.e., multi-vesicular release), the peak concentration of transmitter will increase accordingly. Assuming the clearance rate of transmitter remains constant, the period during which transmitter remains above an effective level will also increase.

A rapidly dissociating competitive antagonist of the NMDA receptor can be used to estimate the time course of the concentration of free glutamate in the synaptic cleft during an epsc (Clements et al., 1992). This is because the degree of inhibition of the epsc will depend on how many binding sites are unoccupied by antagonist at the instant of transmitter release as well as how many binding sites become free of antagonist while transmitter is still elevated in the cleft. If an effective concentration of transmitter is maintained in the cleft for a longer period, as would occur if two nearby vesicles were released simultaneously, the degree of inhibition of the epsc by a rapidly dissociating antagonist will decrease. Using this approach, we find that multi-vesicular release does occur when the probability of transmitter release is raised either by elevated calcium levels, decreased presynaptic inhibition, or paired-pulse facilitation.

RESULTS

Increased calcium concentrations decrease NMDA receptor epsc inhibition by L-AP5

Autaptic NMDA receptor epscs in isolated hippocampal neurons

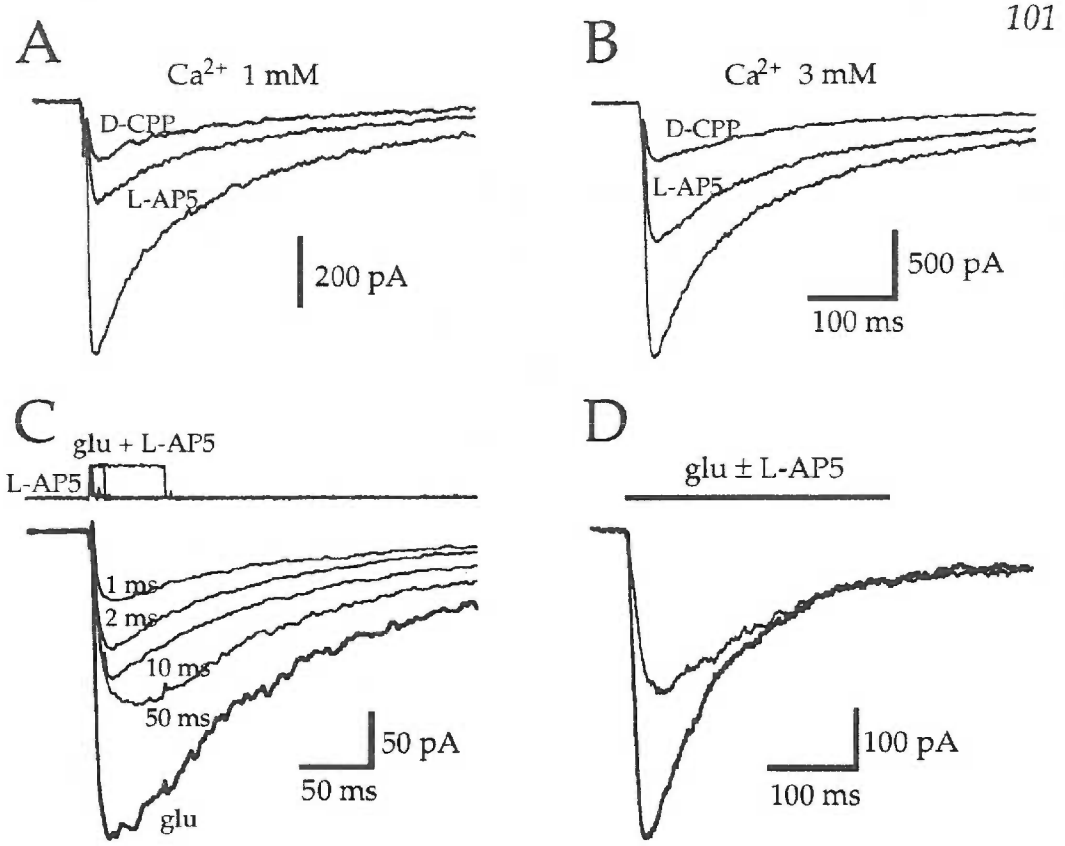

Figure 1. Increasing extracellular concentrations of calcium decreases the degree of inhibition of L-AP5.

(A and B) Superimposed averages of 10 - 15 epscs recorded in 1 mM (A) and 3 mM (B) calcium in the presence of 500 nM D-CPP or 400 μM L-AP5. All the responses are from the same neuron. Note that (A) and (B) are at different gain.
(C) Averages of responses of an outside-out patch to brief applications of glutamate (5 mM) in the presence of 1 mM L-AP5 (thin lines). The largest current (thick line) is the average of responses evoked by 5 mM glutamate (50 ms) in the absence of L-AP5. The upper traces show the open tip responses acquired after rupture of the patch.
(D) The averaged responses of a different patch to 300 ms applications of 5 mM glutamate in the presence (thin line) and absence (thick line) of L-AP5 (1 mM). Note that complete displacement of L-AP5 occurred since the responses asymptote before the end of the application.
In both (C) and (D), L-AP5 was present for many seconds before glutamate was applied and applications were interleaved to nullify any effects of rundown. All recordings in the presence of CNQX 4-5 μM. Holding potential = -60 mV.

(Segal and Furshpan, 1990; Bekkers and Stevens, 1991; Pan et al., 1993) were antagonized by the low affinity (Kd = 40 μM; Olverman et al., 1988) NMDA receptor antagonist L-2-amino-5-phosphonopentanoic acid (L-AP5) or, as a control, by D-carboxypiperazin-propyl-phosphonic acid (D-CPP), a high affinity (Kd = 0.48 μM; Olverman et al., 1988), slowly dissociating antagonist (Benveniste and Mayer, 1991).

Raising the extracellular calcium concentration from 1 to 3 mM increased the autaptic NMDA epsc amplitude 2.5 ± 0.6-fold (n = 7). In both conditions, L-AP5 (400 μM) and D-CPP (500 nM) inhibited the epsc, although L-AP5 was more effective in low calcium (Figure 1A) than in high calcium (Figure 1B). The reduction of the NMDA epsc by 400 μM L-AP5 in 3 mM calcium (46.2 ± 3.3%, mean ± s.e.m.) was significantly less than that in 1 mM calcium (59.6 ± 1.4%, n = 7, $p < 0.01$, paired t-test). Similar results were obtained using another rapidly dissociating NMDA receptor competitive antagonist, D-α-aminoadipic acid (D-AA; Clements et al., 1992). The inhibition of NMDA epscs by 100 μM D-AA in 3 mM calcium (79.7 ± 0.8%) was significantly less than that in 1 mM calcium (86.0 ± 0.7%; n = 6; $p < 0.01$). In both sets of cells, the block by D-CPP was unaffected by calcium concentration (68.0 ± 2.5% inhibition in 3 mM calcium vs. 68.4 ± 2.8% in 1 mM calcium). These data suggest that transmitter remains elevated in the synaptic cleft for longer periods when the calcium concentration is high.

Rapid unbinding of L-AP5 from the NMDA receptor

The degree of inhibition of the NMDA receptor epsc by L-AP5 and

D-AA will depend on the peak transmitter concentration and the rate at which transmitter is cleared from the cleft because L-AP5 and D-AA unbind from NMDA receptors very rapidly (see below and Clements et al., 1992). In contrast, D-CPP should inhibit epscs to the same extent regardless of the transmitter concentration time course because it unbinds very slowly (unbinding rate ~ 1 s^{-1}; Benveniste and Mayer, 1991) relative to the rate of transmitter clearance (time constant ~ 0.3 - 1 ms; Busch and Sakmann, 1990; Clements et al., 1992; Eccles and Jaeger, 1958). Thus, transmitter can only bind and activate those receptors that are unoccupied by D-CPP at the instant of release. If the percent block of the NMDA epsc by D-CPP in conditions of altered release probability varied more than 3% from that in control, poor voltage control was assumed and the data were discarded.

We obtained a qualitative estimate of the dissociation rate of L-AP5 from the NMDA receptor by the application of pulses (1 to 50 ms) of glutamate (5 mM) to outside-out patches in the continuous presence of a saturating concentration of L-AP5 (1 mM) (Figure 1C). Increases in the length of glutamate application reduced the effectiveness of L-AP5 in blocking the response. Unlike previous displacement experiments with the antagonist D-AA (see Clements et al., 1992), complete displacement of antagonist was not observed even with 50 ms pulses. This is probably due to contamination of L-AP5 by D-AP5 (≤ 1%; Tocris Neuramin, personal communication) resulting in a significant concentration of the potent and slowly dissociating D-isomer (dissociation rate = 19.4 s^{-1}; Benveniste and Mayer, 1991). Indeed, longer applications of glutamate

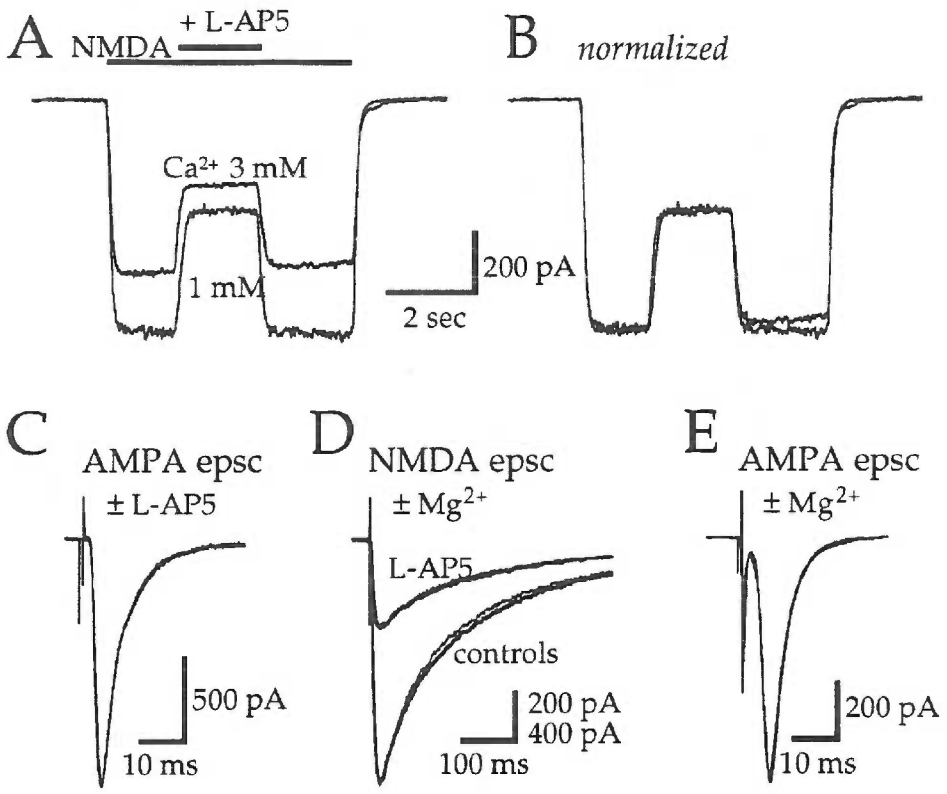

Figure 2. Extracellular calcium does not alter the affinity of L-AP5

(A) Averaged whole cell responses to 5.4 sec applications of NMDA (10 μM) in 1 and 3 mM calcium. L-AP5 (400 μM) was co-applied for 1.8 sec during the NMDA application. The intracellular solution contained 20 mM BAPTA in order to inhibit calcium dependent desensitization of NMDA receptors.
(B) Responses in (A) normalized to their peak amplitudes. (C) Superimposed AMPA receptor epscs recorded in the presence or absence of L-AP5 (400 μM).
(D) Averaged NMDA receptor epscs recorded in the presence and absence of magnesium (60 μM). The efficacy of inhibition by L-AP5 (400 μM) and D-CPP (200 nM; not shown) was unchanged by the postsynaptically mediated reduction of the epsc by magnesium. Responses in magnesium were recorded at the higher gain.
(E) AMPA receptor epscs recorded in the presence and absence of 50 μM magnesium.
Records in (A) (B) and (C) were recorded in the presence of 3 mM calcium. AMPA receptor epscs were isolated with 50 μM D-AP5. NMDA receptor epscs were isolated with 5 μM CNQX. All records are averages of 5 to 15 responses.

evoked currents that did merge with control glutamate currents indicating a biphasic recovery (Figure 1D); the time course of the slow component of recovery is consistent with the dissociation rate of D-AP5. Regardless of this complication, we have used L-AP5 because it increases the sensitivity of the assay over the more slowly dissociating D-AA. The amount of displacement of L-AP5 by 1-2 ms pulses of glutamate greatly exceeded that of D-AA (66 ± 6 % inhibition with 1 ms pulse in 1 mM L-AP5, n = 4; ~90% inhibition with 1 ms pulse in 200 μM D-AA; Clements et al., 1992). An additional indication that L-AP5 dissociates faster than D-AA is that the former slowed the 10 - 90% rise time of the 50 ms pulse of glutamate by only 3.7 ms (control rise time, 7.9 ± 0.6 ms; in L-AP5, 11.6 ± 0.7 ms; n = 9, $p < 0.01$) instead of > 10 ms with 200 μM D-AA (Clements et al., 1992).

The unequal block of the NMDA epsc by L-AP5 in high and low concentrations of calcium was not caused by an altered affinity of L-AP5 for the NMDA receptor. At equilibrium, whole cell responses to 10 μM NMDA were equally blocked by 400 μM L-AP5 in both 1 and 3 mM calcium (Figure 2A, B; 52.4 ± 1.8 % vs 51.9 ± 1.6 % reduction; n = 6). The decrease in current amplitude in 3 mM calcium was due to the calcium-mediated reduction in NMDA channel conductance (Jahr and Stevens, 1987; Ascher and Nowak, 1988). In addition, L-AP5 (400 μM) did not affect transmitter release because it had no effect on the AMPA component of the epsc (Fig. 2C; 99.8 ± 2.0% of control; n = 5). Finally, to control for a change in the effectiveness of L-AP5 due to the absolute size of the epsc, 40 - 60 μM Mg^{2+} was added extracellularly to reduce the epsc to 51 ± 8.1% (n = 4) by partially blocking current flow through NMDA channels (Mayer et

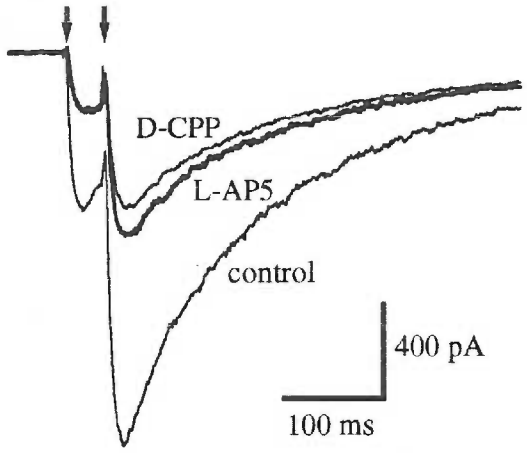

Figure 3. Paired-pulse facilitation decreases the effectiveness of L-AP5.

NMDA epscs evoked by stimuli paired at 40 ms (arrows) in 1 mM calcium in the presence of either D-CPP (250 nM, D-CPP) or L-AP5 (400 μM, L-AP5). Records are averages of 10 sweeps.

al., 1984; Nowak et al, 1984). The degree of block by 400 μM L-AP5 in both control and in Mg^{2+} was the same (Fig. 2D; 55.4 ± 1.3 % in control, 55.6 ± 1.0 % in Mg^{2+}). At these concentrations, Mg^{2+} had no effect on the amplitude of the AMPA component of the autaptic epsc (Fig. 2E; 99.4 ± 0.4% of control; n = 4) indicating that the reduction of the NMDA receptor epsc was caused by channel block rather than by decreased release.

Paired-pulse facilitation decreases inhibition by L-AP5

It is possible that the decreased block of the NMDA receptor epsc by L-AP5 and D-AA in high calcium could be caused by inhibition of transmitter clearance by calcium-dependent changes in reuptake or diffusion barriers. Paired-pulse facilitation is a method of increasing release probability that does not require changes in the extracellular solution. Pairing stimuli at intervals of 30 - 50 ms resulted in a 246 ± 41% increase in the amplitude of the second epsc. L-AP5 (400 μM) and D-CPP (250 nM) inhibited the first epsc to the same extent while L-AP5 was less effective than D-CPP at inhibiting the second epsc (Figure 3). The reduction of the second epsc by L-AP5 (400 μM) (54.9 ± 3.5%) was significantly less than that of the first epsc (62.8 ± 3.3%, n = 6, $p < 0.01$). As this method should not affect clearance mechanisms, these results suggest that increasing the probability of release results in multi-vesicular release.

