Determinants of the Time Course of Excitatory Synaptic Events in the CNS

by

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In memory of

Mary Boos and Grandpa Marshall

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Abstract

The NMDA receptor-mediated excitatory postsynaptic current (EPSC) is one of the clearest examples of an excitatory synaptic event whose time course is determined by the kinetics of the receptor channels. The goal of Section I of this dissertation is to better understand those kinetics. In the central nervous system, vesicular release of the neurotransmitter glutamate quickly increases the concentration of glutamate to millimolar levels at the postsynaptic receptors. We investigated the actions of glutamate on the NMDA class of glutamate receptors under similar conditions using rapid applications of a saturating concentration of glutamate to outside-out patches taken from cultured hippocampal neurons. The behavior of NMDA receptors in response to these concentration jumps is different from their behavior in the presence of a low concentration of transmitter at steady state, conditions that have been used in previous studies. We found that under conditions similar to those at the synapse, the receptor channels open faster than originally thought (in the first 10 - 20 ms) and with a relatively high open probability of 0.3 at the peak of the response.

At most central synapses, after the postsynaptic receptors have been activated, the glutamate concentration rapidly falls due to diffusion out of the cleft and uptake by high affinity transporters. In Sections II and III of this dissertation we investigate these processes at the climbing fiber – Purkinje cell synapse in the cerebellum. Whole-cell recordings in the surrounding Bergmann glial cells revealed that the escaping glutamate resulted in AMPA receptor and glutamate transporter currents in the glial cells. Further investigation using outside-out patch techniques and the drug cyclothiazide (CTZ)

indicated that the glutamate reached a peak concentration of 160-190 μM at the glial membranes and decayed over many milliseconds. These results demonstrate that the fast transmitter glutamate quickly rises to significant levels outside the synaptic cleft, potentially acting as a modulator of synaptic transmission.

Introduction

Throughout the nervous system there are efficient and effective processes which determine the time course of synaptic events. This dissertation examines a few of those processes involved in excitatory transmission in the central nervous system. Knowledge of the time course of synaptic events and their determinants is important for a basic understanding of how the brain functions in health and in disease. The processing of information in the brain involves synaptic integration, and the time course of synaptic inputs is a critical factor in their summation. In this introduction I will first review the general mechanisms which are thought to determine the time course of excitatory synaptic events. Then I will discuss some more specific issues that pertain to the three manuscripts to follow.

The mechanisms determining the time course of synaptic events.

A diversity of mechanisms underlying the time course of synaptic events have evolved at the many different types of synapses in the nervous system. At the prototypical ligand-gated synapse, the neuromuscular junction, an enzyme (acetylcholinesterase) quickly breaks down the transmitter acetylcholine (ACh). The response to a single quanta of transmitter is greatly prolonged by inhibitors of acetylcholinesterase (on average the 1.5 ms decay was increased to 10 ms long) (Katz and Miledi, 1973). When the enzyme is inhibited, diffusion is the primary means of reducing the ACh concentration. The clearance of transmitter appears to be slower than free diffusion partly because of rebinding to receptors along the way (Eccles and Jaeger, 1958; Katz and Miledi, 1973). The enzymatic degradation of ACh is so rapid that the

resulting synaptic current decay follows the deactivation kinetics of the receptors. Analysis of the lifetime of the receptor activations indicate they deactivate with a time course similar to that of the synaptic current (around 1 ms) and have a similar voltage and temperature dependence (Magleby and Stevens, 1972; Anderson and Stevens, 1973; Katz and Miledi, 1973). Therefore, at the neuromuscular junction, the events which determine the time course of the synaptic current include the fast enzymatic degradation of transmitter followed by receptor deactivation (Magleby and Stevens, 1972; Anderson and Stevens, 1973; Katz and Miledi, 1973).

The time course of glutamate in the synaptic cleft at central synapses. In the central nervous system, there is no enzyme in the cleft to breakdown the neurotransmitter glutamate. It appears that at many central excitatory synapses simple diffusion is the primary means of reducing the levels of glutamate in the cleft (Eccles and Jaeger, 1958; Hestrin et al., 1990; Clements et al., 1992; Isaacson and Nicoll, 1993; Sarantis et al., 1993; Clements, 1996). The geometry of central synapses is more favorable than the neuromuscular junction for the rapid clearance of transmitter by diffusion alone. The cleft of a central synapse can be approximated as a disk approximately 15 - 20 nm thick and 0.2 - 1 μm in diameter (Eccles and Jaeger, 1958; Clements, 1996; Rusakov and Kullmann, 1998). In contrast to this simple geometry, the cleft at the neuromuscular junction is made up of several adjacent junctional folds 1 μm deep and 2 - 4 μm across resulting in a much longer diffusional path to the extrasynaptic space (Eccles and Jaeger, 1958; Clements, 1996; Rusakov and Kullmann, 1998). In addition to diffusion, high affinity glutamate transporters aid in the removal of transmitter from the cleft at central synapses (Mennerick and Zorumski, 1994; Tong and Jahr, 1994; Otis et al., 1996;

Diamond and Jahr, 1997). The relative importance of uptake depends on the morphology of the synapse and the level of activation of the synapse (Mennerick and Zorumski, 1994; Tong and Jahr, 1994; Otis et al., 1996; Diamond and Jahr, 1997). The peak concentration of glutamate and its time course has been estimated both theoretically and experimentally. Early estimates of the time course based on a mathematical treatment of free diffusion predict a very rapid clearance of glutamate with a time constant of 0.3 ms (Eccles and Jaeger, 1958). The problem with a mathematical approach is the necessity of several simplifying assumptions about the cleft geometry, the absence of diffusional barriers, and the difficulty in including binding phenomena which can slow transmitter diffusion as shown by Katz and Miledi (1973) at the neuromuscular junction. Monte-Carlo computer analysis has enabled investigators to include all of these details in simulations of the time course of glutamate, and have shown that such details do affect the clearance and spread of glutamate (Clements, 1996). However, there is still considerable uncertainty in many of the parameters used to create the models (Clements, 1996).

The first experimental estimate demonstrated that on average the glutamate concentration peaks at 1.1 mM soon after vesicular release and then falls with an exponential time constant of 1.2 ms (Clements et al., 1992). These experiments were based on the non-equilibrium displacement of a low-affinity antagonist, D-aminoadipate (D-AA), from NMDA receptors by synaptically released glutamate in cultured hippocampal neuron pairs. By driving a kinetic model of the receptor channel complex with a glutamate transient of instantaneous rise and exponential decay, the authors were able to reproduce the observed deviation from equilibrium predictions in the amount of

block produced by D-AA. The original experimental data was reanalyzed in light of the findings of modeling studies that suggest clearance is biphasic with a very fast (~100 μs) component (Clements, 1996). Using a double exponential decay the optimal parameters were 2.7 mM and 0.4 mM for peak concentrations and 100 μs and 2.1 ms for the decay time constants of the two components (Clements, 1996).

The low-affinity antagonist technique has recently been used on AMPA receptor-mediated EPSCs using the rapidly dissociating AMPA receptor antagonist kynurenate (KYN) (Diamond and Jahr, 1997). The faster time-scale of the AMPA receptor response provides for a better resolution measurement. The experimental data was most closely reproduced by a double exponential decay with a large fast component (3.2 mM, tau = 100 μs) and a slower second component (0.525 mM, tau = 1.0 ms) (Diamond and Jahr, 1997). In the presence of KYN, block of transporters slowed the rise time of mEPSCs. This indicates that transporters can speed the clearance of glutamate in the first hundred microseconds after release. Based on the relatively slow turnover time for transporters (12 - 70 ms), it is thought that they may shape the decay of the glutamate transient by binding free glutamate rather than by the subsequent transport step (Tong and Jahr, 1994; Wadiche et al., 1995; Diamond and Jahr, 1997, Bergles and Jahr, 1998). Thus, the experimental evidence suggests that diffusion aided by binding to high affinity transporters leads to a very rapid clearance of glutamate from the cleft.

The rise and fall of transmitter in the cleft is not the main determinant of the time course of a synaptic response. The intrinsic kinetics of the receptor channels that transduce the chemical signal back into an electrical signal can alter the kinetics of the synaptic signal. At central excitatory synapses glutamate acts on AMPA receptors and

NMDA receptors which are found co-localized in the postsynaptic membrane (Bekkers and Stevens, 1989; Hestrin et al., 1990; Traynelis et al., 1993). AMPA receptor-mediated EPSCs have rapid kinetics, rising to a peak in less than 1 ms and decaying in just a few milliseconds. In contrast NMDA receptor-mediated EPSCs have a rise time of 10 - 20 ms and can last for hundreds of milliseconds (Bekkers and Stevens, 1989; Hestrin et al., 1990; Traynelis et al., 1993). Both of these receptors respond to the same or similar (Somogyi, 1998) glutamate transients, and so the difference in their kinetics is attributed to the receptors themselves.

The kinetics of the NMDA receptor-mediated EPSC are determined by the length of the interaction of the ligand with the receptor. Continuous re-binding of transmitter does not underlie synaptic channel activity because a competitive NMDA receptor antagonist (±)-2-Amino-5-phosphonopentanoic acid (AP5) can only block the response if applied before the synapse is activated (Lester et al., 1990). A very brief (1ms) pulse of glutamate applied to an outside-out patch results in a current that lasts over a hundred milliseconds, a time course that closely follows that of the EPSC (Lester et al., 1990). When different affinity NMDA receptor agonists are applied to excised patches they give rise to currents that decay with distinct time courses; the higher affinity ligands resulting in longer responses (Lester and Jahr, 1992). This agonist dependence of the decay was demonstrated at the synapse by loading the vesicles with a false transmitter D-glutamate, which has a lower affinity for the NMDA receptor than L-glutamate (Pan et al., 1993). As expected if the EPSC time course was determined by the length of time the ligand remains bound to the receptor, the EPSCs decayed faster with the false transmitter.

The AMPA receptor-mediated EPSC is much faster than the NMDA receptor-mediated EPSC, and thus closer to the estimated time course of transmitter in the cleft. It is therefore more difficult to discern the primary determinant of its time course. Another aspect of intrinsic channel kinetics, desensitization, may play a role in shaping the AMPA receptor-mediated EPSC at some synapses (Trussell and Fischbach, 1989; Trussell et al., 1993; Trussell et al., 1994; Otis et al., 1996). Desensitized states are bound non-conducting states that may curtail the AMPA receptor response (Trussell and Fischbach, 1989; Trussell et al., 1993; Trussell et al., 1994; Otis et al., 1996). There is a large amount of variation in the speed and extent of desensitization of AMPA receptors found in different brain regions and cell types, giving it a more or less important role in shaping the EPSC depending on the particular receptors being studied (Raman et al., 1994).

An additional mechanism contributing to the shape of excitatory EPSCs is release asynchrony (Van der Kloot, 1988; Van der Kloot, 1988; Diamond and Jahr, 1995; Isaacson and Walmsley, 1995). Synaptic transmission in the central nervous system is quantal in nature as at the nmj. This means that the large EPSC recorded upon synaptic activation is the combined effect of numerous release sites. It has been shown that the evoked AMPA receptor-mediated EPSC is much slower (at least at room temperature) than the responses at individual sites and this is in part because there is some dispersion in the timing of individual release events after the stimulus (Diamond and Jahr, 1995; Isaacson and Walmsley, 1995).

There are regional variations in the determinants of the time course of the AMPA receptor-mediated EPSC. The time course of the glutamate concentration has only been explicitly measured in cultured hippocampal neurons (Clements et al., 1992; Clements,

1996; Diamond and Jahr, 1997). However, the determinants of the time course of the AMPA receptor-mediated EPSC have been studied at a diversity of synapses. The relative importance of channel deactivation, desensitization, cleft geometry, diffusion, uptake, and binding events appears to depend on the particular synapse being studied.

The low-affinity antagonist experiments discussed above demonstrate that at cultured hippocampal synapses the diffusion of glutamate out of the cleft approaches the rate calculated for free diffusion (Eccles and Jaeger, 1958; Clements et al., 1992; Clements, 1996; Diamond and Jahr, 1997). Block of glutamate transporters slows the glutamate transient but does not alter the decay time of either spontaneous miniature or evoked AMPA EPSCs (Tong and Jahr, 1994; Diamond and Jahr, 1997). This suggests that, like the NMDA EPSC, the decay of the AMPA receptor-mediated miniature EPSC is determined by deactivation of the receptors. Diamond and Jahr (1995) have shown that the slower AMPA receptor-mediated EPSC time course reflects the asynchrony of the release process. They argued against the pooling of transmitter from adjacent release sites because the EPSC kinetics were the same at low and high release probabilities (Diamond and Jahr, 1995). However, there is evidence in a similar preparation suggestive of a much longer glutamate transient especially under conditions of high release probability (Mennerick and Zorumski, 1994; Mennerick and Zorumski, 1995). Mennerick and Zorumski (1994, 1996) found that the decay time course of AMPA receptor-mediated EPSCs was correlated with the amount of release and that inhibition of glial uptake could prolong the EPSC decay (Mennerick and Zorumski, 1995). These differences may be explained by morphological differences in the preparations. The plating techniques used by Mennerick and Zorumski promote the envelopment of the

neurites by the accompanying glial cells, while Diamond and Jahr used a two step process plating the neurons on top of a layer of glial cells. In addition the Diamond and Jahr cultures were made from CA1 cells which may have promoted terminals with a single active zone similar to those found at Schaffer collateral synapses onto CA1 cells *in situ*. The Mennerick and Zorumski cultures, which included cells from the entire hippocampus, may have had more boutons with multiple closely spaced active zones, like those of mossy fiber synapses onto CA3 cells *in situ,* promoting the overlap of transmitter domains (Mennerick and Zorumski, 1995).

A large number of studies have examined the physiology of the Schaffer collateral-commissural fiber synapse onto the pyramidal CA1 cells in hippocampal slices. The large effect of temperature on the kinetics of the AMPA receptor-mediated EPSC in these cells argues against diffusion limiting its time course (Hestrin et al., 1990). Uptake blockers did not prolong the EPSC decay, suggesting that glutamate transporters do not determine the EPSC time course (Hestrin et al., 1990; Isaacson and Nicoll, 1993; Sarantis et al., 1993). Measurements in outside-out patches indicated that the desensitization kinetics of these receptors were much slower than the deactivation kinetics (Colquhoun et al., 1992). Furthermore, the deactivation kinetics in patches were similar to the decay of the CA1 EPSCs (Colquhoun et al., 1992). Taken together, this evidence suggests that consistent with the concentration time course estimates made in culture (Clements et al., 1992; Clements, 1996; Diamond and Jahr, 1997), the glutamate transient is very rapid and the AMPA receptor-mediated EPSC decay of CA1 cells in Hippocampal slice reflects channel deactivation.

In the chick auditory nucleus magnocellularis there are specialized giant calyceal synapses in which there are multiple release sites in a single cleft that is enveloped by the presynaptic membrane. At these synapses the glutamate transient appears to be slow enough and the receptor kinetics fast enough that the desensitization of the receptors is a critical determinant of the AMPA receptor-mediated EPSC time course (Trussell et al., 1993; Otis et al., 1996). The decay of an outside-out patch response to a step into glutamate closely matched that of the EPSC when fit with a bi-exponential function (Raman and Trussell, 1992, Trussell et al., 1993, Otis et al., 1996). A large prolongation of the EPSC was observed with the drug cyclothiazide (CTZ), which blocks AMPA receptor desensitization (Trussell et al., 1993). The size of this effect was correlated with the amount of release (Trussell et al., 1993). The decay kinetics of the EPSC and desensitization kinetics in patches were slowed to a similar degree by depolarization, and again the effect was correlated with the amount of release (Otis et al., 1996). Interestingly the decay of mEPSCs closely matched that of the deactivation kinetics in patches, so it appears that a single quantum is cleared from the synaptic cleft before the receptors desensitize. However, with the multiquantal release associated with normal transmission at this synapse there is a delayed clearance of transmitter such that desensitization helps shape the decay (Otis et al., 1996). In fact, an even slower component of clearance limited by glutamate uptake was detected which can lead to rebinding of transmitter and a very slow tail on the EPSC (Otis et al., 1996).

In the cerebellum at both the climbing fiber and parallel fiber inputs to the Purkinje cell there appears to be enough diffusional barriers to prevent the rapid clearance of glutamate. There is release from numerous afferent terminals, which may

promote the pooling of transmitter (Barbour et al., 1994; Takahashi et al., 1995). The synaptic clefts are enveloped by the surrounding Bergmann glial cells, especially at the climbing fiber synapse (Palay and Chan-Palay, 1974). The AMPA receptor-mediated EPSC of both the parallel (τ = 7.3 ms) and climbing fiber (τ = 7.3 ms) inputs decay slower than the receptors desensitize (τ = 4.1 ms) and much slower than they deactivate (τ = 0.8 ms) (Barbour et al., 1994). A partial role of desensitization was indicated by a similar voltage dependence of desensitization and the EPSC decay, as well as a prolongation of the responses by aniracetam or diazoxide (compounds that reduce receptor desensitization) (Barbour et al., 1994; Takahashi et al., 1995). Glutamate uptake blockers (PDC and D-aspartate) have been shown to slow the EPSC decay 10-100 %, with a larger effect found with the parallel fiber EPSC (Barbour et al., 1994; Takahashi et al., 1995; Takahashi et al., 1996). In addition, reducing transmitter release with baclofen or adenosine speeded the decay of the response, while postsynaptic reduction of the EPSC with the AMPA receptor antagonist 6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX) did not change the kinetics (Takahashi et al., 1995). This suggests some pooling of transmitter at these synapses due to either multivesicular release or overlapping transmitter domains combined with restricted diffusion away from the receptors. The result is a relatively slow decline of glutamate in the synaptic cleft and a contribution of receptor desensitization to the decay of the EPSC (Barbour et al., 1994; Takahashi et al., 1995; Takahashi et al., 1996).

Overall, it appears that multiple processes shape the AMPA receptor-mediated EPSC in the central nervous system including diffusion, deactivation, desensitization, binding of glutamate to transporters, synaptic geometry, and glial ensheathment. Which

processes predominate depends on the morphology of the synapse, the type of receptors present, and the intensity of release.

The NMDA receptor kinetics controversy

While it is clear that the decay of the NMDA receptor-mediated EPSC is shaped by the length of time the transmitter remains bound to the receptor (see above) there is disagreement about the underlying microscopic kinetics of the response. In particular, the speed of channel opening after glutamate binds to the receptor (the first latency) is controversial. In Section I of this dissertation this issue is addressed using rapid application techniques and the open channel blocker MK-801.

Two competing schemes describing the activation of NMDA receptors evolved out of contrasting methodologies. One approach has been to study openings and closings of channels in on-cell patch recordings using nanomolar concentrations of agonist under steady-state conditions. These conditions make it possible to observe the single channel openings and closings during the lifetime of a single receptor-ligand interaction (Gibb and Colquhoun, 1991; Gibb and Colquhoun, 1992). Separate binding events are distinguished by very long (>1 s) closed periods which are concentration dependent (Gibb and Colquhoun, 1991; Gibb and Colquhoun, 1992). To obtain information about the first latency of the channels it is necessary to average the receptor-ligand interactions (aligned by their first opening) and then deconvolve this waveform from the average response of a patch to a brief high concentration of glutamate (Edmonds and Colquhoun, 1992). The results of this technique indicated that most channels don't open for many tens of milliseconds, and thus the slow decay of the response is due to these delayed

openings (Edmonds and Colquhoun, 1992). A major assumption of this approach is that the steady state channel behavior at low concentrations is identical to that during a jump into a high concentration of glutamate.

The second approach directly measures the NMDA channel first latency in excised patches under the non-equilibrium conditions of a jump into a saturating concentration of glutamate, similar to synaptic activation of the receptors. In the presence of MK-801, the channels get blocked irreversibly soon after opening for the first time, giving rise to a current which decays with the time course of the first latency distribution (Jahr, 1992; Jahr, 1994). In the first manuscript to follow, we test this interpretation of the current in the presence of MK-801. The distribution decays relatively quickly (τ = 13 ms) indicating that most of the channels open for the first time early in the response (Jahr, 1992; Jahr, 1994). We compare the amount of block produced during a brief application of glutamate in a background of MK-801 to the amount of block when MK-801 is only allowed to block the early part of the response. The similar amount of block found indicates the channels that open do so for the first time early in the response.