4-aminopyridine, baclofen and adenosine affect multi-vesicular release

Previous studies have reported that changing release probability with compounds known to act presynaptically results in proportional

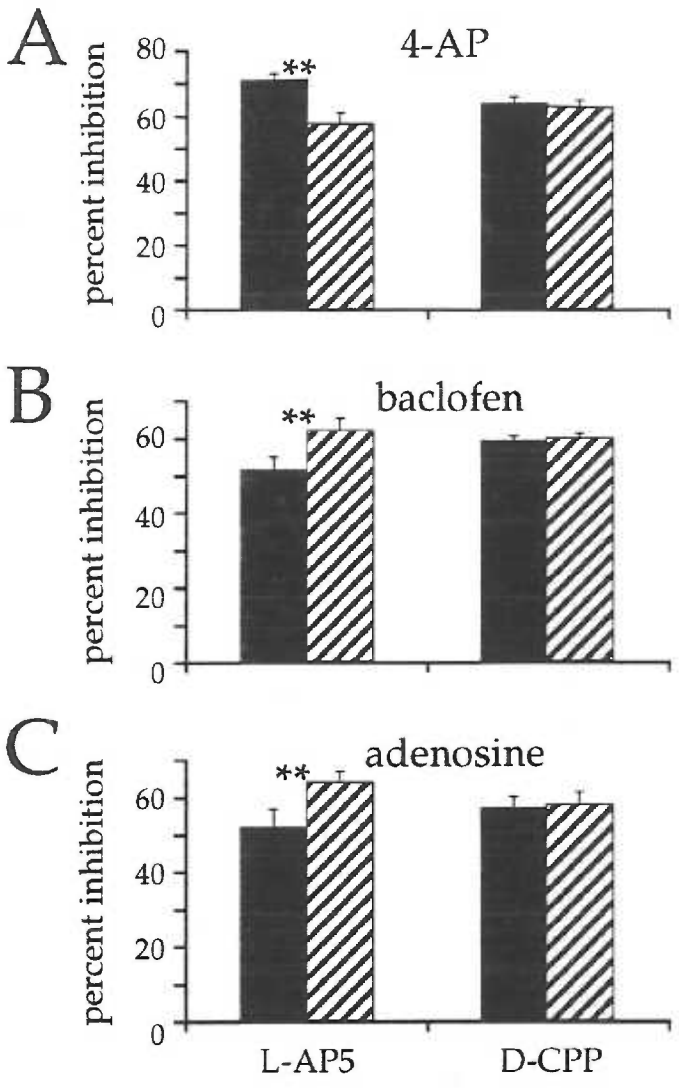

Figure 4. Drugs that change presynaptic release alter L-AP5 inhibition.

Percent inhibition of the NMDA epsc by L-AP5 (400 μM) or D-CPP (200 nM) in control solution (dark bars) and in the presence of 4-AP (300 μM, n = 5), baclofen (10 μM, n = 5) and adenosine (50 μM, n = 5) (hatched bars). The inhibition of the NMDA epsc by L-AP5 in control conditions was significantly different from that in drug treatments (**, $p < 0.01$). The percent inhibition of the NMDA epsc by D-CPP was unchanged by drug treatments. Recordings done in the presence of 1 mM extracellular calcium (4-AP experiments) or 3 mM calcium (baclofen and adenosine).

modifications of both AMPA and NMDA receptor components of the epsc (Kauer et al., 1988; Hestrin et al., 1990; Asztely et al., 1992; Garaschuk et al., 1992; Perkel and Nicoll, 1993; Fleck et al., 1993). These results have been interpreted to mean that release from presynaptic boutons is all-or-none. To directly compare our experiments with these, we have measured the effectiveness of L-AP5 in the presence of the same compounds. 4-aminopyridine (4-AP) increases transmitter release by blocking potassium conductances and thereby increasing calcium influx (Jankowska et al., 1977; Lundh and Thesleff, 1977). In the presence of 4-AP (300 μM), the peak amplitude of the NMDA receptor epsc increased to 257 ± 30%. The reduction of the epsc by L-AP5 (400 μM) in the presence of 4-AP (57.3 ± 3.2%) was significantly smaller than that in control (70.8 ± 1.8%, $n = 5$, $p < 0.01$) (Figure 4A). At this concentration, 4-AP had no effect on whole cell currents induced by application of 10 μM NMDA ($n = 4$). In addition, baclofen (10 μM) and adenosine (50 μM) were used to reduce presynaptic transmitter release (Lanthorn and Cotman, 1981; Olpe et al., 1982; Fredholm and Dunwiddie, 1988; Yoon and Rothman, 1991; Prince and Stevens, 1992). The NMDA receptor epsc was diminished to 48 ± 11% ($n = 5$) and 15 ± 4% ($n = 5$) of control by baclofen and adenosine, respectively. In the presence of baclofen, the reduction of the epsc by L-AP5 (62.0 ± 3.2%) was significantly larger than that in control (51.4 ± 3.4%, $n = 5$, $p < 0.01$, Figure 4B). Adenosine also significantly increased L-AP5 inhibition (63.9 ± 2.8% vs. 51.7 ± 5.1% in control, $n = 5$, $p < 0.01$, Figure 4C). None of these treatments significantly changed the inhibition by D-CPP (62.3 ± 2.0% vs. 63.1 ± 2.1% in 4-AP; 57.0 ± 2.9% vs. 57.7 ± 3.4% in baclofen; 59.2 ±

1.3% vs. 59.9 ± 1.2% in adenosine)

Presynaptic inhibition alters both AMPA and NMDA receptor epscs in parallel

The preceding results suggest that at high release probabilities more than one quantum of transmitter can interact with the same population of postsynaptic receptors. This interpretation is at odds with those of previous findings which suggested that multi-vesicular release does not occur (Perkel and Nicoll, 1993; for review see Redman, 1990). In particular, manipulations that alter release probability were found to produce proportional changes in NMDA and AMPA components of the epsc (Kauer et al., 1988; Hestrin et al., 1990; Asztely et al., 1992; Garaschuk et al., 1992; Perkel and Nicoll, 1993; Fleck et al., 1993). Because both types of receptors are co-localized at the majority of synaptic connections (Bekkers and Stevens, 1989; Jones and Baughman, 1991; Silver et al., 1992) and NMDA receptors have been postulated to be saturated in normal conditions while AMPA receptors are not (Clements et al., 1992; Perkel and Nicoll, 1993), the proportionality of the two epsc components was interpreted to mean that release at single release sites was all or none, i.e., one site, one vesicle. We have repeated these experiments in cultured neurons and obtained the same result. The NMDA and AMPA components of epscs were separable because of their distinct time courses (Figure 5A) and thus the peak amplitude was used as a measure of the AMPA component, whereas the NMDA component was quantified by the charge transfer from 30 to 100 ms. Addition of baclofen (2 μM) or

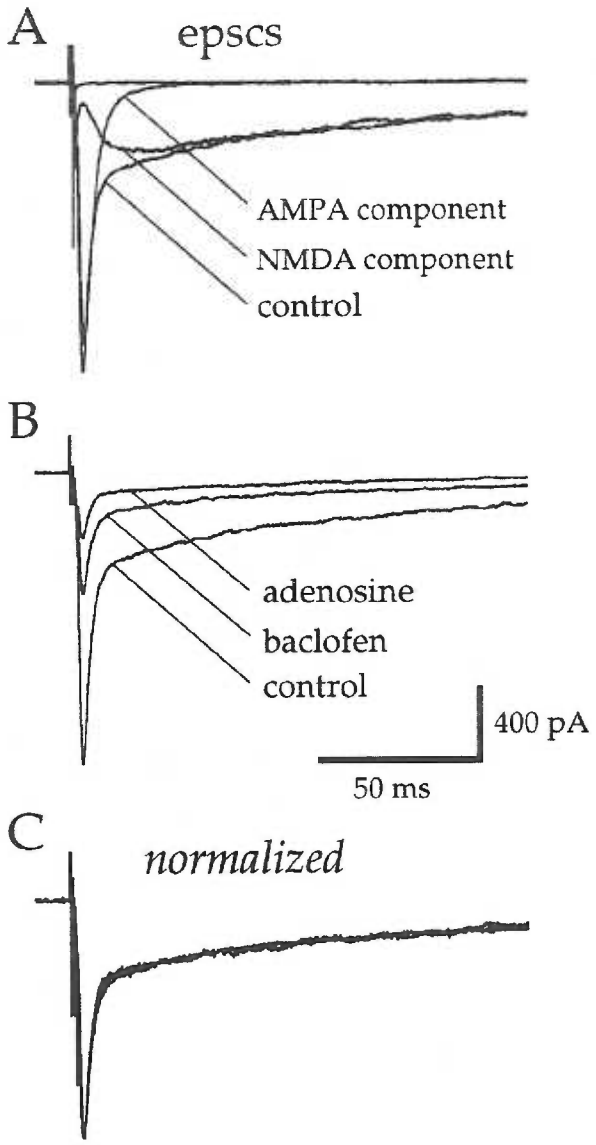

Figure 5. AMPA and NMDA receptor components of the epsc are altered proportionately by changes in release probability.

(A) Averaged epscs recorded in control, 50 μM D-AP5 to reveal the AMPA receptor component, 5 μM CNQX to reveal the NMDA receptor component, or D-AP5 and CNQX together to completely blocked the entire epsc.
(B) Superimposed records of averaged epscs recorded in control solution (3 mM calcium), in 1 μM adenosine or 2μM baclofen. (C) The same records as in (B) normalized to their peak amplitudes.

adenosine (1 μM) resulted in inhibition of both NMDA and AMPA components of the epscs (Figure 5B). When the epsc peak amplitudes were normalized, both components of the epscs were superimposable (Figure 5C) as was found in hippocampal slices (Kauer et al., 1988; Hestrin et al., 1990; Asztely et al., 1992; Garaschuk et al., 1992; Perkel and Nicoll, 1993; Fleck et al., 1993). In baclofen (2 - 10 μM), the non-NMDA and the NMDA receptor epscs were reduced to 31.8 ± 7.1% and 29.5 ± 6.6% of control ($n = 6$, $p > 0.05$). Adenosine (1 - 50 μM) reduced the epscs to 25.2 ± 2.8% and 24.3 ± 2.9% ($n = 6$, $p > 0.05$). At these concentrations neither baclofen nor adenosine affected whole cell responses to NMDA (10 μM) or AMPA (20 μM) ($n = 4$ and 6, baclofen and adenosine, respectively). The adenosine receptor inhibitor, theophylline (100 μM), had no significant effect on epsc amplitude in cultured neurons ($n = 5$). However, it has been found to dramatically increase epscs in hippocampal slices (Haas and Greene, 1988; Garaschuk et al., 1992; Perkel and Nicoll, 1993; Prince and Stevens, 1992) which suggests that basal levels of adenosine in the slice are significant. To compare results from slices with cultured neurons, theophylline was used to reverse the depression of the epscs produced by adenosine application. Theophylline (100 μM) increased the AMPA receptor epsc to 432.3 ± 68% and the NMDA receptor epsc to 420.9 ± 39.1% ($n = 5$, $p > 0.05$) of that in adenosine alone (1 μM). Thus, both AMPA and NMDA components of the epsc are altered proportionately under various conditions of release probability.

Occupancy of NMDA and AMPA receptors

Proportional changes of the NMDA and AMPA receptor epscs are compatible with multi-vesicular release if NMDA and AMPA receptors are both saturated (100% receptor occupancy) by the release of single quanta or if neither is saturated. In the first case, additional release will have no additional effect on epsc amplitude whereas in the second, multi-vesicular release will increase both components of the epsc proportionately if there is a large excess of both types of receptors. Therefore it becomes necessary to determine the degree of occupancy of both receptor types. Because we cannot precisely control the concentration time course of transmitter in the synaptic cleft, we have used short applications of glutamate to outside-out patches to estimate the requirements for full receptor occupancy. This approach requires an accurate determination of the time course of solution change at the surface of the membrane patch. For each patch, after glutamate responses were recorded, the membrane was disrupted and the open pipette response produced by the same solution changes were recorded (top traces in Figure 6A-C). Measurement of this response is critical as small changes in the relative positions of the patch pipette and flow barrels can result in oscillations and/or slow solution exchanges that can alter the kinetics of responses. However, the open pipette response is not necessarily a good indication of the time course of the solution exchange at the membrane surface. To measure the exchange time at the membrane surface, the solution was switched from one containing 160 mM NaCl to one with 400 mM NaCl in the continuous presence of 10 μM NMDA (which evoked a steady-state current). Comparison of the time course of this response to

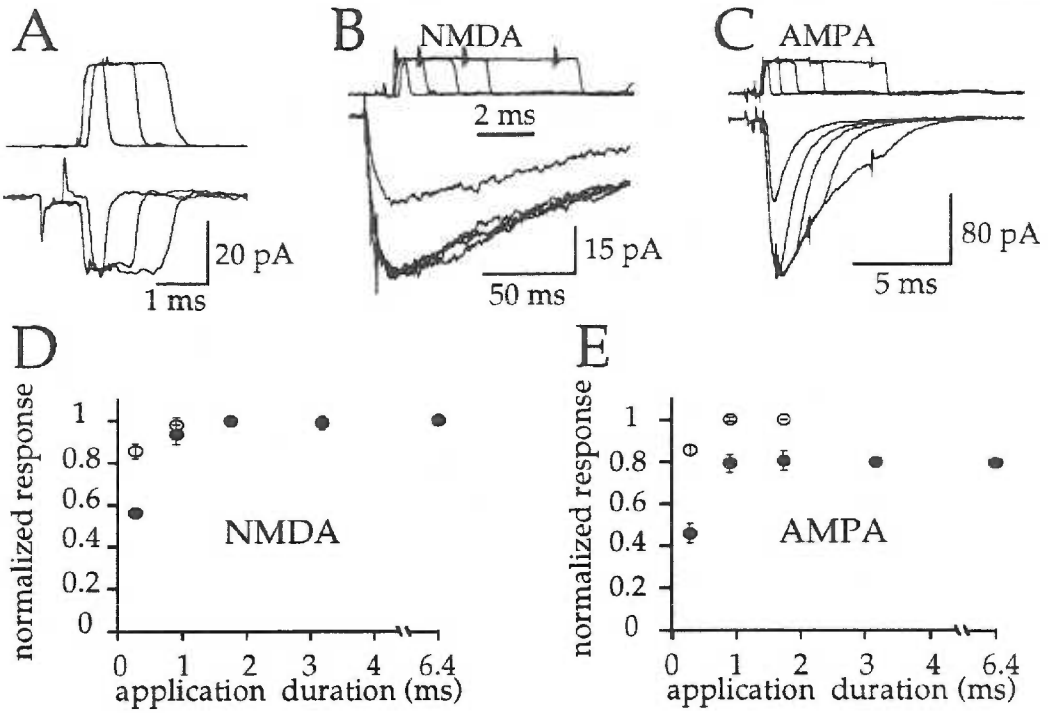

Figure 6. Concentration-time dependence of AMPA and NMDA receptor occupancy by glutamate in outside-out patches.

(A) The rate of solution exchange at the membrane surface of outside-out patches was measured by switching between normal external solution and one augmented with 400 mM NaCl in the continuous presence of 10 μM NMDA (lower traces). This was compared to the time course of solution exchanged measured with the same pipette in the same position after rupture of the membrane patch (upper traces).
(B) Averaged NMDA receptor currents (lower traces) induced by pulses 1 mM glutamate for 0.3, 0.9, 1.7, 3.2 and 6.4 ms (half-height open tip current durations) in the presence of 5 μM CNQX. The 2 ms time calibration bar applies to the open tip currents (upper traces).
(C) Averaged AMPA receptor currents evoked by the same duration pulses of 1 mM glutamate as in (B) to a different patch in the presence of 50 μM D-AP5 and 2 mM magnesium, and no added glycine.
(D and E) Comparison of NMDA (left plot, n = 6, except n = 3 for the 6.4 ms point) and AMPA receptor (right plot, n = 6) responses evoked by 0.3, 0.9, 1.7, 3.2 and 6.4 ms applications of 1 mM (filled circles) and 10 mM (open circles) glutamate to outside-out patches. In both D and E, the responses to 0.3, 0.9 and 1.7 ms applications for 1 and 10 mM glutamate were normalized by the responses evoked by 1.7 ms applications of 10 mM glutamate. The responses to 3.2 and 6.4 ms applications (of 1 mM glutamate) were scaled by the responses evoked by 1 mM glutamate for 1.7 ms. Holding potential = -60 mV.

the open pipette response obtained without changing the proximity of the patch pipette to the flow barrels shows that pulses of test solution can be as short as 0.3 ms (half height duration) and faithfully follow the time course of the open pipette response (Figure 6A). The 10 - 90% rise time of the solution exchange was 99.6 ± 2.6 μs (n = 5).