Monitoring glutamate escaping from the cleft using Bergmann glial cells

As discussed, the clearance of glutamate from the synaptic cleft in the central nervous system involves both diffusion and high affinity transport. The relative importance of these two processes depends on the cleft geometry, the location and density of receptors and transporters, the tortuosity of the extrasynaptic space, and the amount of glial ensheathment, among other factors. In Sections II and III of this

dissertation these processes are investigated at the climbing fiber – Purkinje cell synapse. The Bergmann glial cells provide an ideal sensor of the glutamate that escapes the synaptic cleft, because of the intimate relation of the Bergmann glial cell with these synaptic contacts. They are also large enough to make whole cell and outside-out recordings using the patch clamp technique. In addition, they have both AMPA receptors and glutamate transporters on their surface membrane.

Early in development Bergmann fibers serve as guide wires for migrating granule cells, as other radial glia do throughout the brain (Palay and Chan-Palay, 1974; Muller and Kettenmann, 1995). Later in development Bergmann glia grow into complex lamellar structures which completely envelop Purkinje cells (Palay and Chan-Palay, 1974; Muller and Kettenmann, 1995). Many of the functions of Bergmann glia in the adult brain remain unclear. Originally ascribed solely to the isolation of individual synaptic contacts, they are beginning to be viewed as an integral part of the signaling circuitry of the cerebellum (Ortega et al., 1991; Muller and Kettenmann, 1995). Calcium-permeable AMPA receptors are primarily responsible for the potential signaling role of Bergmann glial cells (Burnashev et al., 1992; Muller et al., 1992). Supporting this possibility, AMPA receptor-mediated and glutamate transporter currents recorded in Bergmann glia are activated by synaptically released glutamate which escapes into the extrasynaptic space (Bergles et al., 1997; Clark and Barbour, 1997; Linden, 1997). In Sections II and III of this dissertation we capitalized on these phenomena, and used the Bergmann glial cell to study the time course and concentration of the glutamate diffusing from the cleft.

References

Anderson, CR, and Stevens, CF (1973) Voltage clamp analysis of acetylcholine produced end-plate current fluctuations at frog neuromuscular junction. J Physiol (Lond) 235: 655-691.

Barbour, B, Keller, BU, Llano, I, and Marty, A (1994) Prolonged presence of glutamate during excitatory synaptic transmission to cerebellar Purkinje cells. Neuron 12: 1331-1343.

Bekkers, JM, and Stevens, CF (1989) NMDA and non-NMDA receptors are co-localized at individual excitatory synapses in cultured rat hippocampus. Nature 341: 230-233.

Bergles, DE, Dzubay, JA, and Jahr, CE (1997) Glutamate transporter currents in Bergmann glial cells follow the time course of extrasynaptic glutamate. Proc Natl Acad Sci U S A 94: 14821-14825.

Bergles, DE, Jahr, CE (1998) Glial Contribution to Glutamate Uptake at Schaffer Collateral-Commissural Synapses in the Hippocampus. J Neuroscience 18: 7709-7716.

Burnashev, N, Khodorova, A, Jonas, P, Helm, PJ, Wisden, W, Monyer, H, Seeburg, PH, and Sakmann, B (1992) Calcium-permeable AMPA-kainate receptors in fusiform cerebellar glial cells. Science 256: 1566-1570.

Clark, BA, and Barbour, B (1997) Currents evoked in Bergmann glial cells by parallel fibre stimulation in rat cerebellar slices. J Physiol (Lond) 502: 335-350.

Clements, JD (1996) Transmitter timecourse in the synaptic cleft: its role in central synaptic function. Trends Neurosci. 19: 163-171.

Clements, JD, Lester, RA, Tong, G, Jahr, CE, and Westbrook, GL (1992) The time course of glutamate in the synaptic cleft. Science 258: 1498-1501.

Colquhoun, D, Jonas, P, and Sakmann, B (1992) Action of brief pulses of glutamate on AMPA/kainate receptors in patches from different neurones of rat hippocampal slices. J Physiol (Lond) 458: 261-287.

Diamond, JS, and Jahr, CE (1995) Asynchronous release of synaptic vesicles determines the time course of the AMPA receptor-mediated EPSC. Neuron 15: 1097-1107.

Diamond, JS, and Jahr, CE (1997) Transporters Buffer Synaptically Released Glutamate on a Submillisecond Time Scale. J Neuroscience 17: 4672-4687.

Eccles, JC, and Jaeger, JC (1958) The relationship between the mode of operation and the dimensions of the junctional regions at synapses and motor end-organs. Proc. Roy. Soc. B 148: 38-56.

Edmonds, B, and Colquhoun, D (1992) Rapid decay of averaged single-channel NMDA receptor activations recorded at low agonist concentration. Proc R Soc Lond B Biol Sci 250: 279-286.

Gibb, AJ, and Colquhoun, D (1992) Activation of *N*-methyl-D-aspartate receptors by l-glutamate in cells dissociated from adult rat hippocampus. J Physiol (Lond) 456: 143-179.

Gibb, AJ, and Colquhoun, D (1991) Glutamate activation of a single NMDA receptor-channel produces a cluster of channel openings. Proc. R. Soc. Lond. B. 243: 39-45.

Hestrin, S (1992) Activation and desensitization of glutamate-activated channels mediating fast excitatory synaptic currents in the visual cortex. Neuron 9: 991-999.

Hestrin, S, Nicoll, RA, Perkel, DJ, and Sah, P (1990) Analysis of excitatory synaptic action in pyramidal cells using whole- cell recording from rat hippocampal slices. J Physiol (Lond) 422: 203-225.

Hestrin, S, Sah, P, and Nicoll, RA (1990) Mechanisms generating the time course of dual component excitatory synaptic currents recorded in hippocampal slices. Neuron 5: 247-253.

Isaacson, JS, and Nicoll, RA (1993) The uptake inhibitor L-trans-PDC enhances responses to glutamate but fails to alter the kinetics of excitatory synaptic currents in the hippocampus. J Neurophysiol 70: 2187-2191.

Isaacson, JS, and Walmsley, B (1995) Counting quanta: direct measurements of transmitter release at a central synapse. Neuron 15: 875-884.

Jahr, CE (1992) High probability opening of NMDA receptor channels by L-glutamate. Science 255: 470-472.

Jahr, CE (1994) NMDA receptor kinetics and synaptic function. Semin Neurosci 6: 81-86.

Katz, B, and Miledi, R (1973) The binding of acetylcholine to receptors and its removal from the synaptic cleft. J Physiol (Lond) 231: 549-574.

Lester, RA, Clements, JD, Westbrook, GL, and Jahr, CE (1990) Channel kinetics determine the time course of NMDA receptor-mediated synaptic currents. Nature 346: 565-567.

Lester, RA, and Jahr, CE (1992) NMDA channel behavior depends on agonist affinity. J Neurosci 12: 635-643.

Linden, DJ (1997) Long-term potentiation of glial synaptic currents in cerebellar culture. Neuron 18: 983-994.

Magleby, KL, and Stevens, CF (1972) The effect of voltage on the time course of end-plate currents. J Physiol (Lond) 223: 151-171.

Mennerick, S, and Zorumski, CF (1994) Glial contributions to excitatory neurotransmission in cultured hippocampal cells. Nature 368: 59-62.

Mennerick, S, and Zorumski, CF (1995) Presynaptic influence on the time course of fast excitatory synaptic currents in cultured hippocampal cells. J. Neurosci. 15: 3178-3192.

Muller, T, and Kettenmann, H (1995) Physiology of Bergmann glial cells. Int Rev Neurobiol 38: 341-359.

Muller, T, Moller, T, Berger, T, Schnitzer, J, and Kettenmann, H (1992) Calcium entry through kainate receptors and resulting potassium-channel blockade in Bergmann glial cells [published erratum appears in Science 1992 Aug 28;257(5074):1190]. Science 256: 1563-1566.

Ortega, A, Eshhar, N, and Teichberg, VI (1991) Properties of Kainate Receptor/Channels on Cultured Bergmann Glia. Neuroscience 41: 335-349.

Otis, TS, Wu, Y, and Trussel, LO (1996) Delayed Clearance of Transmitter and the Role of Glutamate Transporters at Synapses with Multiple Release Sites. J Neuroscience 16: 1634-1644.

Otis, TS, Wu, YC, and Trussell, LO (1996) Delayed clearance of transmitter and the role of glutamate transporters at synapses with multiple release sites. J Neurosci 16: 1634-1644.

Palay, SL, and Chan-Palay, V. (1974). The Neuroglial Cells of the Cerebellar Cortex and The Climbing Fiber. In Cerebellar Cortex Cytology and Organization, S. L. Palay and V. Chan-Plalay, eds. (New York, Heidelberg, Berlin: Springer-Verlag), pp. 288-321.

Pan, ZZ, Tong, G, and Jahr, CE (1993) A false transmitter at excitatory synapses. Neuron 11: 85-91.

Raman, IM, and Trussell, LO (1992) The kinetics of the response to glutamate and kainate in neurons of the avian cochlear nucleus. Neuron 9: 173-186.

Raman, IM, Zhang, S, and Trussell, LO (1994) Pathway-specific variants of AMPA receptors and their contribution to neuronal signaling. J Neurosci 14: 4998-5010.

Rusakov, DA, Kullmann, DM (1998) Extrasynaptic Glutamate Diffusion in the Hippocampus: Ultrastructural Constraints, Uptake, and Receptor Activation. J Neuroscience 18: 3158-3170.

Sarantis, M, Ballerini, L, Miller, B, Silver, RA, Edwards, M, and Attwell, D (1993) Glutamate uptake from the synaptic cleft does not shape the decay of the non-NMDA component of the synaptic current. Neuron 11: 541-549.

Somogyi, P (1998). Precision and variability in the placement of pre- and postsynaptic receptors in relation to transmitter release sites. In Central Synapses: Quantal Mechanisms and Plasticity, D. S. Faber, Korn, H., Redman, S.J, Thompson, S.M., Altman, J.S., ed. (Strasbourg: Human Frontier Science Program), pp. 82-95.

Takahashi, M, Kovalchuk, Y, and Attwell, D (1995) Pre- and Postsynaptic Determinants of EPSC Waveform at Cerebellar Climbing Fiber and Parallel Fiber to Purkinje Cell Synapses. J Neuroscience 15: 5693-5702.

Takahashi, M, Sarantis, M, and Attwell, D (1996) Postsynaptic glutamate uptake in rat cerebellar Purkinje cells. J Physiol. 497: 523-530.

Tong, G, and Jahr, CE (1994) Block of glutamate transporters potentiates postsynaptic excitation. Neuron 13: 1195-1203.

Traynelis, SF, Silver, RA, and Cull-Candy, SG (1993) Estimated conductance of glutamate receptor channels activated during EPSCs at the cerebellar mossy fiber-granule cell synapse. Neuron 11: 279-289.