Applications of 1 mM glutamate as short as 0.9 ms evoked NMDA receptor currents that were 93.4 ± 4.7% of those activated by longer applications of 1 or 10 mM glutamate (Figure 6B, D; 5μM CNQX, 10 μM glycine). This same stimulus (1 mM/0.9 ms) evoked AMPA currents that were only 79.2 ± 4.2% of those activated by 10 mM glutamate (Figure 6C, E; 50 μM D-AP5, 2 mM Mg^{2+}, no glycine). Longer applications of 1 mM glutamate did not increase the amplitude of AMPA receptor currents over those evoked by 0.9 ms pulses. These results suggest that although NMDA receptors were nearly fully occupied by the 1 mM/0.9 ms pulse, AMPA receptors were not. However, in the simplest model that takes desensitization into account, this stimulus would result in at least 80% occupancy of AMPA receptor. Because 10 mM glutamate increased the peak current by only about 20%, doubling the agonist concentration, as might occur in the synaptic cleft if two vesicles were released simultaneously, would have only a small effect on the peak amplitude of the current. These results also imply that if the peak cleft concentration of glutamate released synaptically does not exceed 1 mM, full occupancy of AMPA receptors will not occur regardless of the number of glutamate molecules released or the number of AMPA receptors in the postsynaptic membrane.

DISCUSSION

Transmitter from multiple vesicles can interact with the same population of postsynaptic receptors

We show here that manipulations which increase release probability cause less inhibition of the NMDA receptor epsc by L-AP5 and D-AA. If release were all-or-none at isolated active sites, the control epsc would get larger because more sites would release transmitter, but the percent inhibition by antagonist would not change because at any individual site, the concentration time course of transmitter would be unaltered. The efficacy of antagonist would only decrease if transmitter remained elevated in the cleft for a longer period. This could result from increased release at individual synaptic contacts, i.e., multi-vesicular release, or from a slowing of clearance of transmitter either by increasing diffusion barriers or reducing transmitter reuptake. Our experiments do not directly address the issue of possible changes in diffusion barriers or uptake mechanisms. However, because a variety of manipulations that alter release probability gave the same result, we suggest that raising release probability resulted in increases in the quantal content of those sites where release occurred. An alternative interpretation comes from the recent finding that 24% of presynaptic boutons in the CA1 region of the hippocampus make more than one synaptic contact (Sorra and Harris, 1993). Although the distances between these synapses appears to be large relative to the dimensions of each specialization, it is possible that transmitter released from one site on a bouton could diffuse to the

postsynaptic receptors associated with a neighboring site. This appears to be the case at the specialized excitatory calyceal synapses of the avian nucleus magnocellularis (Trussell et al., 1993) where many active zones can be very closely spaced (Parks, 1981). Diffusion of the inhibitory transmitter, GABA, to distant sites has also been reported (Isaacson et al., 1993). Diffusion of glutamate between synaptic sites in hippocampus has not been reported and adequate electronmicroscopic studies of autaptic connections in culture are not available.

Receptor occupancy

We found that modification of release probability resulted in proportional changes in the size of both AMPA and NMDA receptor components of the epsc, as has been reported by others (Kauer et al., 1988; Hestrin et al., 1990; Asztely et al., 1992; Garaschuk et al., 1992; Perkel and Nicoll, 1993; Fleck et al., 1993). This result has previously been interpreted to mean that release was all-or-none at individual release sites. The basis of this interpretation was that NMDA receptors are substantially closer to being saturated by single quanta than are AMPA receptors. If increased release probability led to multi-vesicular release as well as more sites releasing single vesicles, the AMPA receptor component would increase more than the NMDA receptor component. However, if AMPA receptors are nearly saturated by normal levels of release, both components could increase proportionately as more sites release synchronously.

Our results from outside-out patches provide information

concerning the level of receptor occupancy induced by synaptic release of glutamate. This method is imperfect for several reasons. First, the shape of the pulse of glutamate applied to outside-out pulses is unlikely to exactly mimic that of synaptic release. The rise time may be too long, the falling phase possibly too short, and the peak transmitter concentration in the cleft in the first 100 μs after vesicular opening is unknown. Second, receptors in an outside-out patch may be kinetically unlike those at the synapse even though, to date, the evidence is that they are very similar (Trussell and Fischbach, 1989; Raman and Trussell, 1992; Trussell et al., 1993; Tang et al., 1991; Colquhoun, Jonas and Sakmann, 1992; Lester et al, 1990; Lester and Jahr, 1992; Hestrin, 1992; Carmignoto and Vicini, 1992). Third, there is an inexhaustible supply of glutamate in the patch experiments whereas at the synapse, there are on the order of 2000 molecules of glutamate per vesicle (based on 100 mM intravesicular concentration and 40 nm vesicular diameter; Burger et al., 1989; Villanueva et al., 1990). As there appear to be relatively few AMPA and NMDA receptors per synaptic site (Bekkers and Stevens, 1989; Jahr, 1992; Silver et al., 1992; Robinson et al., 1991; Traynelis et al., 1993), this last concern may not be important unless there is a large population of other glutamate binding sites in the cleft, such as glutamate transporters, which could buffer the concentration of glutamate.

Despite these reservations, NMDA receptors are almost certainly close to being saturated by normal levels of release because even with the relatively slow solution exchange at patches, an application of nominally 1 mM glutamate lasting less than 1 ms resulted in binding of over 90% of

receptors (see also Clements et al., 1992; Perkel and Nicoll, 1993). The same pulse of glutamate probably resulted in about 80% of AMPA receptors being bound. Thus, further increases in the concentration time course of glutamate at the synapse will cause only small changes in the occupancy of either receptor type and similar small changes in their respective epscs. The increases in current due to multi-vesicular release may, then, be too small to cause a measurable change of the ratio of AMPA to NMDA receptor epscs.

Previous estimates of the concentration time course of transmitter in the synaptic cleft (Clements et al., 1992) reflected the average behavior of all active synaptic contacts and did not have the time resolution necessary to ascertain the concentration profile in the first hundred microseconds after release. At central excitatory synapses, the synaptic cleft is quite narrow, about 20 nm, and synaptic vesicles are about 40 nm in diameter and probably contain at least 100 mM glutamate (Burger et al., 1989; Villanueva et al., 1990). If release of transmitter from vesicles were instantaneous, a very high concentration of glutamate would be reached briefly at the postsynaptic membrane just across from the release site. However, release of the contents of small vesicles may require at least 100 μs (Almers and Tse, 1990) which would slow the rise of transmitter across the cleft and decrease its peak concentration. It remains possible that glutamate attains a concentration higher than 1 mM at a restricted postsynaptic area and that AMPA receptors are primarily sequestered in this area. In addition, subunit composition of individual receptors (Seeburg, 1993; Silver et al., 1992; Colquhoun et al., 1992; Trussell et al.,

1993) and temperature, both of which can affect glutamate affinity, may also change receptor occupancy.

Consequences for synaptic transmission

If nearly all postsynaptic NMDA and AMPA receptors are occupied after release of a single quantum, then multi-vesicular release will have little effect on the strength of transmission. In this case, any increase in one component of the epsc without a concomitant increase in the other component would require a postsynaptic mechanism of enhancement (Muller et al., 1988; Kauer et al., 1988). However, if different methods of altering release probability could preferentially target synaptic sites containing predominantly one receptor type or the other, changes in postsynaptic sensitivity may not be needed to explain alterations in the ratio of the two synaptic components.

Instead of producing an increase in synaptic strength, multi-vesicular release may merely be the consequence of terminals with high release probabilities (Rosenmund et al., 1993; Hessler et al., 1993). The utility of multi-vesicular release, then, may be to obviate a mechanism to limit release to one vesicle per stimulus.

EXPERIMENTAL PROCEDURES

Tissue culture

Experiments using outside-out patches were performed on hippocampal neurons dissociated from 1-3 day postnatal rats and maintained in cell culture for 1-3 weeks as described previously (Lester et al., 1989). Autaptic recordings were obtained from isolated hippocampal neurons obtained using the same procedures as above but plated on collagen/poly-D-lysine "microdots" as described by Bekkers and Stevens (1991).

Outside-out patch recordings

Outside-out patch recordings (Axopatch 1C or 1D, Axon Instruments) were obtained using pipettes containing either Na or Cs gluconate, 140 mM; NaCl, 10 mM; HEPES 10 mM; EGTA 10 mM; MgATP 2-4 mM; adjusted to pH 7.2 with either NaOH or CsOH. Control external solutions contained 160 mM NaCl; KCl 3 mM; $CaCl_2$ 0.2-2 mM; HEPES 5 mM; adjusted to pH 7.4 with NaOH. To isolate NMDA receptor currents, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) was added at 4 μM and glycine at 20 μM. To isolate AMPA receptor currents, D-AP5 (50 μM) and $MgCl_2$ (2 mM) were added to solutions nominally free of glycine. Solution exchanges were made with glass flow tubes attached to piezoelectric bimorphs (Vernitron, Bedford, OH) as previously described (Lester and Jahr, 1992; Lester et al., 1993). Open tip currents were enhanced by adding 10 mM NaCl to agonist solutions except when 10 mM glutamate was

used. Artifacts caused by voltage jumps applied to the piezo bimorph were isolated by turning off the flow, averaged and subtracted from the open tip averages. Two lots of L-AP5 were obtained from Tocris Neuramin. Lot 4, which was used in most of the experiments, was slightly less contaminated by D-AP5 than lot 5 as judged by displacement experiments (figure 1C, D). This accounts for the greater block of the NMDA receptor epsc by L-AP5 in the 4-AP experiment than in the calcium concentration experiments and the paired-pulse experiments.

Whole cell experiments

Autaptic currents were recorded (Axopatch 1D) with low resistance patch pipettes (0.5 - 2.5 MΩ) filled with an internal solution containing K gluconate, 140 mM; NaCl, 10 mM; HEPES, 10 mM; EGTA, 10 mM; adjusted to pH 7.4 with KOH. Control extracellular solution contained NaCl, 160 mM; KCl, 3 mM; HEPES, 10 mM; $CaCl_2$, 1-3 mM; glycine, 20 μM; picrotoxin 50 μM; adjusted to pH 7.4 with NaOH. CNQX at 5 μM was present in the extracellular solution unless otherwise stated. Autaptic epscs were evoked with 0.5 - 2 ms voltage jumps to -20 or 0 mV from a holding potential of -60 mV. The currents were low-pass filtered at 0.5 to 5 kHz and digitally sampled at 1 - 5 kHz. Series resistance compensation (80 - 100 %) was used in all experiments. For most illustrations, the partially clamped action potential and associated capacitative currents evoked by the stimulating voltage jump were isolated with CNQX and D-AP5 or D-CPP and subtracted from the averaged epsc. For whole cell responses to exogenous NMDA, K_4BAPTA at 20 mM replaced internal

EGTA to block the Ca^{2+}-dependent NMDA receptor desensitization (Legendre et al., 1993). In addition, 1 μM tetrodotoxin was added to the extracellular solution to block spontaneous action potentials. Solution changes were made with gravity-fed flow tubes as described previously (Lester and Jahr, 1992; Lester et al., 1993).

In both patch and synaptic experiments, responses in different conditions were interleaved in time to nullify any time-dependent changes. If rundown of responses from a patch were so fast that excluding the first response in the average significantly changed the size or kinetics of the average, the data were discarded. Data are expressed as mean ± s.e.m. All experiments were performed at room temperature.

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BLOCK OF GLUTAMATE UPTAKE INCREASES POSTSYNAPTIC EXCITATION

Running title: Glutamate uptake and synaptic excitation

Gang Tong and Craig E. Jahr
Vollum Institute and Department of Cell Biology and Anatomy
Oregon Health Sciences University
Portland, OR 97201-3098

Correspondence: Craig E. Jahr
Vollum Institute, L474
OHSU
3181 SW Sam Jackson Park Road
Portland, OR 97201-3098
voice: 503-494-5471
Fax: 503-494-6972
E-mail: jahr@ohsu.edu

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SUMMARY

We have studied the effects of blockers of glutamate transporters on the strength of excitatory synaptic transmission in cultured rat hippocampal neurons. The transporter blockers Li^+ and THA increased the amplitude, but not the decay time, of spontaneous AMPA receptor EPSCs recorded at 34°C but not 24°C. Evoked AMPA receptor EPSCs were similarly affected by THA. The rapidly dissociating AMPA receptor competitive antagonist PDA, but not the high affinity antagonist NBQX, inhibited evoked AMPA receptor EPSCs less in the presence of THA. We suggest that cleft glutamate concentration is elevated longer in the absence of functional transporters and that at 34°C, AMPA receptors are not saturated by synaptically released glutamate.

INTRODUCTION

At many synapses in the CNS, excitatory neurotransmission is mediated by the release of glutamate from presynaptic terminals into the synaptic cleft and subsequent binding to two classes of ligand-gated ion channels in the postsynaptic membrane, NMDA and non-NMDA receptors (Mayer and Westbrook, 1987; Collingridge and Lester, 1989). Unlike neuromuscular transmission which is limited by both enzymatic hydrolysis and diffusion of acetylcholine from the cleft (Katz and Miledi, 1973; Magleby and Terrar, 1975), termination of the actions of glutamate results from its removal from the cleft by diffusion and possibly by uptake. Although physiological and molecular studies have demonstrated the existence of transporter proteins capable of uptake of extracellular glutamate (Iversen, 1971; Garthwaite, 1985; Pines et al., 1992; Kanai and Hediger, 1992; Storck et al., 1992; Isaacson and Nicoll, 1993; Sarantis et al., 1993; Arriza et al., 1994), there was until recently no evidence for the involvement of transporters in shaping the time course of synaptic events (Hestrin et al., 1990; Isaacson and Nicoll, 1993; Sarantis et al., 1993). Instead, diffusion of glutamate out of the cleft was thought to be the rate limiting factor in clearance, while a distant, slower but capacious uptake is required to maintain a large diffusional gradient. Mennerick and Zorumski (1994) recently reported that blockade of glutamate uptake in hippocampal neuronal cultures results in a decreased amplitude but increased time course of decay of NMDA receptor EPSCs and, in the presence of cyclothiazide, AMPA receptor EPSCs. This suggests that free glutamate may remain elevated in the synaptic cleft for many hundreds of

milliseconds continually rebinding and reactivating postsynaptic ion channels. In contrast to this, we have found that at near physiological temperatures, inhibitors of glutamate transporters increase the amplitude of AMPA receptor EPSCs without altering the decay time constant whether or not cyclothiazide is present. We suggest that in our conditions, clearance of free glutamate is delayed only a fraction of a millisecond by inhibition of glutamate transporters. This slight prolongation is apparently sufficient to potentiate EPSCs by 20 to 30%.

RESULTS

Glutamate transporter blocker, THA, increases amplitude of AMPA receptor spontaneous EPSCs in cultured hippocampal neurons

DL-threo-β-hydroxyaspartic acid (THA) is an inhibitor of the three recently cloned glutamate transporters with Ki's in the 10-30 μM range (Pines et al., 1992; Kanai and Hediger, 1992; Arriza et al., 1994) and has been shown to inhibit uptake by competing with glutamate for transport (Barbour et al., 1991;Arriza et al., 1994). THA (300 μM) increased the mean amplitude (19.7 ± 1.6%; n=7) but not the decay time constant of spontaneous AMPA receptor excitatory postsynaptic synaptic currents (sEPSCs) at 34°C (Figure 1B; Table 1) but not at 24°C (Figure 1C; Table 1). This suggests that at near physiological temperatures, THA-sensitive uptake of glutamate may be more important for its clearance from the synaptic cleft than at room temperature. sEPSCs were blocked by CNQX

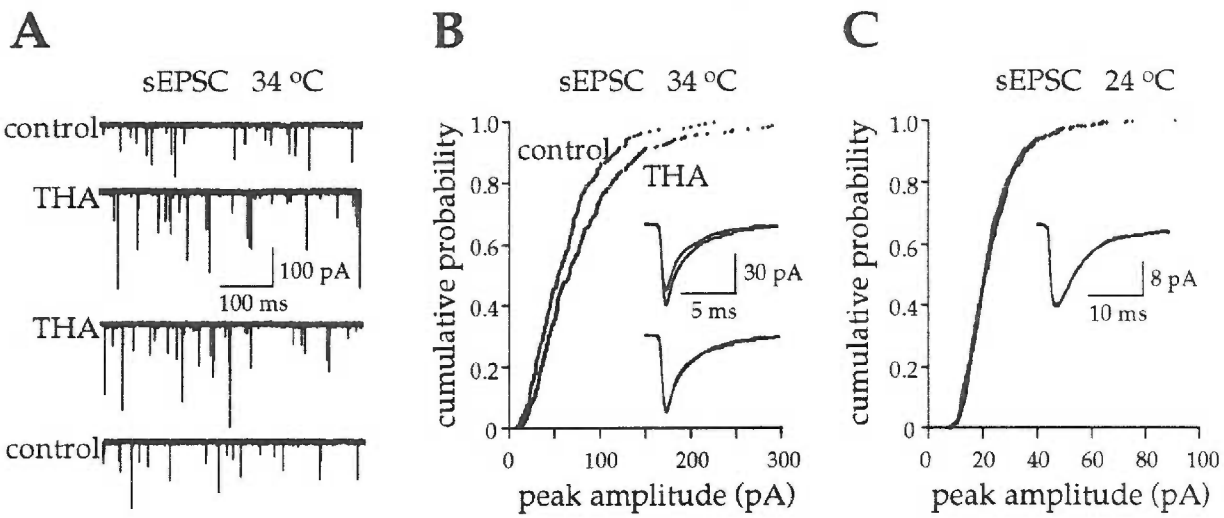

Figure 1. THA increases amplitude of spontaneous AMPA receptor EPSCs.

(A) Representative current traces in control and in the presence of THA (300 μM). Holding potential = -90 mV. sEPSCs were recorded in the presence of tetrodotoxin (1 μM), picrotoxin (100 μM), magnesium (1 mM) and D-CPP (20 μM) at 34 °C.
(B). Cumulative probability plots of sEPSC amplitudes in control and in the presence of THA (300 μM) at 34 °C. The inset shows the averages of 192 sEPSCs in control and 289 sEPSCs in the presence of 300 μM THA. The lower traces are normalized to their peak amplitudes. (A) and (B) are from the same cell.
(C). Cumulative probability plots of sEPSCs amplitudes in control and in the presence of THA at 24 °C from another neuron. The inset shows the averages of 529 sEPSCs in control and 538 sEPSCs in the presence of 300 μM THA.

Table 1. Amplitude and decay time constants of AMPA receptor responses.

	34 °C		24 °C	
sEPSC	control	THA	control	THA
	(n = 7)		(n = 6)	
τ decay (ms)	2.41 ± 0.28	2.27 ± 0.27	4.51 ± 0.60	4.58 ± 0.68
amplitude (pA)	36.69 ± 6.73 **	43.78 ± 7.98	22.32 ± 3.74	22.27 ± 3.73
sEPSC	control	Li^+	control	Li^+
	(n = 6)		(n = 6)	
τ decay (ms)	2.20 ± 0.14	2.30 ± 0.22	3.82 ± 0.37	3.88 ± 0.38
amplitude (pA)	54.60 ±10.76 ++	65.09 ±12.51	22.49 ± 3.30	21.73 ± 3.13
eEPSC	control	THA	control	THA
	(n = 10)		(n = 4)	
τ decay (ms)	3.92 ± 0.41	3.90 ± 0.39	6.25 ± 0.93	6.19 ± 0.95
amplitude (nA)	1.45 ± 0.27 ***	1.84 ± 0.32	0.85 ± 0.23	0.86 ± 0.24
O/O patch	control		control	
	(n = 6)		(n = 10)	
τ decay (ms)	0.93 ± 0.13		1.51 ± 0.20	

sEPSCs were collected in control or drug (THA or Li^+) at 24 °C or 34 °C. Amplitude and decay time constant measurements are of means of 143 to 1637 sEPSCs each from seven (THA, 34°C), six (THA, 24 °C), six (Li^+, 34 °C), and six (Li^+, 24 °C) neurons. ** $p = 0.0074$, ++ $p = 0.016$, paired t-tests. The Kolmogorov-Shirnov test was used to compare sEPSCs at a single temperature in control and drug. In 6 of 7 neurons, $p < 0.05$ for THA at 34°C. In 5 of 6 neurons, $p < 0.05$ for Li^+ at 34°C. In 6 of 6 neurons, $p > 0.05$ for THA at 24 °C. In 6 of 6 neurons, $p > 0.05$ for Li^+ at 24 °C. All values are mean ± s.e.m. Evoked EPSCs (eEPSCs) were recorded from autapses. *** $p = 0.0005$, paired t-test (n=10). Outside-out (O/O) patch responses to 0.3 ms applications of 10 mM glutamate.

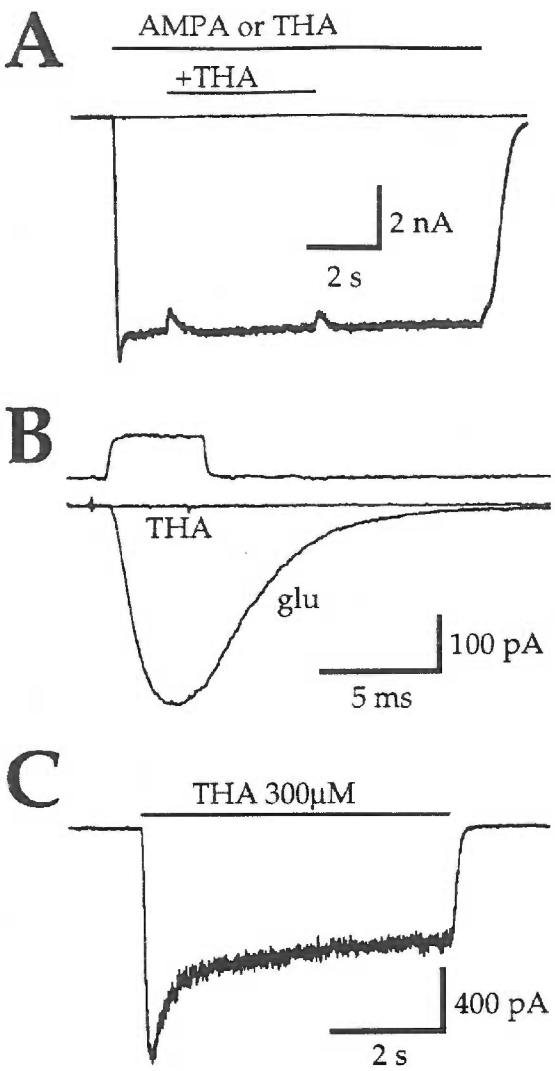

Figure 2. THA has no effect on AMPA receptors.

(A) Whole-cell responses to 10 s applications of AMPA (20 μM, thick line) or THA (300 μM, thin flat line) in the presence of magnesium (1 mM) and D-CPP (20 μM) with no added glycine. THA (300 μM) was also coapplied for 4 s during the AMPA applications.
(B) Averaged AMPA receptor currents (lower traces) induced by 4 ms applications of THA (300 μM) or glutamate (1 mM) to a outside-out patch in the presence of magnesium and D-CPP (20 μM) with no added glycine. The upper most trace is open tip current recorded after patch breakdown.
(C) Whole-cell response to 5.8 s application of THA (300 μM) in the presence of glycine (10 μM) and NBQX (5 μM). Records in (A) and (C) are single sweep responses from the same cell. Records in (B) are averages of ten sweeps.

or NBQX (5 μM).

THA (300 μM) had no effect on whole cell responses to AMPA (20 μM; n = 7), nor did it by itself evoke any whole cell current in the presence of NMDA receptor blockers (Figure 2A, n=7). In addition, short pulses of THA (300 μM) did not evoke any AMPA receptor current in outside-out patches where solution exchange is fast relative to desensitization even in cases where glutamate evoked very large AMPA responses (Figure 2B; n = 4). THA (300 μM) did directly activate NMDA receptor currents in the presence of NBQX (4 μM), glycine (20 μM) and no added Mg^{2+} (Figure 2C; n = 7).

THA potentiates evoked AMPA receptor EPSCs

As with spontaneous AMPA receptor EPSCs, the amplitude of the non-NMDA receptor EPSC evoked autaptically was potentiated by THA to 132.2 ± 5.6% of control at 34°C (Figure 3A; n=10) but not at 24°C (101.0% ± 0.9% of control; Table 1; n = 4) without alteration of its time course (Figure 3B; control τ decay = 3.92 ± 0.41 ms, THA τ decay 3.90 ± 0.39 at 34 °C; control τ decay = 6.245 ± 0.93 ms, THA τ decay 6.185 ± 0.95 at 24 °C; Table 1). Paired-pulse facilitation was unaffected by THA (Figure 3B; n=4) suggesting that the potentiation by THA was of postsynaptic origin.

A method used previously to determine the time course of free glutamate in the synaptic cleft (Clements et al., 1992; Tong and Jahr, 1994) relied on the displacement of rapidly dissociating antagonists of the NMDA receptor by synaptically released glutamate. Prolonging the presence of glutamate in the cleft or at outside-out patches resulted in less

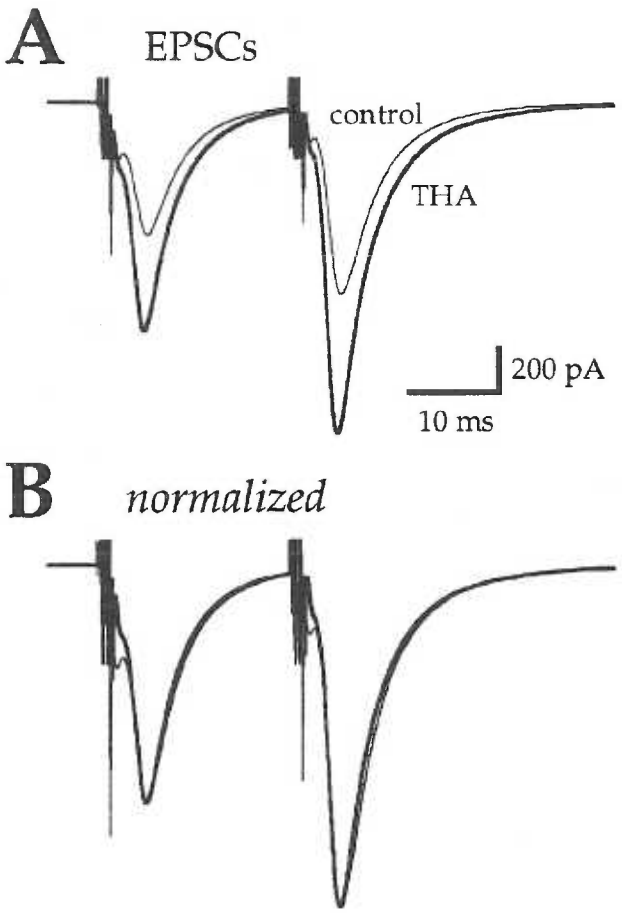

Figure 3. THA potentiates evoked AMPA receptor EPSCs .

(A) Averaged EPSCs evoked by stimuli paired at 20 ms in control and in the presence of THA (300 μM).
(B) The same records as in (A) normalized to their first peak amplitudes. Records are averages of ten sweeps.

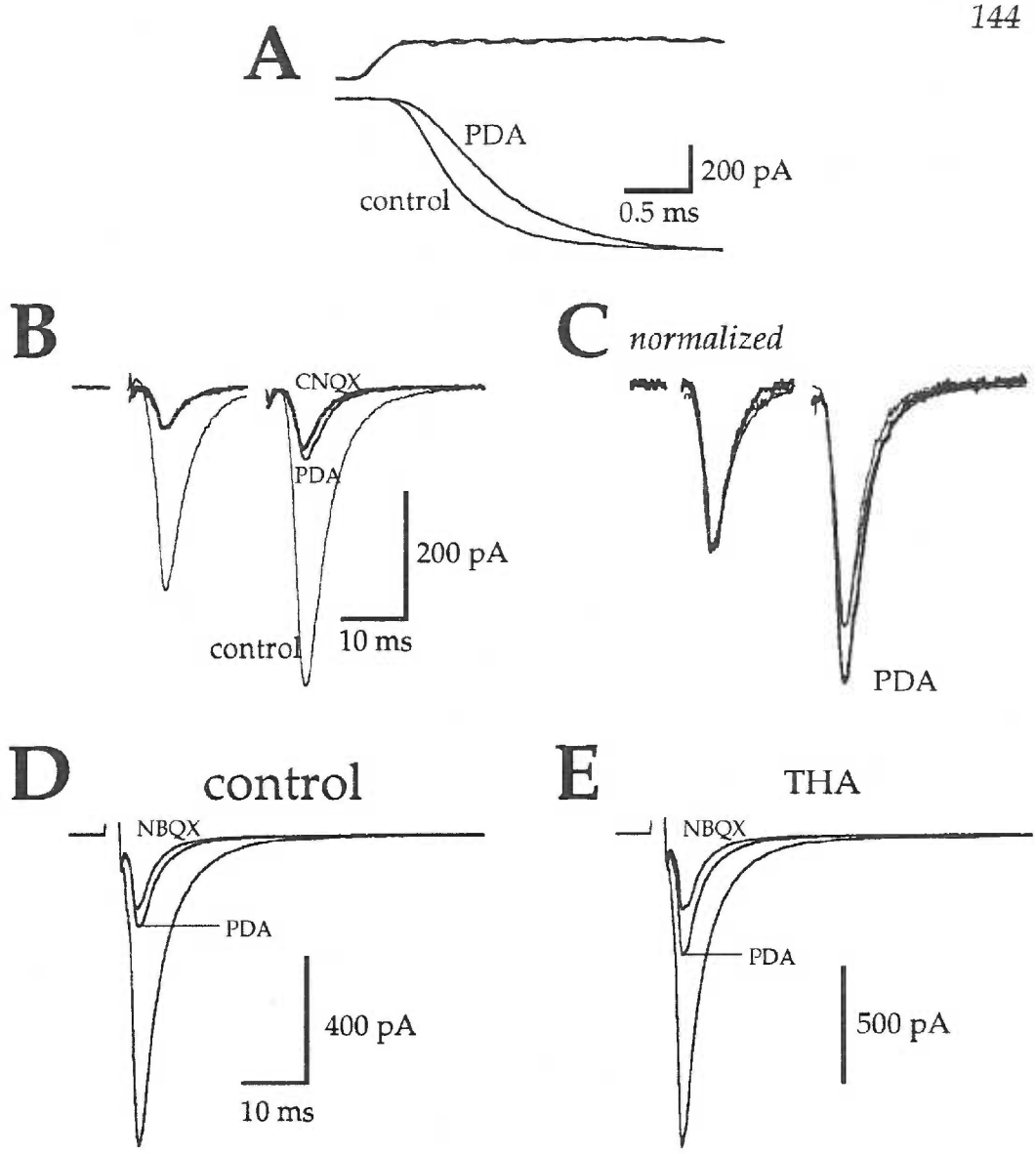

Figure 4. THA decreases evoked AMPA receptor EPSC inhibition by PDA.