Trussell, LO, and Fischbach, GD (1989) Glutamate receptor desensitization and its role in synaptic transmission. Neuron 3: 209-218.

Trussell, LO, Raman, IM, and Zhang, S (1994) AMPA receptors and rapid synaptic transmission. Sem. Neurosci. 6: 71-79.

Trussell, LO, Zhang, S, and Raman, IM (1993) Desensitization of AMPA receptors upon multiquantal neurotransmitter release. Neuron 10: 1185-1196.

Van der Kloot, W (1988) Estimating the timing of quantal releases during end-plate currents at the frog neuromuscular junction. J Physiol (Lond) 402: 595-603.

Van der Kloot, W (1988) The kinetics of quantal releases during end-plate currents at the frog neuromuscular junction. J Physiol (Lond) 402: 605-626.

Wadiche, JI, Arriza, JL, Amara, SG, and Kavanaugh, MP (1995) Kinetics of a human glutamate transporter. Neuron 14: 1019-1027.

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Kinetics of NMDA channel opening

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Abstract

The period required for *N*-methyl-D-aspartate (NMDA) channels to open for the first time after agonist binding, the first latency, was estimated in outside-out patch recordings from rat hippocampal neurons using fast application techniques and the open channel blocker MK-801. In the presence of MK-801, brief applications of L-glutamate or the low affinity agonist L-cysteate resulted in a similar amount of block despite the much shorter period of channel activation by L-cysteate. A brief co-application of L-glutamate and MK-801 resulted in a block similar to that found with an application of L-glutamate in a background of MK-801. These results, along with our findings that MK-801 does not block desensitized receptors, indicate that NMDA channels have a mean first latency of about 10 ms, consistent with a peak open probability near 0.3. If NMDA channels at synapses behave similarly, relatively few channels would be required to produce the postsynaptic calcium transient associated with synaptic plasticity and developmental regulation.

[Key words: ion channels, NMDA , kinetics, open probability, first latency, EPSC time course]

In the vertebrate central nervous system, synaptic release of the excitatory neurotransmitter L-glutamate results in activation of NMDA receptor channels in the postsynaptic membrane that can last for several hundred milliseconds (Hestrin et al., 1990; Lester et al., 1990) . The NMDA receptor's high affinity for L-glutamate results in this prolonged channel activity because of the slow unbinding of agonist (Lester et al., 1990; Patneau and Mayer, 1990; Clements and Westbrook, 1991; Gibb and Colquhoun, 1991; Gibb and Colquhoun, 1992; Lester and Jahr, 1992) . Two schemes describing the single channel events which underlie a macroscopic response to a brief pulse of L-glutamate have developed in parallel.

In the first scheme NMDA channels open, on average, about 10 ms after agonist binding. The scheme is based on experiments using MK-801 (Jahr, 1992) which blocks NMDA channels very rapidly after they open but unblocks very slowly at negative holding potentials, and then only when agonist is bound (Huettner and Bean, 1988). In the presence of 20 μM MK-801, a brief application of a saturating concentration of L-glutamate results in channel activity reflecting the first openings of individual channels. This is because any channel that opens for more than about 2 ms will be blocked essentially irreversibly by 20 μM MK-801. Therefore, in a patch containing many NMDA channels, the macroscopic current recorded in MK-801 approximates a first latency distribution (Jahr, 1992).

This distribution can be fitted with a single exponential with a time constant of about 13 ms, indicating that most channels open for the first time soon after agonist binding. If this first latency distribution is deconvolved from a response in the absence of MK-801, the resulting distribution represents the conditional probability that a channel is open at time t given that it was open at $t = 0$. This distribution decays slowly, lasting several hundred milliseconds. Taken together, these distributions describe channel behavior in which openings occur with moderately high probability soon after agonist binding and repeatedly open and close until dissociation of agonist hundreds of milliseconds later (Jahr, 1994). It was also estimated that at the peak of the patch response to L-glutamate about 30% of the channels in the patch were simultaneously open. This agrees well with other patch measurements (Lin and Stevens, 1994; Benveniste and Mayer, 1995; Rosenmund et al., 1995; Wyllie et al., 1997) and some whole cell (Benveniste and Mayer, 1995) and synaptic measurements (Hessler et al., 1993).

In the second scheme most NMDA channels open after a considerable delay. The distribution describing the conditional probability that a channel is open at time t given it was open at $t = 0$ was constructed for this scheme by aligning groups of channel

openings, called "super-clusters" (Gibb and Colquhoun, 1991; Gibb and Colquhoun, 1992) , recorded at low agonist concentrations (Edmonds and Colquhoun, 1992) . In contrast to the first scheme, this conditional open probability distribution decays quickly, from 1 to 0.26 in 10 ms, indicating that after the initial burst or cluster of openings, the likelihood of subsequent openings is very low (Edmonds and Colquhoun, 1992) . Deconvolving the conditional open probability distribution from the response of a patch to a brief pulse of L-glutamate gives rise to a first latency distribution which predicts that the majority of the channels open for the first time over 100 milliseconds after binding agonist and that the channels have a very low P_o. The low open probability is consistent with estimates using steady state agonist applications (Huettner and Bean, 1988; Traynelis and Cull-Candy, 1990) and some synaptic measurements (Rosenmund et al., 1993; Rosenmund et al., 1995) .

The present experiments were undertaken to determine which of these schemes best describes NMDA channel behavior in outside-out patches.

Materials and Methods

Cell culture. Experiments were conducted on outside-out patches of rat hippocampal neurons grown in primary culture. Cells were taken from P1-3 rats and maintained in culture for 1-3 weeks (Lester et al., 1989) .

Solutions. Recording pipettes were filled with a solution containing 140 mM cesium gluconate, 10 mM NaCl, 10 mM HEPES, 10 mM EGTA, and 5 mM MgATP, adjusted to pH 7.2 with CsOH and kept on ice until use. External solutions contained 160 mM NaCl, 3 mM KCl, 10 mM HEPES, 0.2 mM $CaCl_2$, 5 μM NBQX, and 20 μM glycine, adjusted to pH 7.4 with NaOH. High purity salts were used in the external solution to minimize contaminating divalents. Internal and external solution osmolalities were 305-315 mosms. Sources of chemicals: L-glutamate, L-cysteate, HEPES, EGTA,

Mg-ATP, and gluconic acid, SIGMA (St. Louis, MO); NaCl, Mallinckrodt (Paris, Kentucky); high purity (Gold Label) NaCl, KCl and cesium hydroxide, Aldrich Chemical (Milwaukee, WI); $CaCl_2$, Johnson Matthey (Ward Hill, MA); glycine, Bio-Rad Laboratories (Hercules, CA); MK-801, RBI (Natick, MA). NBQX was a gift from Novo Nordisk (Denmark).

Recording and perfusion techniques. Outside-out patch recordings were made with borosilicate glass pipettes (WPI, Sarasota, FL) pulled to a "bubble number" of 7.4 - 7.8 and occasionally lightly polished to final tip resistance of 1-4 MOhms. Currents were sampled at 2 kHz and low-pass filtered at 1 kHz using an Axopatch 200A, AxoBasic software and a TL-1 DMA interface (Axon Instruments, Foster City, CA). Solution exchanges were made with flow tubes attached to piezoelectric bimorphs (Vernitron, Bedford, OH), as described previously (Lester and Jahr, 1992; Tong and Jahr, 1994) . Open tip measurements were made at the end of each experiment to test the speed and consistency of the solution exchange. Data from patches with questionable exchange were not analyzed. The open tip solution exchanges had a 10-90% rise time of < 500 μs, the sampling interval. Patches were held at 0 mV between agonist applications and at -60 mV during responses to agonists. Trials were separated by 15-20 s to allow recovery from desensitization. Statistical analysis was performed using InStat (Graph Pad Software, San Diego, CA). Reported values are given as mean ± standard deviation. All experiments were performed at room temperature.

Results

Block by MK-801 is independent of agonist affinity

NMDA channels activated by short pulses of high affinity agonists remain active for longer periods than when bound by low affinity agonists (Lester and Jahr, 1992) because ligand-gated ion channels can generally open only while agonist is bound (Hille, 1992) . Responses activated by brief applications of the low affinity agonist L-cysteate

decay much faster (τ_1 = 31 ms, 95%; τ_2 = 164 ms) than responses to L-glutamate (τ_1 = 68 ms, 80%; τ_2 = 553 ms, from Lester and Jahr, 1992). If NMDA channels open only after prolonged bound times (the long first latency scheme), then far fewer channels would be blocked by MK-801 during an L-cysteate response than during an L-glutamate response because L-cysteate would unbind before most channels could open for the first time. However, if NMDA channels open soon after agonist binding (the short first latency scheme), receptor activation by L-cysteate and L-glutamate should result in a similar degree of block. This prediction requires the behavior of the channels to be comparable while either agonist is bound, as evinced by the similarity of responses to long applications of the two agonists (Lester and Jahr, 1992) .

It was necessary to determine the minimum application duration of saturating L-cysteate required to give a maximal response because the unbinding rate of L-cysteate is much faster than that of L-glutamate. A response with maximal amplitude was achieved with a 15 ms pulse of 10 mM L-cysteate (Figure 1). The charge transfer during the first 20 ms of the response was 89 ± 10% of that produced by a 5 ms pulse of 10 mM L-glutamate, while the total charge transfer with L-cysteate was only 23 ± 9% of that with L-glutamate (n=6). L-cysteate appears to be slightly less efficacious than L-glutamate at NMDA receptors since 10 mM L-cysteate is a saturating concentration (Patneau and Mayer, 1990) .