(A). Averages of responses of an outside-out patch to brief applications of glutamate (10 mM) in the presence and absence of 1 mM PDA. The upper traces show the open tip responses acquired after rupture of the patch.
(B and C). Paried-pulse facilitation decreases the effectiveness of PDA. (B). AMPA receptor EPSCs evoked by stimuli paired at 20 ms in the presence of either CNQX (400 nM) or PDA (1.5 mM) at 34 ^{o}C. (C) The same records in (B) normalized to their first peak amplitudes.
(D and E) Superimposed averages of AMPA receptor EPSCs recorded in control (D) and in the presence of THA (300 μM, E) in the presence of 200 nM NBQX and 1.5 mM PDA. Note that (D) and (E) are at different gain.

inhibition by these antagonists because as the antagonist dissociates, glutamate can bind to and activate the newly available receptors. The same method can be used with antagonists of the non-NMDA receptor. Piperidine dicarboxylic acid (PDA) is a low affinity non-NMDA receptor antagonist (Collingridge and Lester, 1989). In the presence of PDA (1 mM), responses of outside-out patches to 2 ms applications of 10 mM glutamate are depressed and the rising phase of the response is slowed. The slowing of the rising phase can be more clearly shown in the absence of desensitization (Figure 4A) after addition of 20 μM cyclothiazide (Patneau et al., 1992; 1993; Yamada and Tang, 1993). As judged by the time required for the current to reach a plateau, equilibration of PDA and glutamate with the receptors is very rapid and thus on and off rates for both ligands very fast.

As shown previously with NMDA receptor competitive antagonists (Tong and Jahr, 1994), PDA inhibits the first EPSC of a pair evoked 20 ms apart more (70.4 ± 6.0%) than the second (64.0 ± 6.3%; n=6; $p<0.002$) indicating that effective concentrations of free glutamate are present at postsynaptic receptors longer after the second stimulus. If THA potentiates non-NMDA EPSCs by slowing clearance of glutamate from the cleft, then PDA should inhibit the EPSC proportionately less in the presence of THA. PDA inhibited EPSCs by 68.7 ± 2.2% in the presence of THA compared with 74.2 ± 2.2% in its absence (n=5; $p<0.005$).

Li^+ potentiates spontaneous non-NMDA EPSCs

Substitution of extracellular Li^+ for Na^+ blocks glutamate uptake by

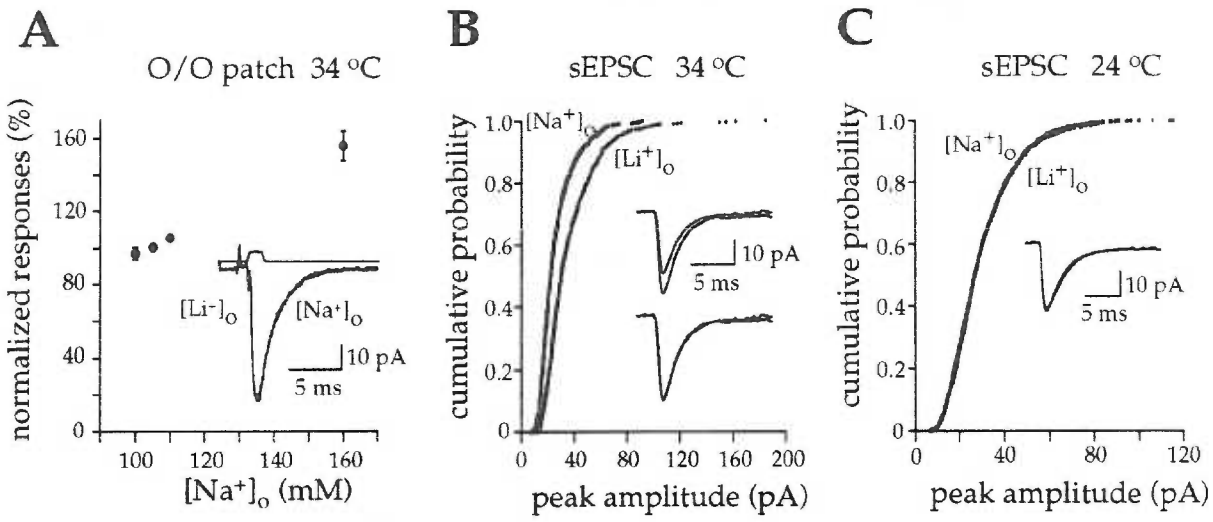

Figure 5. Extracellular lithium increases the amplitude of sEPSCs.

(A). Comparison of the permeability of lithium and sodium through AMPA receptors (n = 4 - 8 for each point). The concentration of lithium was 167 mM. To totally eliminate Na^+, LiOH was used to pH extracellular solutions and glutamic acid. The inset is the superimposed averages of AMPA receptor responses induced in an outside-out patch by 1.5 ms applications of 10 mM glutamate in the presence of 105 mM sodium and 167 mM lithium. The upper trace is the open tip response. Records are averages of 30 responses.
(B). Cumulative probability plots of sEPSC amplitudes in 105 mM sodium and in 167 mM lithium at 34 °C. The inset shows the averages of 755 sEPSCs in sodium (105 mM) and 751 sEPSCs in lithium (167 mM). The lower traces are same averages normalized to their peak amplitudes.
(C). Cumulative probability plots of sEPSC amplitudes in sodium (105 mM) and in 167 mM lithium at 24 °C from another neuron. The inset shows the averages of 1591 mEPSCs in sodium (105 mM) and 1637 mEPSCs in lithium (167 mM).

binding to Na^+ binding sites of the transporter (Schwartz and Tachibana, 1990; Barbour et al., 1991; Wyllie et al., 1991). Because AMPA receptors are less permeable to Li^+ than to Na^+, external Na^+ was reduced to 105 mM to match the amplitudes of responses of outside-out patches evoked by 10 mM glutamate in 167 mM Li^+ (Figure 5A). Spontaneous non-NMDA epscs recorded in 167 mM Li^+ were significantly larger than those in 105 mM Na^+ at 34 ^oC but not at 24 ^oC (Figure 5B, C; Table 1) suggesting that glutamate transporters can alter clearance of glutamate from the synaptic cleft. As with the THA results, the time course of decay was not altered by Li^+ (Figure 5B, C, inset; Table 1).

Occupancy of non-NMDA receptors is decreased by raising temperature

Because glutamate transporter activity can be clearly demonstrated at room temperature (Hestrin et al., 1990; Isaacson and Nicoll, 1993; Sarantis et al., 1993; Mennerick and Zorumski, 1994), it may be that the lack of effect of blockers on EPSC amplitudes at 24^oC is due, at least in part, to another effect of temperature. At room temperature, synaptic release of glutamate may nearly saturate postsynaptic AMPA receptors (Jonas et al., 1993; Tong and Jahr, 1994). In a binding reaction that is exothermic, the unbinding rate will be more sensitive to increases in temperature than the binding rate because the energy of activation of the unbinding rate is greater than that of the binding rate. Thus, receptor occupancy may be lower at 34^oC than at 24^oC. This was tested by applying either 1 mM or 10 mM glutamate to outside-out patches for increasing periods at 34^oC. As shown previously (Tong and Jahr, 1994),

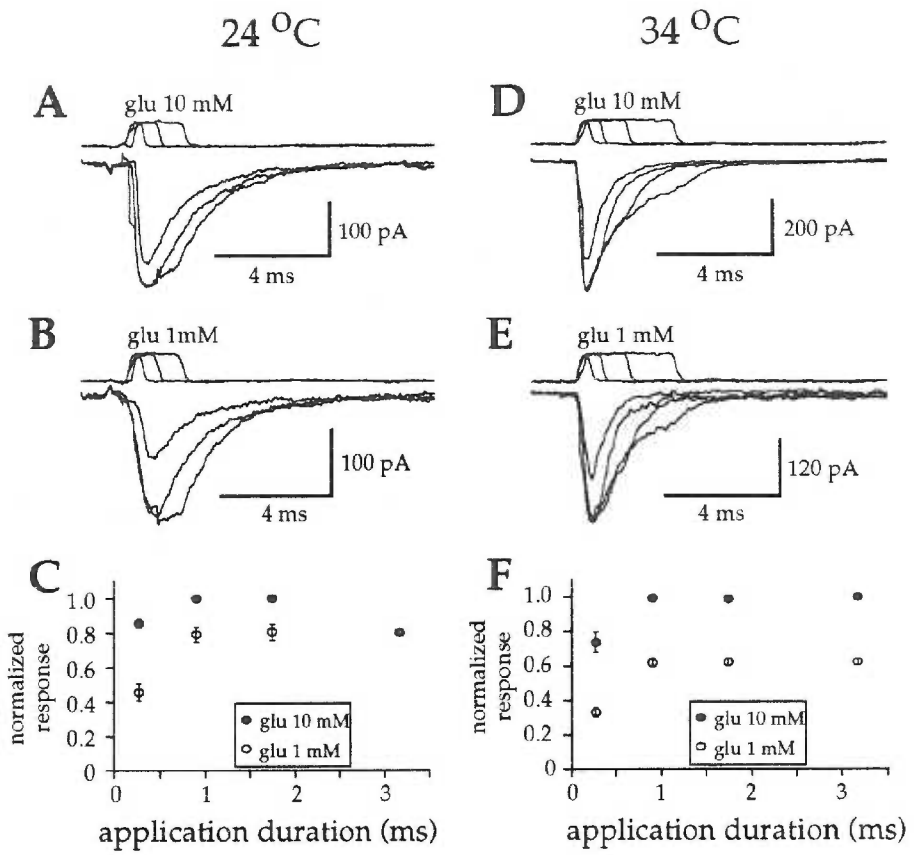

Figure 6. Occupancy of AMPA receptors is decreased by raising temperature.

(A) Averaged AMPA receptor currens (lower traces) induced by pulses of 10 mM glutamate for 0.3, 0.9, and 1.7 ms (half-height open tip current durations) in the presence of 1 mM magnesium, 20 μM D-CPP and no added glycine at 24 °C. The upper traces are open tip responses recorded after patch breakdown. (B) Averaged AMPA receptor currents evoked from the same patch as in (A) by 1 mM glutamate pulses at 24 °C. (D) Averaged AMPA receptor currents induced by pulses of 10 mM glutamate for 0.3, 0.9, 1.7 and 3.2 ms at 34 °C. (E) Averaged AMPA receptor currents evoked from the same patch as in (D) by 1 mM glutamate pulses at 34 °C. (C and F) Comparison of AMPA receptor responses evoked by 0.3, 0.9, 1.7, and 3.2 ms applications of 1 mM (open circles) and 10 mM (closed circles) glutamate to outside-out patches at 24 °C (C; n=6) and at 34 °C (F; n=6). In (C), the responses to 0.3, 0.9, and 1.7 ms applications for 1 and 10 mM glutamate were normalized by the responses evoked by 1.7 ms applications of 10 mM glutamate. The response to 3.2 ms applications of 1 mM glutamate was scaled by the responses evoked by 1 mM glutamate for 1.7 ms. In (F), the responses to 1 and 10 mM glutamate applications were normalized by the responses evoked by 3.2 ms application of 10 mM glutamates. The data in (C) was from a previous study (Tong and Jahr, 1994).

at 24°C, a 0.9 ms application of 1 mM glutamate results in a current that is about 80% of that of a similar application of 10 mM glutamate suggesting that most receptors are bound (Figure 6A-C). At 34°C, however, a 0.9 ms, 1 mM glutamate application evoked currents only 61.8 ± 2.1% of those produced by 10 mM glutamate (Figure 6D-F; n=6). Longer applications of 1 mM glutamate did not increase AMPA receptor currents. These results suggest that prolongation of effective concentrations of glutamate in the cleft can be translated into larger EPSCs only when AMPA receptors are not close to saturation to begin with.

DISCUSSION

Transport blockers potentiate AMPA receptor EPSCs

We have found that blockers of glutamate transporters potentiate evoked and spontaneous AMPA receptor EPSCs in cultured hippocampal neurons. Potentiation did not occur at room temperature (24°C) in agreement with previous reports using brain slice preparations (Isaacson and Nicoll, 1993; Sarantis et al., 1993). These authors reported, however, that transporter blockers caused potentiation of responses to exogenously applied glutamate and increased tonic levels of extracellular glutamate. Recently, glial uptake of synaptically released glutamate has also been demonstrated at room temperature in culture (Mennerick and Zorumski, 1994). Even at room temperature, then, glutamate transporters are functional. Unlike the results from slices and possibly in culture under some conditions (Mennerick and Zorumski, 1994), we find no evidence that transporter blockers cause glutamate build-up.

The speed of solution exchange may account for these differences. In culture, solutions can be exchanged in the synaptic cleft in about 20 ms as judged by the time required to affect NMDA receptor EPSCs by changing concentrations of Mg^{2+} or glycine site agonists in the superfusate (Lester et al., 1990; Lester et al., 1993). In these conditions, released glutamate will be washed away within a few milliseconds even in the presence of uptake blockers. In slice preparations, solution changes require minutes and the consequent build-up of micromolar concentrations of glutamate in uptake blockers may result in two

phenomena: desensitization of postsynaptic receptors (Trussell and Fischbach, 1989; Tang et al., 1991; Vyklicky et al., 1991) and presynaptic inhibition of glutamate release (Forsythe and Clements, 1990). Both phenomena will result in smaller evoked EPSCs and desensitization will diminish spontaneous EPSCs. In hippocampal slice (Isaacson and Nicoll, 1993; Sarantis et al., 1993) and recent culture (Mennerick and Zorumski, 1994) experiments, evoked AMPA receptor EPSCs are variably reduced in amplitude by the transporter blocker trans-pyrrolidine-2,4-dicarboxylic acid (trans-PDC), while in cerebellar slices EPSCs were decreased by about 20% (Sarantis et al., 1993). These results suggest that in slice preparations and in some culture conditions, elevation of tonic concentrations of glutamate by uptake blockers may cause receptor desensitization and presynaptic inhibition that could overwhelm any potentiation caused by decreased uptake. We suggest that the increase in amplitude of EPSCs caused by transporter blockers in culture were masked in slice experiments by a combination of receptor saturation (see below), receptor desensitization, and presynaptic inhibition.

Potentiation of EPSC amplitude but not decay time

Both THA and Li^+ substitution resulted in increases in the amplitude of AMPA receptor EPSCs but had no measurable effect on the decay time of either spontaneous EPSCs or, in the case of THA, evoked EPSCs. This is easily explained if desensitization controlled the decay time course of the EPSC. However, in most preparations including this one, it seems that the decay rate of AMPA receptor EPSCs is too fast to be

determined solely by desensitization because the decay of patch responses to very short applications of glutamate is faster than that of responses to prolonged applications (see Figure 6; Hestrin, 1992; Colquhoun et al., 1992; Livsey and Vicini, 1992; Jonas et al., 1993; but see Trussell and Fischbach, 1989; Trussell et al., 1993). Thus, the EPSC either follows the lifetime of free glutamate in the cleft in which case clearance is much slower than the equilibration time of transmitter with receptors, or, like the neuromuscular junction (Kordas, 1969; Magleby and Stevens, 1972), transmitter is cleared very quickly and the lifetime of bound receptors determines the EPSC decay time. In either case, both processes have to be very fast at least at some synaptic contacts, because the fastest spontaneous EPSCs have half-decay times of about 0.5 ms. Recently, Bartol and Sejnowski (1993) have shown with Monte Carlo modeling of the excitatory synapse, that about 90% of free glutamate (peak concentration of 2 mM) will be cleared from the cleft with a time constant of about 100 μs (see also Eccles and Jaeger, 1958). Using the state diagram and rate constants from Jonas et al. (1993), increasing the time constant of decay of free glutamate in the cleft from 100 to 140 μs will have little effect on the mean decay rate of the sEPSC (about 2 ms; see Table 1), but, if only half of the AMPA receptors at a given synapse are doubly occupied, this increase in clearance time would increase the amplitude of the EPSC by 20-40%.

In our hands, the mean decay time constant of sEPSCs is about double that of AMPA receptor currents in outside-out patches evoked by sub-millisecond pulses of glutamate. This suggests either that the two

populations of receptors are different or that dendritic cable properties prolong the sEPSCs as recorded from the soma. If the population of briefest sEPSCs represent the true time course of the conductance, it would suggest that cable filtering further obscures broadening of spontaneous AMPA receptor EPSCs due to small increases in the clearance time of transmitter.

Temperature dependence of potentiation by transporter blockers

We have seen potentiation of AMPA receptor sEPSCs by transporter blockers only at temperatures significantly above room temperature. Two effects of temperature could account for these observations. First, a 10°C elevation increases transporter rate 2 - 2.5-fold (Schwartz and Tachibana, 1990; M. Kavanaugh, personal communication) and would accelerate glutamate removal. The second effect of raising temperature is on AMPA receptor occupancy. At 34°C, occupancy of AMPA receptors by the transmitter will be lower than at 24°C at which temperature AMPA receptors are close to saturation. Because of the thermodynamics of glutamate binding to AMPA receptors, at 34 °C, fewer receptors will be occupied which will allow increases in the glutamate concentration time course to be translated into larger EPSCs. Thus, while transporter block at room temperature may slow clearance, the effect on synaptic responses may be minimal.

Clearance by transport or by binding

If transporter turnover is slow but clearance of free transmitter from

the cleft is fast, then the actual uptake of glutamate cannot be important in the potentiation of the EPSC and the first effect of temperature, i.e., to increase turnover rate, cannot be important. A faster effect of transporters would be to buffer free glutamate by binding which is very fast (Schwartz and Tachibana, 1990), followed by slower uptake. For binding to be important on the microsecond time scale, many transporters would have to be present in the cleft or at least very close to release sites. There are as yet no reports of transporter density or localization. Rapid buffering of transmitter by binding is at odds with the potentiation by Li^+. Li^+ apparently binds to transporter sites where Na^+ normally binds but does not support uptake (Schwartz and Tachibana, 1990; Barbour et al., 1991; Wyllie et al., 1991). If binding alone adds significantly to the clearance of glutamate, Li^+ must in addition substantially decrease the affinity of glutamate binding. Preliminary evidence suggests that substitution of Li^+ for Na^+ decreases the transporter affinity for glutamate about 10-fold (Wadiche and Kavanaugh, personal communication). Even if transporter turnover is very fast (1000 - 10000 s^{-1}; Attwell et al., 1993; Schwartz and Tachibana, 1990), to be effective on the time scale of 100 µs would still require many transporters to be present in the cleft.

Conclusions

We suggest that , in our experimental conditions, glutamate released into the cleft is removed by two processes with similar rates. First, diffusion across small distances is very rapid. As most excitatory synapses have diameters on the 100 nm scale (Harris and Stevens, 1989),

diffusion alone can be very rapid. Second, ligand binding can also be very fast with rate constants on the order of 10^7 $M^{-1}s^{-1}$ (Hille, 1992). If many copies of glutamate transporter are located close to presynaptic release sites, binding (followed by uptake) could effectively remove free glutamate from the cleft on a 100 μs time scale.

EXPERIMENTAL PROCEDURES

Tissue culture

Experiments using outside-out patches and whole-cell recordings were performed on hippocampal neurons from 1-3 day postnatal rats maintained in cell culture for 1-3 weeks as described previously (Lester et al., 1989). Autaptic recordings were obtained from isolated hippocampal neurons obtained using the same procedures as above but plated on collagen/poly-D-lysine "microdots" as described by Bekkers and Stevens (1991).

Outside-out patch recordings

Outside-out patch recordings (Axopatch 1D, Axon Instruments) were obtained using pipettes containing Na or Cs gluconate, 140 mM; NaCl, 10 mM; HEPES, 10 mM; EGTA, 10 mM; MgATP 4 mM; adjusted to pH 7.4 with NaOH or CsOH. Control external solutions contained 160 mM NaCl; KCl 3 mM; $CaCl_2$ 0.2 - 2 mM; HEPES 5 mM; adjusted to pH 7.4 with NaOH. To isolate AMPA receptor currents, D-AP5 (100 μM) or D-CPP (20 μM) was added to solutions nominally free of glycine. Solution exchanges were made with glass flow tubes attached to piezoelectric bimorphs (Vernitron, Bedford, OH) as previously described (Lester and Jahr, 1992; Lester et al., 1993; Tong and Jahr, 1994). Open tip currents were enhanced by adding 10 mM NaCl to agonist solutions except when 10 mM glutamate was used. Artifacts caused by voltage jumps applied to the piezoelectric bimorph were isolated by turning off the flow, averaging

5 - 20 records, and subtracting these averages from the open tip and receptor-mediated current averages. To change the temperature of the flowing solutions, the shaft and tips of the flow tubes were superfused with external solutions at 34 °C. The temperature of the solutions at the opening of the flow tubes was measured with a small thermistor (Fluke, Everett, WA). The currents were digitally sampled at 1 - 50 kHz, and low pass filtered at 0.5 - 10 kHz.

Whole-cell experiments

Whole-cell recordings of autaptic currents and sEPSCs were made (Axopatch-1D) with low resistance patch pipettes (0.5 - 2.5 MΩ) containing K or Cs gluconate, 140 mM; NaCl, 10 mM; HEPES, 10 mM; EGTA, 10 mM; MgATP 4 mM adjusted to pH 7.4 with KOH or CsOH. Control extracellular solution contained 160 mM NaCl, 3 mM KCl, 5 mM HEPES, 1-3 mM $CaCl_2$, and 50 μM picrotoxin, adjusted to pH 7.4 with NaOH. To isolate AMPA receptor currents, 1 mM magnesium and either D-AP5 (100 μM) or D-CPP (20 μM) were added to solutions nominally free of glycine. To isolate NMDA receptor currents, CNQX (10 μM) or NBQX (5 μM) was added to solutions containing 20 μM glycine. Autaptic EPSCs were evoked with 0.3 - 2 ms voltage jumps to -20 or 0 mV from a holding potential of -60 to -90 mV. The currents were low pass filtered at 0.5 - 10 kHz and digitally sampled at 1 - 50 kHz. Series resistance compensation (90 - 100%) was used in all experiments. For most illustrations, the partially clamped action potential and associated capacitative currents evoked by the stimulating voltage jump were isolated with CNQX and D-

AP5 or D-CPP and subtracted from the averaged EPSC. For sEPSC experiments, 1 - 2 μM tetrodotoxin was added to the extracellular solution to block spontaneous action potentials and recordings were made from neurons on microdots containing 3 - 10 neurons. Solution changes were made with gravity-fed flow tubes as described previously (Lester and Jahr, 1992; Lester et al., 1993). To change the temperature, the flow tubes were enclosed in a temperature-controlled circulating water jacket. The temperature of the external solution that exposed to cells was measured with a small thermistor (Fluke, Everett, WA) before and after each experiment.

Detection of spontaneous excitatory postsynaptic currents

Spontaneous EPSCs were detected by the first derivative of the currents (dI/dt). The first derivative of 2 contiguous digitized intervals had to exceed a threshold of 2 - 3 times the s.d. of the background dI/dt. The sEPSCs thus identified were verified by visual inspection to exclude electrical artefacts. The peak amplitude of the sEPSC was then calculated. Events arising from the summation of individual sEPSCs were excluded from estimates of peak amplitude by requiring a minimal interval of time between the peak of one event and the rise of a succeeding event. To determine whether the amplitude distribution of the two populations differed, the nonparametric Kolmogorov-Smirnov test was used (Van der Kloot, 1991; Manabe et al., 1992).

Analysis

AxoBasic software and the TL-1-125 interface (Axon Instruments) were used for data acquisition and some analysis. In addition, Igor (Wavemetrics, Lake Oswego, OR) and KaleidaGraph (Synergy, Reading, PA) software were used for analysis. Kinetic modeling was performed with SCoP (Simulation Resources, Berrien Springs, MI). Data are expressed as mean ± s.e.m.

In both patch and synaptic experiments, responses in different conditions were interleaved in time to nullify any time-dependent changes. If rundown of responses from a patch were so fast that excluding the first response in the average significantly changed the size or kinetics of the average, the data were discarded.

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A FALSE TRANSMITTER AT EXCITATORY SYNAPSES

Running title: A false transmitter at excitatory synapses

Zhi Zhong Pan[1], Gang Tong[2] and Craig E. Jahr[2]
The Vollum Institute and the Departments of Physiology[1] and Cell Biology and Anatomy[2]
Oregon Health Sciences University L474
3181 SW Sam Jackson Park Road
Portland, OR 97201-3098

Correspondence: Craig E. Jahr
Vollum Institute L474
3181 SW Sam Jackson Park Road
Portland, OR 97201-3098
Phone: 503-494-5471
FAX: 503-494-6972
email: jahr@ohsu.edu

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SUMMARY

We used the low affinity NMDA receptor agonist D-glutamate to study the time course of synaptic activation of NMDA receptors. Repetitive stimulation of cultured hippocampal neurons loaded with D-glutamate caused a dramatic shortening of both the rising and decaying phases of NMDA receptor excitatory postsynaptic currents (epscs) evoked by autaptic stimulation. The epsc time course was mimicked by NMDA receptor currents evoked in outside-out patches by 1-4 millisecond applications of D-glutamate. Thus, D-glutamate can be released as a false transmitter. The results show that both the rise and fall of the NMDA receptor epsc are normally controlled by the slow unbinding rate of the natural neurotransmitter, and that the concentration of free transmitter is elevated in the cleft for only a few milliseconds after release.

INTRODUCTION

Synaptic activation of N-methyl-D-aspartate (NMDA) receptors at central synapses results in a long lasting excitatory postsynaptic response (Dale and Roberts, 1985; Wigstrom et al., 1985). The slow time course of the NMDA receptor epsc (Forsythe and Westbrook, 1988; Hestrin et al., 1990; Lester et al., 1990; Randall et al., 1990) is thought to result from prolonged binding of the transmitter, L-glutamate, to the receptors, causing repetitive reopening of the bound channels (Hestrin et al., 1990; Lester et al., 1990; Gibb and Colquhoun, 1992; Lester and Jahr, 1992). On a shorter time scale, this is true for the prototypic ligand-gated ion channel, the nicotinic receptor; its open burst duration increases with the affinity of the agonist (Hille, 1992). However, as NMDA receptors can remain activated 100 times longer than the nicotinic receptor, it is possible that deactivation of NMDA receptors is controlled by a time-dependent process other than unbinding not unlike inactivation of voltage-gated channels. If this were the case, the decay rate of the response would be independent of the affinity of the agonist. However, NMDA receptor currents activated in outside-out patches by low affinity agonists decay much more quickly than those evoked by high affinity agonists (Lester and Jahr, 1992; Sather et al., 1992). In addition, partial agonists of the glycine-site that decrease the affinity of NMDA receptors for L-glutamate (Kemp and Priestley, 1991) accelerate the decay of NMDA receptor epscs (Lester et al., 1993) further suggesting that transmitter unbinding controls the epsc duration. It has not yet been possible to directly test the kinetic behavior of synaptic NMDA receptors with lower affinity agonists because

patches of membrane have not been excised exclusively from synaptic sites. Replacing L-glutamate in synaptic vesicles with a lower affinity agonist would address this point and, as a result of a faster unbinding rate, would also place limits on the time course of free transmitter in the synaptic cleft.

To be effective, a false transmitter must be concentrated in synaptic vesicles and must activate postsynaptic glutamate receptors. The uptake mechanism in vesicles is very selective for L-glutamate; excitatory amino acids including L-aspartate, D-aspartate and L-cysteate do not interfere with ^{3}[H]-L-glutamate uptake (Naito and Ueda, 1985; Villanueva et al., 1990; Tabb and Ueda, 1991; Fykse et al., 1992). The optical isomer of L-glutamate, D-glutamate, does inhibit vesicular uptake (Naito and Ueda, 1985; Maycox et al., 1988; Tabb and Ueda, 1991; Fykse et al., 1992), suggesting that it may be taken up. D-glutamate also excites CNS neurons (Curtis and Watkins, 1960; Grimwood et al., 1991), induces neuronal excitotoxicity (Garthwaite, 1985; Rosenberg et al., 1992) and binds to rat brain NMDA receptors with an affinity 36 to 74 times lower than L-glutamate (Monaghan and Cotman, 1986; Olverman et al., 1988; Grimwood et al., 1991). Therefore, D-glutamate should act as a false transmitter at glutamatergic synapses.

Unlike the neuromuscular junction where precursors of false transmitters are taken up from the extracellular medium by the plasma membrane choline transporter (Colquhoun et al., 1977), in the central nervous system D-glutamate is only weakly transported by the plasmalemmal glutamate transporter (Balcar and Johnston, 1972; Kanai

and Hediger, 1992; Eliasof and Werblin, 1993). Therefore it must be loaded into presynaptic neurons through the recording pipette. We show that D-glutamate is packaged and released as a false transmitter and can activate NMDA receptor epscs with a time course that is consistent with its low affinity for NMDA receptors.

RESULTS

Intracellular loading with D-glutamate causes faster NMDA receptor epscs

Whole cell recordings were obtained from isolated hippocampal neurons with patch pipettes containing 100 mM D-glutamate. As loading and turnover of releasable stores of transmitter required very long recording times, dual whole cell recordings were impractical. Therefore, we monitored autaptic excitation in single hippocampal neurons grown in isolation on "microdots" in culture (Segal and Furshpan, 1990; Bekkers and Stevens, 1991). Neurons were stimulated at 0.33 to 1 Hz by 1 ms voltage jumps to -20 mV from a holding potential of -60 mV in the presence of CNQX (3-5 μM), picrotoxin (50 μM) and glycine (20 μM). This evoked a poorly voltage-clamped Na^+ current followed in many recordings by autaptic stimulation of an NMDA receptor epsc (Figure 1A, "early"; Segal and Furshpan, 1990; Bekkers and Stevens, 1991). For a variable but brief period after obtaining the whole cell recording, the NMDA receptor epsc had a normal time course with a slow rise to peak

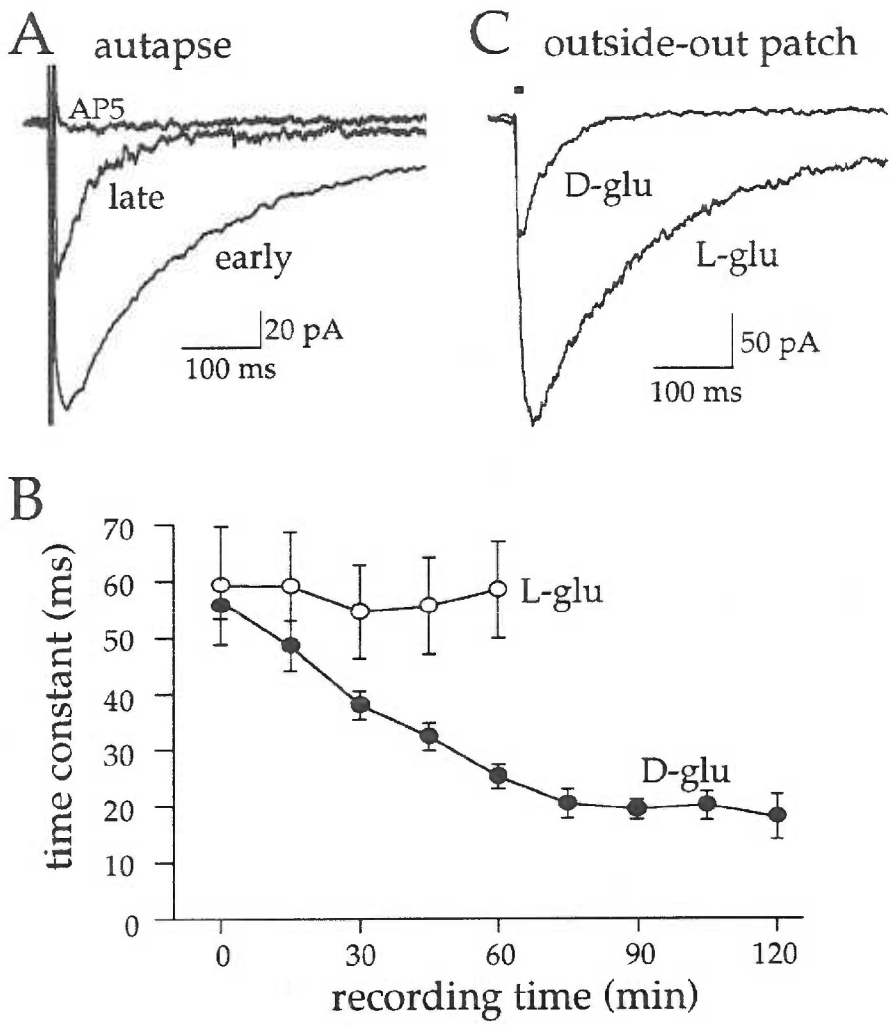

Figure 1. Time course changes of NMDA receptor epsc caused by internal D-glutamate.