Control responses were elicited with a 5 ms pulse of 10 mM L-glutamate before and then after a single trial consisting of a 15 ms pulse of 10 mM L-cysteate applied after equilibrating the patch in 20 μM MK-801 (Figure 2). L-glutamate was used for the control responses to allow comparison with the block using L-glutamate (Jahr, 1992) . Charge transfer (Q) was measured by integrating the averages of five of the control responses before and after the MK-801 trial, and the percent block was calculated as $(Q_{before}-Q_{after})/Q_{before}$. Consistent with the short first latency scheme, the block

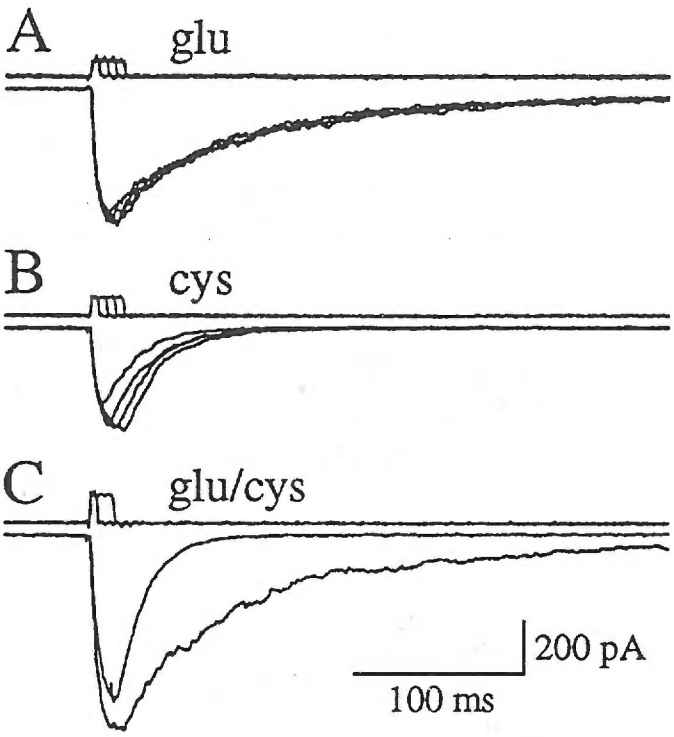

Figure 1. ***Decay time of NMDA channel responses depends on the agonist.***

(**A**) Superimposed responses of a patch to 5, 10, 15, and 20 ms applications of 10 mM L-glutamate (glu). Each trace is an average of five sweeps; the different length applications were made successively and then repeated in a cyclic manner. (**B**) Superimposed responses to 5, 10, 15, and 20 ms applications of 10 mM L-cysteate (cys) using the same protocol as in (A). (**C**) The average NMDA receptor response to a 15 ms application of 10 mM L-cysteate superimposed on the average response to a 5 ms application of 10 mM L-glutamate in the same patch. Parts (A), (B), and (C) are from different patches. Open tip traces of the various length applications are superimposed above the patch responses. V_h = -60 mV.

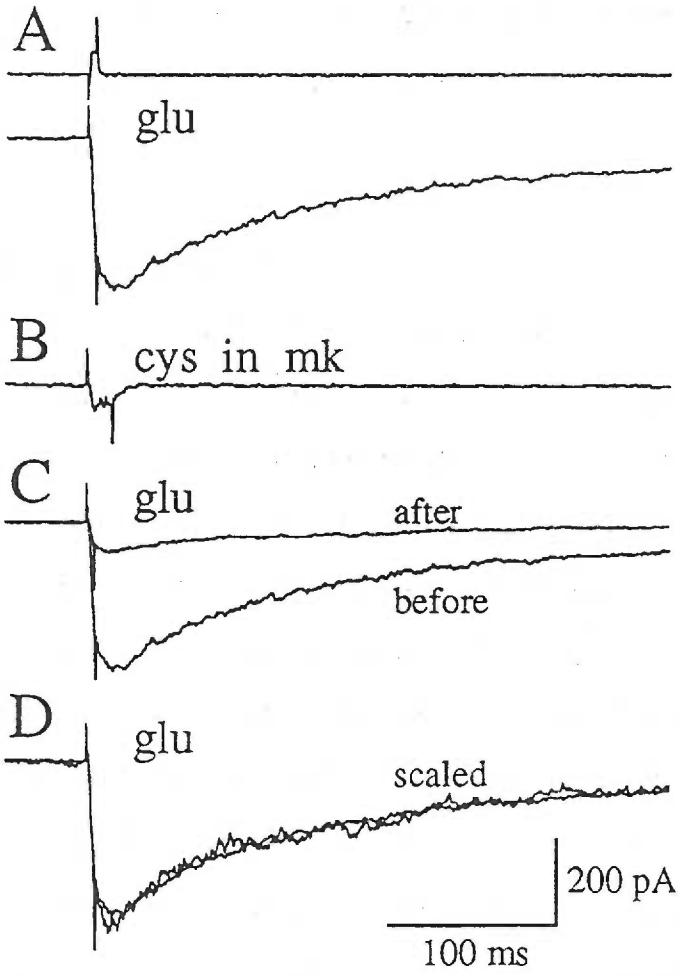

Figure 2. ***The magnitude of block by MK-801 is comparable for L-cysteate and L-glutamate responses.***

(**A**) The average NMDA receptor response of an outside-out patch to a 5 ms pulse of 10 mM L-glutamate (glu) prior to MK-801 exposure. Above the response is the open tip trace measured at the end of the experiment. (**B**) A 15 ms co-application of 10 mM L-cysteate in a background of 20 μM MK-801: the blocking trial. (**C**) Averaged responses to 5 ms pulses of 10 mM L-glutamate before and after the MK-801 exposure, superimposed. (**D**) Responses in (C) scaled to their peaks. All traces are from the same patch.

observed using L-cysteate (79 ± 11%, n=7) was not significantly different ($p>0.1$, Student's unpaired two tailed t-test) to that using L-glutamate (70 ± 10%, n=8; from Jahr, 1992).

MK-801 does not block desensitized channels

A concern with the previous experiments, in which MK-801 was continuously present, is the possibility that MK-801 may block receptors that are bound by ligand but in a nonconducting (e.g., desensitized) state, thereby resulting in an overestimate of the number of channels that open before agonist unbinding. To address this concern, a 1 s application of L-glutamate (10 mM) was used to desensitize a population of receptors. An application of this length results in an NMDA response that decays markedly while agonist is present (Figure 3A, top trace). During this long application of L-glutamate, 20 μM MK-801 was co-applied for 20 ms either 905 ms into the application (Figure 3A) or 5 ms into the application (Figure 3B). Five control responses to L-glutamate before and after the MK-801 trial were averaged and integrated to calculate the reduction in charge transfer caused by the exposure to MK-801 at the two times. The amount of block ($(Q_{before}-Q_{after})/Q_{before}$) was significantly less when MK-801 was applied at 905 ms (19 ± 16% block) than at 5 ms (51 ± 17% block; $p=0.009$, Student's two tailed paired t-test , n=6). The amount of block was correlated with the charge transfer at the time of the block, as would be expected if MK-801 could only block open receptors. The ratio of the block at 905 ms to the block at 5 ms (38 ± 32%) was not significantly different from the ratio of the charge transfer from 905-925 ms to the charge transfer from 5-25 ms (26 ± 14%; $p=0.27$, Student's two tailed paired t-test, n=6). These results indicate that desensitized receptors are unavailable for block by MK-801.

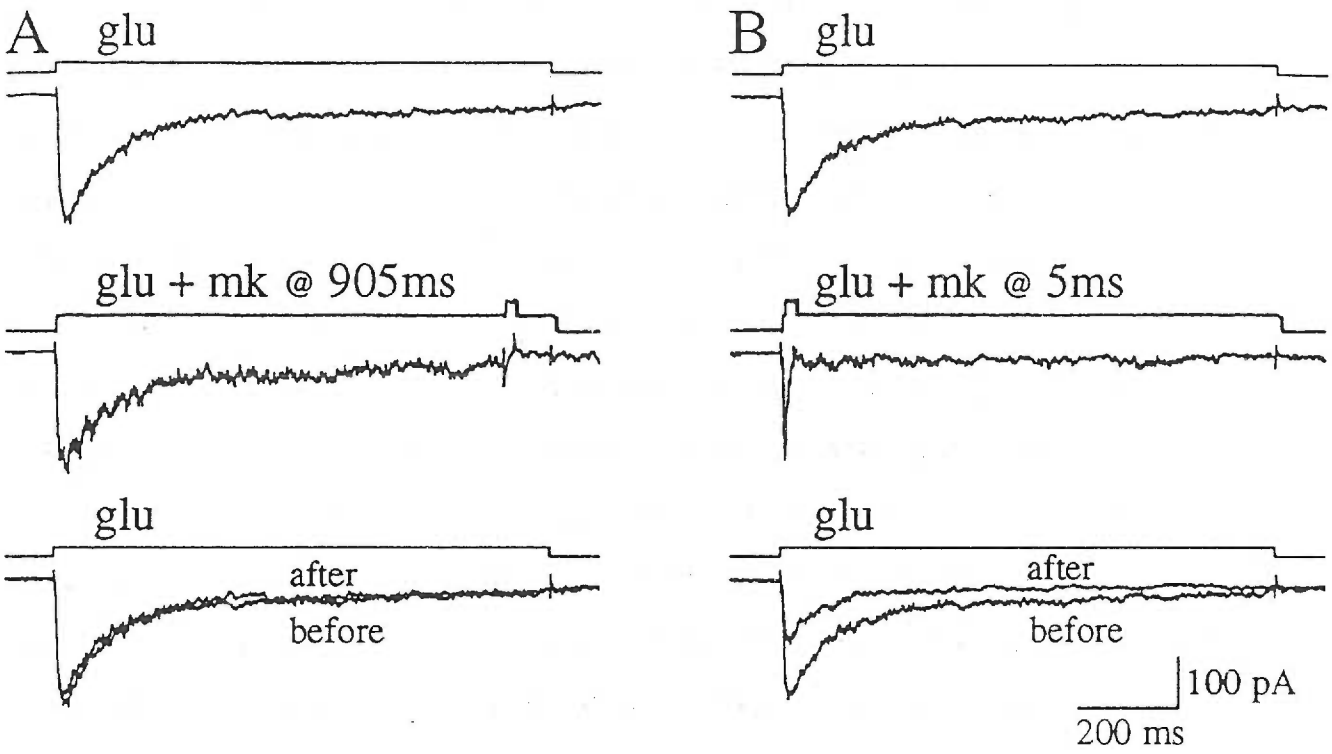

Figure 3. ***MK-801 does not block desensitized NMDA channels.***

(**A**) top: The average NMDA receptor response of an outside-out patch to a 1 second application of 10 mM L-glutamate (glu). middle: a 1 second application with a concomitant 20 ms jump into 10 mM L-glutamate plus 20 μM MK-801 (mk), 905 ms after the beginning of the long application. The open-tip current is displayed above the response. bottom: The superimposed averages of 5 responses to 1 s applications of 10 mM L-glutamate before and after the single MK-801 sweep. (**B**) The same experiment as shown in (A) except the 20 ms jump into L-glutamate plus MK-801 is 5 ms after the start of the long L-glutamate application. The responses in this figure are all from a single patch.

MK-801 blocks most channels in the first 10 ms

To estimate more directly the average first latency of NMDA channels, patches were exposed to MK-801 only during a brief agonist application. If the short first latency scheme is correct, a transient simultaneous exposure to L-glutamate and MK-801 should produce a significant block. However, if the long first latency scheme is correct, the majority of openings occur later in the response and little block should occur. The amount of block caused by a single 10 ms pulse of 10 mM L-glutamate and 20 μM MK-801 was measured as above by integrating the charge transfer under control conditions (5 ms pulse of 10 mM L-glutamate, five sweeps in each average) before and then after the exposure to MK-801 (Figure 4). This protocol resulted in an average block of 62 ± 10% measured in ten patches. This amount of block is comparable ($p>0.10$, Student's two tailed unpaired t-test) to the 70 ± 10% (n=8; from Jahr, 1992) measured when the MK-801 was present throughout the blocking trial, and indicates that a large percentage of channels open within 10 ms of binding L-glutamate.

It is possible that MK-801 can bind to a low affinity site on the closed channel (e.g., in the external vestibule) from which it could block the channel once the pore opens tens of milliseconds after the end of the application. This would result in an underestimate of the average first latency. To address this possibility, a 4 ms application of 10 mM L-glutamate was preceded at decreasing intervals by a 4 ms pulse of 20 μM MK-801 (Figure 5). With each patch, three types of applications were made in succession and then repeated in a cyclical fashion three to six times: first a control trial with no pulse of MK-801, followed by a trial with a pulse of MK-801 completed 30 ms before the start of the L-glutamate pulse, and finally a test trial with a pulse of MK-801 ending either 20, 15, 10, 5, or 3 ms before the pulse of L-glutamate. Except for two long-lived patches, only one of the test times was attempted per patch.

In 17 patches, the trials with an application of MK-801 ending 30 ms before the L-glutamate test pulse were 101 ± 9.6% of the control response. A one way ANOVA

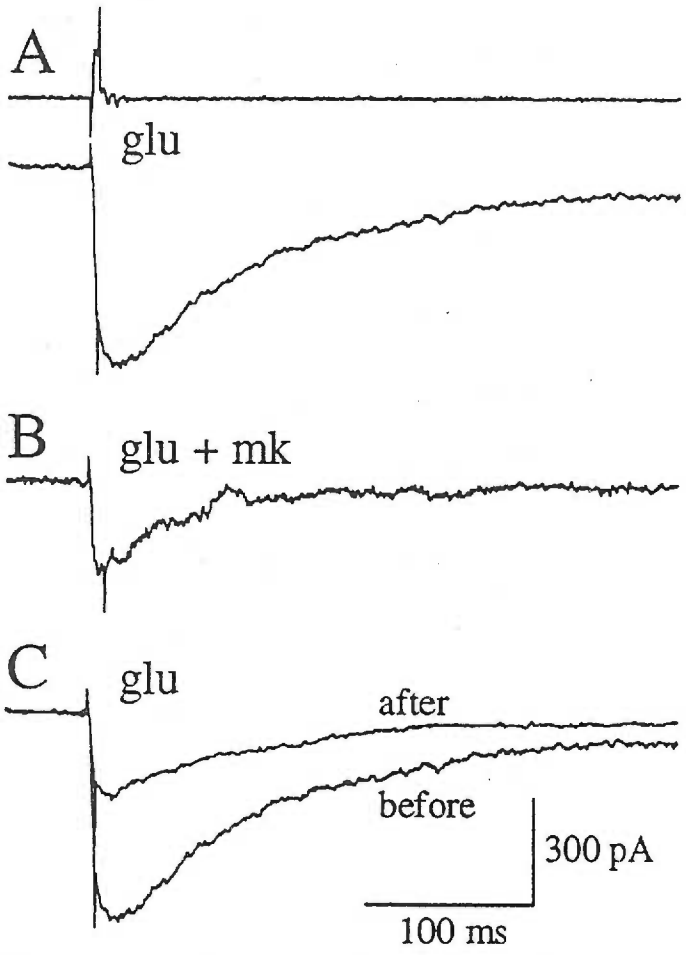

Figure 4. ***Brief exposure to L-glutamate plus MK-801 produces an amount of block similar to that found by applying L-glutamate in a background of MK-801.***

(**A**) An average of five NMDA receptor responses of an outside-out patch to 5 ms pulses of 10 mM L-glutamate (glu) before exposure to MK-801. The open-tip current is displayed above the response. (**B**) The response of the same patch to a 10 ms pulse of 10 mM L-glutamate and 20 μM MK-801 (mk). (**C**) The average in (A) and an average of five responses after exposure to MK-801, superimposed.

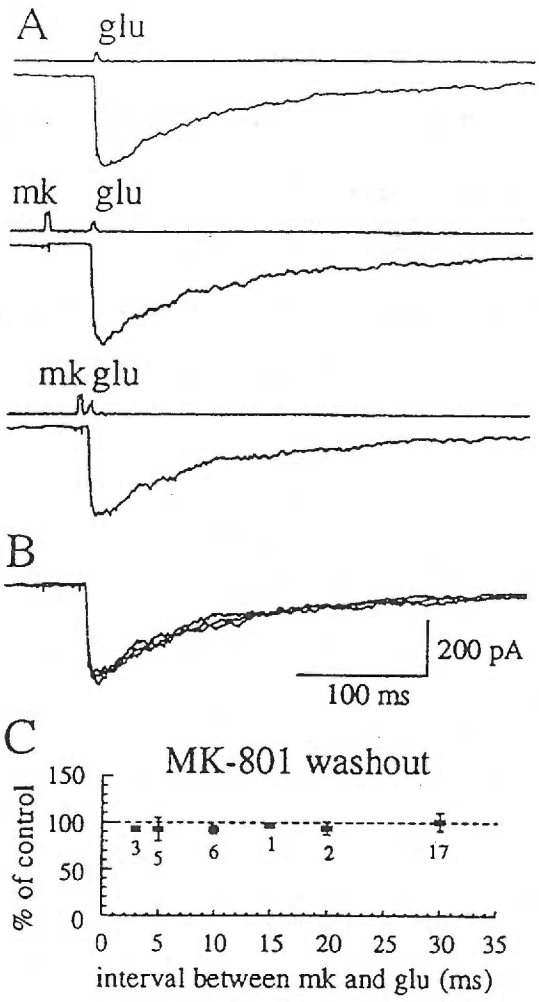

Figure 5. ***Rapid washout of MK-801.***

(A) Three types of trials made in succession and then repeated cyclically: (top) control trial, a single 4 ms pulse of 10 mM L-glutamate (glu), (middle) a 4 ms pulse of 20 μM MK-801 (mk) followed by a 4 ms pulse of 10 mM L-glutamate with a 30 ms interval between the two pulses, (bottom) same as the middle trace but with a 3 ms interval between the MK-801 pulse and the L-glutamate pulse. Each trace is the average of three sweeps. The open-tip currents are displayed above each trace. (B) The three traces shown in (A), superimposed. All traces are from the same patch. (C) A plot of the mean response for each time interval, expressed as a percent of the control response in the patch the measurement was made. The numbers below the data points indicate the number of patches contributing to each mean, and the error bars are standard deviations.

was performed with post hoc comparisons between the decreasing test intervals and the responses of the 30 ms interval, all expressed as percentages of the control response in the same patch. The ANOVA gave a p value of 0.182 indicating the difference among the means was not significant. In addition, the Bonferroni p values for individual comparisons with the 30 ms interval were not significant.

Discussion

Our results using concentration jumps with outside-out patches support the short first latency scheme presented above. We have found that block by MK-801 is independent of agonist affinity; transient NMDA channel activity evoked by L-cysteate results in a block by MK-801 comparable to that of longer lasting L-glutamate induced activity. We have also shown that a brief co-application of MK-801 and L-glutamate is sufficient to produce a block similar to that found when MK-801 is present throughout the trial. In addition, we have demonstrated that the block of desensitized receptors by MK-801 is unlikely to account for the large block produced by MK-801 during a patch response. These results indicate that most of the channels that open before agonist dissociation do so for the first time within ten milliseconds, and that the long decay of NMDA channel responses to brief pulses of L-glutamate is due to repeated openings of channels and not to long first latencies. This is consistent with the channels having a peak open probability near 0.3 in response to brief saturating concentrations of agonist (Jahr, 1992; 1994) .

The conclusions of this study contrast with those of low concentration steady-state studies which require a low probability of opening and long first latencies to explain the slow NMDA response (Edmonds and Colquhoun, 1992) . The disparity may be due to differences in the behavior of the channels under contrasting recording conditions. As has been suggested previously (Edmonds and Colquhoun, 1992) , quick jumps into high concentrations may give rise to activations with a higher P_o than those found in steady

state low concentration experiments . Preliminary evidence to this effect was reported by Edmonds and Colquhoun (1992) and was explained by possible differences in the initial occupancies of the various kinetic states. An example of a kinetic model which could account for the differences between the experimental results is one which includes openings of receptors with one ligand bound.

Mono-liganded receptor openings are not likely to occur at physiological concentrations of transmitter (Patneau and Mayer, 1990; Clements and Westbrook, 1991). However, the low agonist concentrations used in the steady state experiments may increase the likelihood of such events, analogous to what is found with acetylcholine receptors (Dionne et al., 1978, Colquhoun and Sakmann, 1985; Colquhoun and Ogden, 1988) and $GABA_A$ receptors (Twyman et al., 1990) . The saturating concentrations of agonists used in the present study result in doubly-liganded receptors which may be more likely than mono-liganded receptors to enter into the "high P_o periods" seen by many researchers (Jahr and Stevens, 1987; Howe et al., 1988; Gibb and Colquhoun, 1991; Gibb and Colquhoun, 1992) . These periods of intense activity, with a P_o of about 0.8 (Gibb and Colquhoun, 1992) , can last for hundreds of milliseconds and may contribute significantly to the macroscopic response evoked by brief applications of high concentrations of L-glutamate. In steady state experiments using low concentrations of L-glutamate but high concentrations of glycine, the periods of high P_o occurred about once a minute during continuous recording and yet contributed 22% of the openings (Gibb and Colquhoun, 1991) . It is not known whether high P_o periods are dependent on agonist concentration, but this could explain the brevity of the aligned single channel activations observed by Edmonds and Colquhoun (1992). In that study high P_o periods were not reported, perhaps because of the very low concentrations of both L-glutamate and glycine used. These periods of intense activity could result in significant charge transfer late in the response, slowing the decay of the conditional open probability distribution.