(A) Superimposed averages of autaptic epscs recorded in 3 μM CNQX and 20 μM glycine just after obtaining the whole cell recording (early), 1 hour later in the same conditions (late) and in D-AP5 (50 μM) without added glycine (AP5). The internal solution contained 100 mM D-glutamate. Holding potential = -60 mV.
(B) The first time constant of a double exponential fit of NMDA receptor epscs plotted versus recording time. Recordings were made with either 100 mM D-glutamate (filled circles; n = 4 - 13) or 100 mM L-glutamate (open circles; n = 4) in the internal solution.
(C) Superimposed averages of currents evoked in an outside-out patch by 2 ms applications of 10 mM L-glutamate and 10 mM D-glutamate in 3 μM CNQX and 20 μM glycine.

and a biexponential decay with time constants of 55.9 ± 2.3 ms (mean ± S.E.; accounting for 48 ± 4% of the amplitude) and 302 ± 19 ms (n = 11). After a longer period of stimulation, however, the NMDA receptor epsc became smaller and began to decay much more quickly (Figure 1A, "late"). In those cells from which recordings lasted at least one hour, the decay phase of the NMDA receptor epsc was well described by a double exponential with time constants of 22.6 ± 2.0 ms (69 ± 4%) and 185 ± 31 ms (n = 11). This remaining epsc was blocked by the addition of D-AP5 (Figure 1A) consistent with NMDA receptor activation.

The change in the time course of the NMDA receptor epsc occurred gradually and reached steady-state in about 75 minutes (figure 1B; filled circles). In recordings obtained with pipettes in which L-glutamate (100 mM) was substituted for D-glutamate, no change in the decay time of the NMDA receptor epsc was observed (Figure 1B, open circles) suggesting that long recording times were not responsible for the change in the rate of decay.

D-glutamate is a low affinity NMDA receptor agonist

As previously reported (Lester et al., 1990), the time course of the "early" NMDA receptor epsc was similar to currents evoked in outside-out patches by short applications of L-glutamate (Figure 1C). The decay phase of the L-glutamate response was fitted with a double exponential with time constants of 66.6 ± 2.7 ms and 421 ± 34 ms (n = 11). Unlike the "late" epsc, the decay phase of currents evoked in outside-out patches by short applications of D-glutamate were usually well described by a single

exponential with a time constant similar to the fast phase of the "late" epsc (τ = 28.6 ± 1.7 ms; n = 9; Figure 1C). The faster "late" epsc could not be attributed to high cytoplasmic concentrations of D-glutamate because currents evoked in outside-out patches by short applications of L-glutamate had normal time courses when 100 mM D-glutamate was present in the patch pipette (n = 4).

The fast decay time of currents evoked by D-glutamate is expected for an agonist with a low affinity for the NMDA receptor (Lester and Jahr, 1992; Sather et al., 1992). The dose-response relationship of D-glutamate at NMDA receptors was determined in outside-out patches by applying 250 ms applications of 4 concentrations of D-glutamate to each patch (Figure 2AB). The data were fitted with the logistic equation (smooth curve) which yielded an EC_{50} of 75 μM and a Hill coefficient of 1.1. D-glutamate was found to have an EC_{50} of 80 μM in whole cell recordings (Hill coefficient of 1.2; 7 cells) in which Ca-dependent desensitization was greatly diminished by using 20 mM BAPTA internally and 0.2 mM calcium in the drug solution. Therefore, D-glutamate was at least 33-fold less potent than L-glutamate (EC_{50} = 2.3 μM; Patneau and Mayer, 1990). However, at saturating concentrations, D-glutamate was only slightly less efficacious than L-glutamate at NMDA receptors; currents evoked by 10 mM D-glutamate had peak amplitudes 83 ± 1% (n = 5) of those evoked in the same patches by 10 mM L-glutamate (Figure 2C). In contrast, D-glutamate was found to be a very weak agonist of AMPA receptors. In the absence of CNQX and glycine but in the presence of 50 μM D-AP5, 2 - 10 ms applications of 10 mM D-glutamate to outside-out patches activated

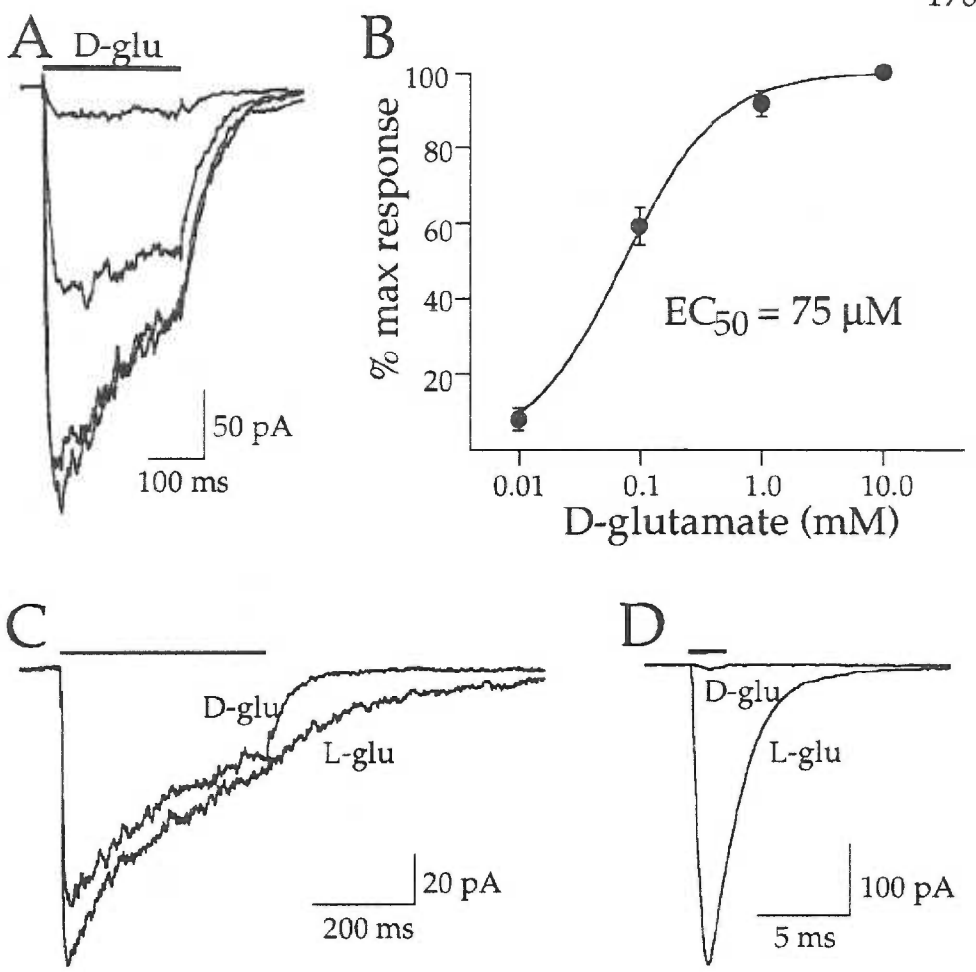

Figure 2. Activation of NMDA and AMPA receptors by D-glutamate.

(A) Averaged responses of an outside-out patch to 250 ms applications of 0.01, 0.1, 1 and 10 mM D-glutamate in 20 μM glycine and 3 μM CNQX.
(B) D-glutamate dose-response curve of peak responses from 4 patches. Currents were normalized to those activated at 10 mM. The smooth curve is the least-squares fit by the logistic equation $I = I_{max}/\{1+(EC_{50}/[dose])^n\}$ where n = 1.1 and EC_{50} = 75 μM.
(C) Averaged currents evoked in an outside-out patch by 400 ms applications of 10 mM D- or L-glutamate in 20 μM glycine and 3 μM CNQX.
(D) Averaged currents activated in an outside-out patch by 2 ms applications of 10 mM D-glutamate or L-glutamate in the absence of glycine and presence of 50 μM D-AP5.

currents that were only 3 ± 3% (n = 8) of those evoked in the same patches by 10 mM L-glutamate (Figure 2D).

Properties of NMDA receptor currents activated by D- and L-glutamate

Two pieces of evidence suggest that even in the longest recordings of autaptic NMDA receptor epscs some L-glutamate was still released. First, the "late" epscs decayed with a double exponential time course rather than the single exponential decay of currents evoked by D-glutamate in patches (Figure 3A) although this could be also attributed to differences in channel kinetics in the two preparations. Second, removal of CNQX from the external solution always revealed an AMPA receptor epsc that was larger than might be expected from the low efficacy of D-glutamate at AMPA receptors in outside-out patches. Figure 3B illustrates the AMPA receptor component of the epsc at a time when the fast decay of the NMDA receptor epsc indicated that the exchange of D- for L-glutamate was substantial.

To understand the interaction of L- and D-glutamate with NMDA receptors, the agonist actions of the two enantiomers were analyzed more carefully. We compared responses of outside-out patches activated by short pulses of high concentrations (1 or 10 mM) of either D- or L-glutamate. Increasing the duration of the application of L-glutamate from 1 to 4 ms had no effect on the 10 - 90% rise time (8.5 ± 0.6 ms and 8.7 ± 0.7 ms, respectively) or the decay time course of the averaged currents (Figure 4A; n = 9). The peak amplitudes of currents evoked by 1 ms pulses were 96 ± 2% of those evoked by 4 ms pulses. These results indicate that even during the shortest applications, L-glutamate saturated (100% occupancy)

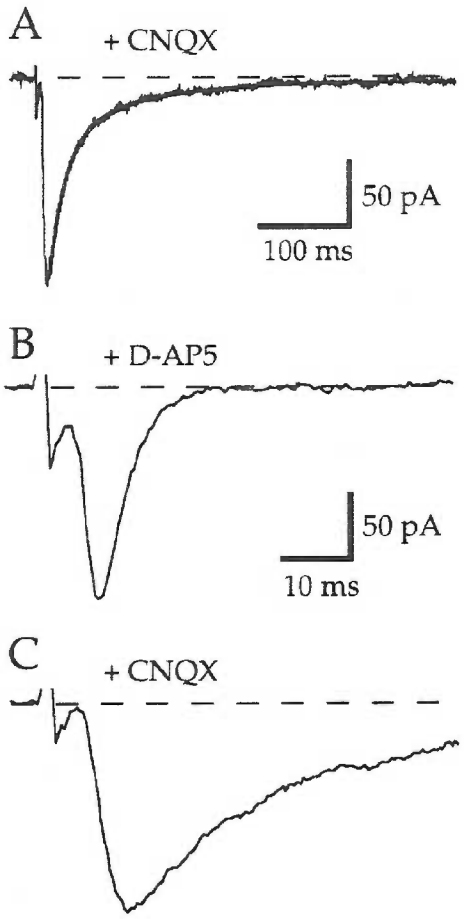

Figure 3. Autaptic currents evoked after 80 minutes of whole cell recording with D-glutamate in the internal solution.

(A) Average of currents recorded in 3 μM CNQX and 20 μM glycine. The decay phase was fitted with a double exponential (bold line). The time constants are 18 ms (78% of amplitude) and 87 ms (22%).
(B) Average of currents recorded in 50 μM D-AP5 and no added glycine.
(C) Same record as in A at the faster time scale of B to show the rising phase. The stimulus artifact and the uncontrolled sodium current recorded in the presence of D-AP5 and CNQX (no glycine added) were subtracted from all records.

the NMDA receptors in the patch and that there was very little unbinding of L-glutamate in the first few milliseconds after binding.

In the same patches, D-glutamate evoked currents that decayed much faster than those evoked by L-glutamate (Figure 4A). The decay phases of these currents were well described by a single exponential ($t = 28.6 \pm 1.7$ ms, $n = 9$) indicating that D-glutamate unbinds much more rapidly than L-glutamate. Longer applications of D-glutamate resulted in currents with larger peak amplitudes and longer rising phases, although their decay times did not change appreciably (Figure 4A,B). The same relative increases in current amplitudes were obtained with either 1 or 10 mM D-glutamate. These results suggest that 1 ms applications of D-glutamate resulted an initial 100% receptor occupancy which did not saturate the response because many receptors became unbound before they opened. Only with prolonged applications of saturating concentrations of D-glutamate (10 mM) did the currents begin to resemble those activated by L-glutamate (see Figure 2C).

The relationship between application duration and rise times of D- and L-glutamate currents is shown in Figure 4C. A comparison of this relationship with the rise time of "late" NMDA receptor epscs (i.e., evoked by D-glutamate) provides an approximation of the time course of free transmitter in the synaptic cleft. A "late" epsc was judged suitable for comparison only if its decay time was fast (first time constant ≤ 26 ms) and subtraction of currents recorded in the presence of both CNQX and D-AP5 resulted in an unobscured rising phase of the NMDA receptor epsc. Because of the prolonged recording times required for substantial

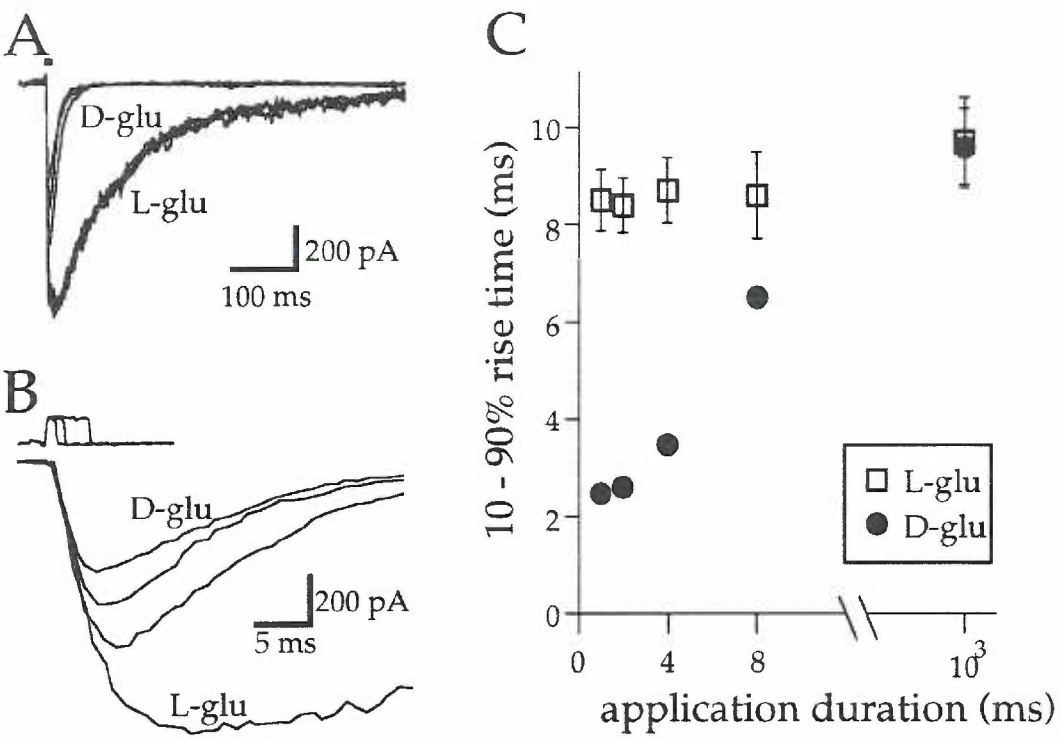

Figure 4. Comparison of currents evoked by D- and L-glutamate in outside-out patches.

(A) Averages of currents evoked by 1, 2 and 4 ms applications of D- or L-glutamate (10 mM) in an outside-out patch.
(B) The same averages as in A of currents evoked by D-glutamate and by the 1 ms application of L-glutamate on a faster time scale. Longer applications of D-glutamate resulted in larger responses, the rising phases of which were superimposable on the L-glutamate response. The upper traces in B were recorded with the same patch pipette as the lower traces after the patch broke down. The deflections reflect the solution exchange times at the tip of the pipette although at the surface of the membrane patch the exchange will be somewhat slower (see Lester and Jahr, 1992).
(C) Comparison of the 10 - 90% rise times of currents evoked by 1, 2, 4 ms and 1 s applications of D-glutamate (filled circles) and L-glutamate (open squares) to outside-out patches. Lack of error bars indicates that the SEM was smaller than the plotting symbol.

replacement of L-glutamate by D-glutamate, only three recordings met these criteria. Their 10 - 90% rise times were 2.1, 3.9 and 4.0 ms as compared to 8.0 ± 0.6 ms for epscs observed in early recordings (n = 9). An example is shown in Figure 3C (4 ms rise time). Assuming that the concentration time course of free transmitter in the synaptic cleft is comparable to the filtered square pulse at the surface of an outside-out patch (Lester and Jahr, 1992), a 2 - 4 ms rise time of the epsc would correspond to a period of ≤ 4 ms of elevated transmitter (Figure 4C). Because the concentration time course of free transmitter in the cleft probably is not a square pulse but more likely rises in a few tens of microseconds and decays with an approximately exponentially time course, this estimate is only an approximation.