Correlations in channel activity apparent in single channel recordings (Gibb and Colquhoun, 1992, Edmonds et al., 1995) may be indicative of channel properties which could result in the discrepancy between the concentration jump experiments and those under steady state conditions. Strong correlation between adjacent openings, between adjacent shut periods, and an inverse correlation between adjacent open and shut periods indicate that long openings are more often found near other long openings and brief closings. The extreme of this trend would be the high P_o periods mentioned above, and this behavior may result from a kinetic state favored by jumps into high agonist concentrations.

A recent study by Benveniste and Mayer supports the short first latency / high P_o scheme. Using co-applications of the open channel blocker 9-aminoacridine (9-AA) and L-glutamate they measured an absolute limit of 75 ms on the first latency of NMDA receptors (Benveniste and Mayer, 1995) . Co-applications of longer durations collected no additional channels in the open-blocked state, as measured by the amplitude of tail currents evoked by a depolarizing pulse at the end of the application. In addition, after a 20 ms co-application, they noted a rise in inward current upon returning to the control solutions which they interpreted as first openings of channels, similar to a response to L-glutamate in the absence of 9-AA. This response was only 27% of control which suggests that 73% of the channels that would open in response to L-glutamate, opened within 20 ms of agonist presentation.

The single channel behavior in agreement with our findings consists of an average first latency of about 10 ms, a peak P_o near 0.3, and a significant number of channels exhibiting long lasting bursting. If synaptic NMDA channels behave similarly to those in outside-out patches (Hessler et al., 1993; but see Rosenmund et al., 1993; Rosenmund et al., 1995) , this short first latency / high P_o scheme suggests that relatively few channels (c. 5 - 30) are required at individual synaptic sites to account for the small NMDA component of miniature excitatory synaptic currents (Bekkers and Stevens, 1989;

Robinson et al., 1991; McBain and Dingledine, 1992; Silver et al., 1992) . Very recently, it has been reported that calmodulin can regulate the P_o of NMDA channels (Ehlers et al., 1996) . This may account for the differences in estimates of P_o in different preparations (Jahr, 1992; Hessler et al., 1993; Rosenmund et al., 1993; Rosenmund et al., 1995) .

References

Bekkers JM, Stevens CF (1989) NMDA and non-NMDA receptors are co-localized at individual excitatory synapses in cultured rat hippocampus. Nature 341: 230-233.

Benveniste M, Mayer ML (1995) Trapping of glutamate and glycine during open channel block of rat hippocampal neuron NMDA receptors by 9-aminoacridine. J Physiol (Lond) 483.2: 367-384.

Clements JD, Westbrook GL (1991) Activation kinetics reveal the number of glutamate and glycine binding sites on the *N*-methyl-D-aspartate receptor. Neuron 7: 605-613.

Colquhoun D, Ogden DC (1988) Activation of ion channels in the frog end-plate by high concentrations of acetylcholine. J Physiol (Lond) 395: 131-159.

Colquhoun D, Sakmann B (1985) Fast events in single channel currents activated by acetylcholine and its analogues at the frog muscle end-plate. J Physiol (Lond) 369: 501-557.

Dionne VE, Steinbach JH, Stevens CF (1978) An analysis of the dose-response relationship at voltage-clamped frog neuromuscular junctions. J Physiol (Lond) 281: 421-444.

Edmonds B, Colquhoun D (1992) Rapid decay of averaged single-channel NMDA receptor activations recorded at low agonist concentration. Proc. R. Soc. Lond. B. 250: 279-286.

Edmonds B, Gibb AJ, Colquhoun D (1995) Mechanisms of activation of glutamate receptors and the time course of excitatory synaptic currents. Annu. Rev. Physiol. 57: 495-519.

Ehlers MD, Zhang S, Bernhardt JP, Huganir RL (1996) Inactivation of NMDA receptors by direct interaction of calmodulin with the NR1 subunit. Cell 84: 745-755.

Gibb AJ, Colquhoun D (1991) Glutamate activation of a single NMDA receptor-channel produces a cluster of channel openings. Proc. R. Soc. Lond. B. 243: 39-45.

Gibb AJ, Colquhoun D (1992) Activation of *N*-methyl-D-aspartate receptors by L-glutamate in cells dissociated from adult rat hippocampus. J Physiol (Lond) 456: 143-179.

Hessler NA, Shirke AM, Malinow R (1993) The probability of release at a mammalian central synapse. Nature 366: 569-572.

Hestrin S, Sah P, Nicoll RA (1990) Mechanisms generating the time course of dual component excitatory synaptic currents recorded in hippocampal slices. Neuron 5: 247-253.

Hille B (1992) Ionic Channels of Excitable Membranes. Sunderland, MA: Sinauer.

Howe JR, Colquhoun D, Cull-Candy SG (1988) On the kinetics of large-conductance glutamate-receptor ion channels in rat cerebellar granule neurons. Proc. R. Soc. Lond. B. 233: 407-422.

Huettner JE, Bean BP (1988) Block of *N*-methyl-D-aspartate-activated current by the anticonvulsant MK-801: Selective binding to open channels. Proc. Natl. Acad. Sci. USA 85: 1307-1311.

Jahr CE (1992) High probability opening of NMDA receptor channels by L-glutamate. Science 255: 470-472.

Jahr CE (1994) NMDA receptor kinetics and synaptic function. Semin Neurosci 6: 81-86.

Jahr CE, Stevens CF (1987) Glutamate activates multiple single channel conductances in hippocampal neurons. Nature 325: 522-525.

Lester RAJ, Clements JD, Westbrook GL, Jahr CE (1990) Channel kinetics determine the time course of NMDA receptor-mediated synaptic currents. Nature 346: 565-567.

Lester RAJ, Jahr CE (1992) NMDA channel behavior depends on agonist affinity. J. Neurosci. 12: 635-643.

Lester RAJ, Quarum ML, Parker JD, Weber E, Jahr CE (1989) Interaction of 6-cyano-7-nitroquinoxaline-2,3-dione with the *N*-methyl-D-aspartate receptor-associated glycine binding site. Mol. Pharm. 35: 565-570.

Lin F, Stevens CF (1994) Both open and closed NMDA receptor channels desensitize. J. Neurosci. 14: 2153-2160.

McBain C, Dingledine R (1992) Dual-component miniature excitatory synaptic currents in rat hippocampal CA3 pyramidal neurons. J. Neurophysiol. 68: 16-27.

Patneau DK, Mayer ML (1990) Structure-activity relationships for amino acid transmitter candidates acting at N-methyl-D-aspartate and quisqualate receptors. J. Neurosci. 10: 2385-2399.

Robinson HPC, Sahara Y, Kawai N (1991) Nonstationary fluctuation analysis and direct resolution of single channel currents at postsynaptic sites. Biophys. J. 59: 295-304.

Rosenmund C, Clements JD, Westbrook GL (1993) Nonuniform probability of glutamate release at a hippocampal synapse. Science 262: 754-757.

Rosenmund C, Feltz A, Westbrook GL (1995) Synaptic NMDA receptor channels have a low open probability. J Neurosci 15: 2788-95.

Silver RA, Traynelis SF, Cull-Candy SG (1992) Rapid-time-course miniature and evoked excitatory currents at cerebellar synapses *in situ*. Nature 355: 163-166.

Tong G, Jahr CE (1994) Multivesicular release from excitatory synapses of cultured hippocampal neurons. Neuron 12: 51-59.

Traynelis SF, Cull-Candy SG (1990) Proton inhibition of *N*-methyl-D-aspartate receptors in cerebellar neurons. Nature 345: 347-350.

Twyman RE, Rodgers CJ, Macdonald RL (1990) Intraburst kinetic properties of the GABAA receptor main conductance state of the mouse spinal cord neurones in culture. J Physiol (Lond) 423: 193-220.

Wyllie, D, Edmonds, B, Colquhoun, D (1997) Single activations of recombinant NMDA NR1a/NR2A receptors recorded in one-channel patches. J Physiology 501.P: 13P-14P.

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Glutamate transporter currents in Bergmann glial cells follow the time course of extrasynaptic glutamate

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Abbreviations: CF, climbing fiber; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate; NBQX, 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(F)quinoxaline; D-CPP, R(-)-3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid; gabazine, SR-95531;THA, D,L-threo-β-hydroxyaspartic acid; DHK, dihydrokainate; GYKI-52466, 1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5H-2,3-benzodiazepine hydrochloride.

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Abstract

Glutamate transporters in the central nervous system are expressed in both neurons and glia, they mediate high affinity, electrogenic uptake of glutamate, and they are associated with an anion conductance that is stoichiometrically uncoupled from glutamate flux. Although a complete cycle of transport may require 50 to 100 milliseconds, previous studies suggest that transporters can alter synaptic currents on a much faster time scale. We find that application of L-glutamate to outside-out patches from cerebellar Bergmann glia activates anion-potentiated glutamate transporter currents which activate in less than a millisecond, suggesting an efficient mechanism for the capture of extrasynaptic glutamate. Stimulation in the granule cell layer in cerebellar slices elicits all or none AMPA receptor and glutamate transporter currents in Bergmann glia which have a rapid onset, suggesting that glutamate released from climbing fiber (CF) terminals escapes synaptic clefts and reaches glial membranes shortly after release. Comparison of the concentration dependence of both AMPA receptor and glutamate transporter kinetics in patches with the time course of CF-evoked responses indicates that the glutamate transient at Bergmann glial membranes reaches a lower concentration than attained in the synaptic cleft and remains elevated in the extrasynaptic space for many milliseconds.

Introduction

Termination of the actions of synaptically released glutamate requires uptake by high affinity glutamate transporters. These transporters are expressed by both neurons and glia and maintain low extracellular glutamate levels by coupling translocation to the electrochemical gradients for Na^+, K^+, and H^+ (Robinson and Dowd, 1997). The importance of these transporters in restricting glutamate neurotoxicity is evidenced by the physiological, behavioral and anatomical abnormalities which result when their expression is reduced (Rothstein et al., 1996) or eliminated (Tanaka et al., 1997). On a

faster time scale, glutamate transporters appear to be important in limiting the duration of synaptic excitation at some synapses (Sarantis et al., 1993; Mennerick and Zorumski, 1994; Otis et al., 1996; Takahashi et al., 1996; Tanaka et al., 1997) by rapidly lowering the concentration of glutamate in the synaptic cleft following exocytosis; however, transporter antagonists do not prolong EPSCs at all synapses (Hestrin et al., 1990; Isaacson and Nicoll, 1993; Sarantis et al., 1993) suggesting that other factors which vary between synapses such as receptor kinetics, location and density of transporters, and diffusional barriers may also be important in shaping the glutamate transient in the cleft. Glutamate transporters located near release sites have also been shown to slow the activation of postsynaptic ionotropic receptors (Tong and Jahr, 1994; Diamond and Jahr, 1997) suggesting that glutamate may bind to transporters within a millisecond after release. Such rapid binding kinetics have recently been demonstrated for glutamate transporters expressed in Purkinje cells (Otis et al., 1997). However, the lack of subtype-selective antagonists has hampered assessment of the relative contribution of neuronal and glial transporters to the uptake of glutamate on this time scale.