Binding rates of D- and L-glutamate to NMDA receptors

If D- and L-glutamate are co-released in the late recordings, they must compete for postsynaptic binding sites. Because free transmitter is present in the cleft in significant concentrations only for a few milliseconds (Clements et al., 1992), steady-state equilibration of the binding of the two agonists will not occur, and thus, the relative binding rates of the two agonists will determine which agonist activates more receptors. If one enantiomer binds ten times faster than the other, its unbinding rate will be the predominant determinant of the epsc decay time course assuming equal concentrations of the two agonists. The binding rate of L-glutamate has been reported to be 5 μM^{-1} s^{-1} from the activation rate of NMDA receptors in outside-out patches using low

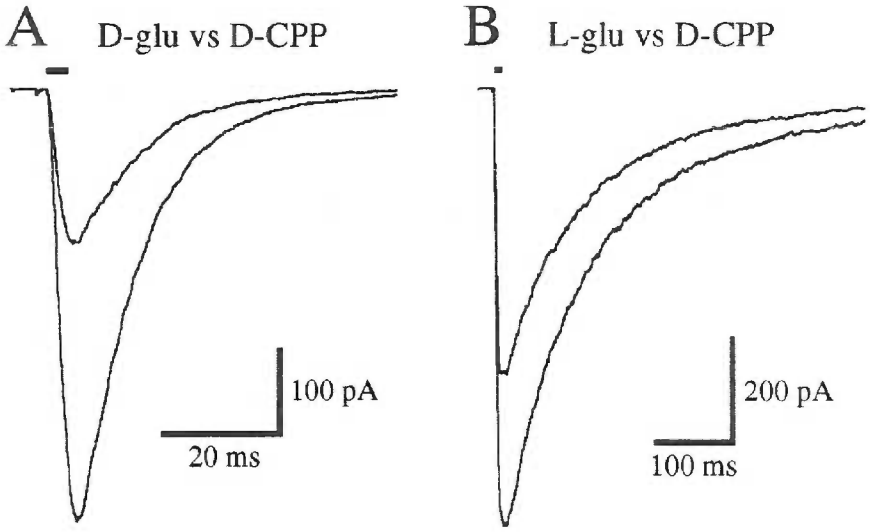

Figure 5. Binding rates of D- and L-glutamate determined by the simultaneous application of either agonist with D-CPP.

(A) Averaged currents evoked in an outside-out patch by 4 ms pulses of 2 mM D-glutamate with or without the simultaneous addition of 2 mM D-CPP.
(B) Same as in A except L-glutamate was used in a different patch. Different time scales in A and B. In both A and B, the smaller currents were those evoked in the presence of D-CPP.

concentrations of the agonist (Clements and Westbrook, 1991). This approach cannot be used with D-glutamate because its equilibrium binding affinity is so low that very little current is activated at the low concentrations necessary to measure activation rates accurately. Therefore, the binding rates of the two glutamate enantiomers were compared using D-carboxypiperazin-propyl-phosphonic acid (D-CPP), a competitive NMDA receptor antagonist. Equal and saturating concentrations (2 mM) of D-glutamate and D-CPP were applied to outside-out patches for brief periods (4 ms). Because two agonist molecules must bind to an NMDA receptor for the channel to open (Benveniste and Mayer, 1991; Clements and Westbrook, 1991), if D-glutamate and D-CPP had equal binding rates, 25% of the channels should become doubly bound by agonist and thus the current produced should be only 25% of that activated by a similar application of D-glutamate alone. In four patches, the mixed application activated currents with amplitudes that were 46 ± 3% of those activated by D-glutamate alone (Figure 5A). When this experiment was performed with L-glutamate in place of D-glutamate, the mixed application resulted in currents with 63 ± 2% of the amplitude of those activated by L-glutamate alone (Figure 5B; n = 6). These experiments indicate that D-glutamate binds more slowly to NMDA receptors than L-glutamate (approximately 70% as fast) although this is probably a slight underestimate because some D-glutamate will unbind during the 4 ms application. These results indicate that if the two agonists are released at equal concentrations, L-glutamate will bind to more NMDA receptor binding sites than D-

glutamate. However, because it is likely that more D-glutamate is released after prolonged recording times than L-glutamate, more NMDA receptor binding sites will be occupied by the D-isomer.

DISCUSSION

Clearance of transmitter from the synaptic cleft

In the present study, we have shown that when transmitter pools are turned over in the presence of high cytoplasmic concentrations of D-glutamate, the D-isomer can apparently become the predominant agonist released upon presynaptic stimulation. As expected for a low affinity agonist of the NMDA receptor, both the decay phase of the epsc and the response of outside-out patches to short applications of agonist are fast. These experiments also confirm the results of previous studies that estimated the time course of clearance of free transmitter in the cleft (Clements et al., 1992) by providing direct evidence that transmitter does not remain at significantly elevated concentrations for more than a few milliseconds. The present results err on the side of over-estimation of the transmitter time course because 1) the rise time of D-glutamate evoked currents is relatively insensitive to application durations shorter than about 3 or 4 ms because the finite dissociation rate of D-glutamate becomes rate limiting; 2) the rise time of "late" epscs would be prolonged if there was significant co-release of L-glutamate; and 3) if reuptake plays a role in the removal of transmitter from the cleft, D-glutamate will be cleared more slowly than the native transmitter L-glutamate because the former is a poor substrate for the known high affinity plasma membrane transporters (Balcar and Johnston, 1972; Kanai and Hediger, 1992; Eliasof and Werblin, 1993). On the other hand, if transporters are present at high density in the cleft, they could rapidly buffer the D-glutamate

concentration merely by binding.

Co-release of D- and L-glutamate

Even in the longest autaptic recordings there apparently was not a complete elimination of L-glutamate from the releasable pool of transmitter. First, there was always a small slow component to the "late" NMDA receptor epsc that was not observed in D-glutamate evoked patch currents. Second, the AMPA receptor component of the epsc was not eliminated despite the very low efficacy of D-glutamate at AMPA receptors. From the rate of decay of the NMDA receptor epsc it seems that more D- than L-glutamate was released after prolonged stimulation. Preliminary kinetic modeling supports this contention -- only when the ratio of D- to L-glutamate approaches 5 does the first time constant of decay decrease to 20 ms and account for about 80% of the response. The residual AMPA receptor epsc would be expected even with an overwhelming release of D-glutamate as it does not effectively compete with L-glutamate for the AMPA receptor. The ratio of D- to L-glutamate of 5 may not be as high as expected for the dramatic change in time course observed. However, if an NMDA receptor is bound by one of each of the enantiomers, its opening behavior will be dominated by the unbinding of D-glutamate since presumably only one ligand has to unbind for the channel to become inactive.

False transmitters can identify rate limiting steps in synaptic transmission

The use of a false transmitter at the neuromuscular junction provided direct evidence that the time course of the endplate current is determined by the affinity of the transmitter for the nicotinic receptor, not by some intrinsic property of the receptor channel complex (Colquhoun et al., 1977). NMDA receptor channels appear to operate in a similar fashion to the nicotinic receptor. Agonists with lower affinity for the receptor activate the channel for shorter periods of time (Lester and Jahr, 1992; Sather et al., 1992). However, even with low affinity agonists such as L-cysteate or D-glutamate, the channel can open and close multiple times before the agonist unbinds, apparently because there are only certain states of the channel from which agonist can unbind. Thus, although the microscopic affinity (unbinding rate/binding rate) of the agonist influences the period during which the channel can open, it is not the only determinant of the bound period. If the channel spends significant periods in states (open and closed) from which agonist cannot unbind, a very fast unbinding rate is not rate limiting. This may be the case at the NMDA receptor where the complex bursts, clusters and superclusters of openings (Howe et al., 1988; Gibb and Colquhoun, 1992) suggest a tortuous path of conformational states the protein can take between agonist binding and unbinding.

EXPERIMENTAL PROCEDURES

Tissue culture

Experiments using outside-out patches were performed on hippocampal neurons from 1-3 day postnatal rats maintained in cell culture for 1-3 weeks as described previously (Lester et al., 1989). Autaptic recordings were obtained from isolated hippocampal neurons obtained using the same procedures as above but plated on collagen/poly-D-lysine "microdots" as described by Bekkers and Stevens (1991).

Outside-out patch recordings

Outside-out patch recordings (Axopatch 1C or 1D, Axon Instruments) were obtained using pipettes containing either Na or Cs gluconate, 140 mM; NaCl, 10 mM; HEPES 10 mM; EGTA 10 mM; MgATP 2-4 mM; adjusted to pH 7.2 with either NaOH or CsOH. Control external solutions contained 160 mM NaCl; KCl 3 mM; $CaCl_2$ 0.2 mM; HEPES 5 mM; adjusted to pH 7.4 with NaOH. To isolate NMDA receptor currents, CNQX was added at 3-4 μM and glycine at 20 μM . To isolate AMPA receptor currents, D-AP5 was added at 50 μM to solutions nominally free of glycine. Solution exchanges were made with flow tubes attached to piezoelectric bimorphs (Vernitron, Bedford, OH) as previously described (Lester and Jahr, 1992; Lester et al., 1993).

Synaptic experiments

Autaptic currents were recorded (PC-501; Warner Instruments,

Hamden, CT or Axopatch 1C) with whole cell pipettes containing either K D-glutamate or L-glutamate, 100 mM; K gluconate, 50 mM; NaCl 10 mM; HEPES 10 mM; EGTA 10 mM; MgATP 3 mM; adjusted to pH 7.2 with KOH. External solutions were the same as in the patch experiments except that 2 - 3 mM $CaCl_2$ was present. Autaptic currents were evoked with 1 - 2 ms voltage jumps to -20 to 0 mV from a holding potential of -60 mV. Solution changes were made with gravity-fed flow tubes as described previously (Lester and Jahr, 1992; Lester et al., 1993).

In most autaptic recordings where D-glutamate was the predominant internal anion, substantial rundown of the synaptic currents (both NMDA and AMPA receptor-mediated) occurred. Several factors may have contributed to this rundown, including: 1) fast unbinding of D-glutamate from NMDA receptors and the very low efficacy of D-glutamate at AMPA receptors; 2) poor plasmalemmal reuptake of D-glutamate so that any re-loading of vesicular stores was from cytoplasmic stores supplied by the pipette (whereas normally, at least some of the released L-glutamate may be recycled by the presynaptic terminal); 3) less efficient vesicular uptake of D-glutamate than L-glutamate (Naito and Ueda, 1985; Maycox et al., 1988; Tabb and Ueda, 1991; Fykse et al., 1992); and 4) an unspecified rundown that occurs even when L-glutamate is present in the recording pipette.

Analysis

AxoBasic software and the TL-1-125 interface (Axon Instruments) were used for data acquisition and some analysis. In addition, Igor

(Wavemetrics, Lake Oswego, OR) and KaleidaGraph (Synergy, Reading, PA) software packages were used for analysis. Kinetic modeling was performed with SCoP (Simulation Resources, Berrien Springs, MI). All data are expressed as mean ± SEM. All experiments were performed at room temperature.

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DISCUSSION

More than one quantum of transmitter can interact with the same population of postsynaptic receptors.

Our results suggest that hippocampal excitatory synapses can release more than one vesicle of transmitter from each synaptic contact after each action potential (Tong and Jahr, 1994). These findings fit reasonably well with recent advances in our understanding of synaptic transmission. Studies have shown that the size of excitatory synapses can vary more than 50 fold (Harris and Stevens, 1989), and the probability of release at synapses can vary about 10 fold (Rosenmund et al., 1993; Hessler et al., 1993). Thus, a larger synapse could have more docking and release sites, leading to a higher probability of release. If vesicle docking sites at an individual release site function independently, multivesicular release could occur at high release probability sites. A simple calculation reveals that the probability of releasing two vesicles from individual release sites could be as high as 0.25 since the probability of release at the high release sites is about 0.5 (Rosenmund et al., 1993; Hessler et al., 1993).

Much of the evidence supporting single vesicular release of transmitter has been based on quantal analysis of transmission at an inhibitory synapse, or an aspiny excitatory synapse (Korn and Faber, 1991; Gulyas et al., 1993). Therefore, whether a synapse is capable of multivesicular release may vary from synapse to synapse and may depend on the size of presynaptic specializations, the number of docked vesicles, the identity of the neurotransmitter and may be correlated with

the morphology of the postsynaptic specializations.

Clearance of transmitter from the synaptic cleft.

Like many other neurotransmitters, clearance of glutamate from the cleft depends on the activity of glutamate transporters and on simple diffusion. Our data suggest that the clearance of free glutamate from the cleft may have an extremely fast phase (on the 100 μs time scale) during which the glutamate concentration in the cleft will drop to a level insufficient to activate AMPA receptors. Early studies (Eccles and Jaeger, 1958) suggested that free diffusion alone would result in a very rapid decline of transmitter concentration at central excitatory synapses (τ = 300 μs). Recently, Bartol and Sejnowski (1993) have shown that the time course of glutamate concentration in the cleft following release has a rapid component decreasing from ~2 mM to 0.5 mM in less than 100 μs and a slower component which kept glutamate concentration at ~0.1 μM for over 1 ms. There are at least two possible mechanisms in which glutamate transporters can have a significant impact on the concentration profile of glutamate in the synaptic cleft. First, if transporter turnover is slow, glutamate transporters may rapidly buffer glutamate merely by binding. If this is the case, within a very short period of time after release, the glutamate concentration in the synaptic cleft will drop to a level insufficient to activate AMPA receptors since the binding of glutamate to transporters is very fast (Schwartz and Tachibana, 1990). The buffering capacity will depend on the density and location of glutamate transporters at synapses. Second, if the turnover is very fast (10, 000 s^{-1};

Schwartz and Tachibana, 1990), glutamate transporters may actually transport glutamate out of the cleft during synaptic transmission. Again, for this scenario to work, many transporters would be required. In both cases, the diffusion of glutamate out of the cleft need to be very rapid since, in our hands, the transporter blockers do not prolong the decay time course of AMPA receptor EPSCs.

Occupancy of the synaptic AMPA receptor.

Depending on the model used, synaptic AMPA receptors may or may not be saturated by synaptically release glutamate at room temperature (Clements et al., 1992; Perkel and Nicoll, 1993; Jonas et al., 1993). Our results support the notion that the AMPA receptors are saturated or nearly saturated by synaptic release at room temperature. However, the AMPA receptors may not be saturated at near physiological temperature. In this case, increasing the number of synapses that release multiple vesicles per action potential will potentiate postsynaptic response.

The unbinding rate of transmitter and intrinsic properties of NMDA receptors defines the time course of the NMDA EPSC.

The use of a false transmitter at the neuromuscular junction provided direct evidence that the time course of the endplate current is determined by the affinity of transmitter for the nicotinic receptors (Colquhoun et al., 1977). Similarly, the time course of the NMDA receptor EPSCs is also determined by the affinity of the transmitter (Lester and

not the AMPA receptors. The potentiation of the AMPA receptor response could be due in part to increasing multiple vesicular release of transmitter, or decreasing transporter activity from potentiated individual synaptic contacts. Further experiments need to be done to test this hypothesis.

CONCLUSION

Neurotransmission at excitatory synapses is a complicated process. Central excitatory synapses have delicate machinery for precise regulation of their functions. More than one vesicle of transmitter can be released from individual synaptic contacts after each action potential, and can interact with the same population of postsynaptic glutamate receptors. The released glutamate is removed from the synaptic cleft by both uptake and simple diffusion. The transmitter is present in the synaptic cleft for at most a few milliseconds. Both synaptic NMDA and AMPA receptors are normally saturated by synaptic release at 24 ^{o}C; however, synaptic AMPA receptors are not saturated at physiological temperature (37 ^{o}C). Furthermore, the slow time course of the NMDA receptor EPSCs is controlled by the slow unbinding rate of the natural transmitter. A better understanding of these basic processes of excitatory synaptic transmission should provide valuable knowledge for studying more complicated neuronal activity of the central nervous system.

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