In the cerebellum, Bergmann glial processes ensheath excitatory synapses on Purkinje cells (Palay and Chan-Palay, 1974; Spacek, 1985), express high levels of the glutamate transporter GLAST (Rothstein et al., 1994; Lehre et al., 1995), and accumulate radiolabelled glutamate (de Barry et al., 1982); they are therefore ideally positioned to capture glutamate that escapes from the synaptic cleft. Synaptic activation of glutamate transporters in Bergmann glia has been recently demonstrated in cerebellar slices (Clark and Barbour, 1997) and are similar to the glutamate transporter currents elicited in cultured glial cells following neuronal stimulation (Mennerick and Zorumski, 1994; Linden, 1997). These synaptic transporter currents begin shortly after stimulation suggesting that glutamate reaches sites on glial membranes within a millisecond after exocytosis. This observation is consistent with estimates of the diffusion rate of glutamate (Wahl et al., 1996) as well as the decay rate of the glutamate transient in the

cleft (Clements et al., 1992; Diamond and Jahr, 1997). However, the amount of glutamate which escapes the cleft and the time that it remains elevated in the extrasynaptic space are not known. We describe the intrinsic kinetics of glial transporters in outside-out patches from Bergmann glial cells and compare these to AMPA receptor and transporter currents activated through CF stimulation in cerebellar slices to estimate the time course of glutamate in the extrasynaptic space. Our results indicate that the glutamate concentration at glial membranes peaks at a level much lower than the 1 - 3 mM achieved in the synaptic cleft (Clements et al., 1992; Diamond and Jahr, 1997) and persists in extrasynaptic regions for more than 10 milliseconds following release.

Materials and methods

Whole cell recordings and outside-out patches were obtained from Bergmann glia in cerebellar slices (300 μm) prepared from postnatal day (P) 11 - P15 rats. Bergmann glia were visualized using a 40x water-immersion objective on an upright microscope (Zeiss Axioskop) equipped with IR/DIC optics. Patch pipettes had resistances of 2 - 4 MΩ when filled with K gluconate. The bath solution contained (in mM) 119 NaCl, 2.5 KCl, 2.5 $CaCl_2$, 1.3 $MgCl_2$, 1 NaH_2PO_4, 26.2 $NaHCO_3$, and 11 glucose, saturated with 95% O_2/5% CO_2. Pipette solutions contained (in mM) 130 K^+ A^-, 20 HEPES, 10 EGTA, and 1 $MgCl_2$, pH 7.2. A^- denotes NO_3^-, SCN^-, gluconate or methanesulfonate. Isolated AMPA responses were recorded in patches with an internal solution composed of (in mM) 100 Cs_2SO_4, 20 HEPES, 10 EGTA, 4 MgATP, and 0.3 GTP, pH 7.2. CFs were stimulated (30 - 140 μA, 100 μs) with a theta glass pipette filled with bath solution that was placed in the granule cell layer. Synaptic currents were filtered at 1 - 2 kHz and digitized at 10 kHz, and patch currents were filtered at 5 kHz and digitized at 30 - 50

kHz. Current-clamp responses were recorded with an Axoclamp-2A, while synaptic and patch currents were recorded with an Axopatch-200A. When recording synaptic currents, access resistance was monitored throughout the experiment and averaged 5.5 ± 2.3 MΩ; if it changed more than 20%, the experiment was discarded. Holding potentials have been corrected for junction potentials. For morphological identification, Bergmann glia were filled with 1.5 mM Cy5-EDA (Amersham Life Science) in KNO_3 via the patch pipette and imaged on a confocal microscope (Noran Odyssey XL). Rapid agonist applications to outside-out patches were performed as described previously (Tong and Jahr, 1994) using an extracellular solution containing (in mM) 135 NaCl, 5.4 KCl, 5 HEPES, 1.8 $CaCl_2$, 1.3 $MgCl_2$, at pH 7.2. Concentration-response was performed on individual patches by connecting a 4-barrel miniature manifold (Warner Instruments) to one side of a theta pipette. Artifacts arising from voltage steps applied to the bimorph have been blanked. Values are given as mean ± S.D. All experiments were performed at room temperature (22 - 24°).

Results

Bergmann glial cell bodies were initially identified in slices of rat cerebellum by their small size (~ 10 μm in diameter) and their location in the Purkinje cell layer. Whole-cell recordings from these cells revealed their characteristic low input resistances (30 ± 21 MΩ, n = 24), high resting membrane potentials (-87 ± 6 mV, n = 24), and lack of voltage dependent currents (Fig. 1*A*). Biexponential fits to the passive membrane responses to current steps yielded time constants of: τ_{fast} = 0.43 ± 0.25 ms, 51 ± 12%; τ_{slow} = 2.4 ± 0.4 ms; n = 6). Cells exhibiting these electrophysiological properties were filled the dye, Cy5-EDA, revealing that they typically had 5 or more thin, apically projecting processes which were interrupted along their length by irregular protrusions

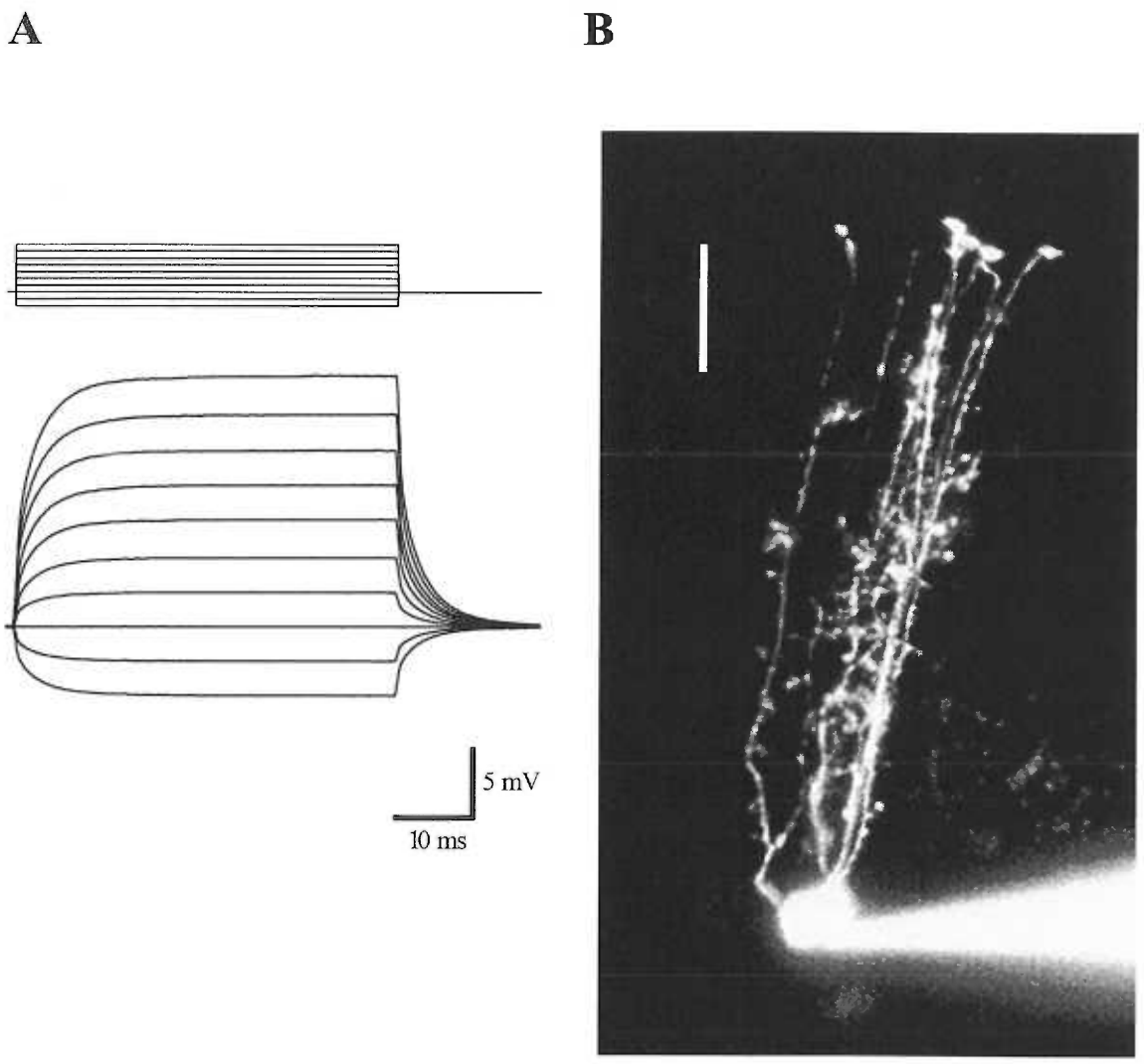

Figure 1. ***Identification of a Bergmann glial cell.***

(A) Voltage responses of a Bergmann glia cell to 50 ms current steps (-200 pA to 700 pA) from a resting potential of -87 mV. Fitting the rising phases of these responses with two exponentials gave a fast time constant of 0.36 ms (47.3 % amplitude) and a slower time constant of 3.3 ms. The time constants for the rise and decay phases were not significantly different ($p > 0.05$). This cell had an input resistance of 27 MΩ. *(B)* The cell was recorded with a patch pipette filled with 1.5 mM Cy5-EDA in KNO_3 internal solution and simultaneously imaged on a confocal microscope. Image is a composite of 11 optical sections taken at 5 μm steps. Bar = 25 μm.

before terminating at the pial surface with rounded expansions (Fig. 1*B*). These morphological features are characteristic of Bergmann glia (Palay and Chan-Palay, 1974). Rapid application of L-glutamate (10 mM) to outside-out patches from Bergmann glial cells activated both an AMPA receptor-mediated current that was blocked by NBQX and GYKI-52466, and a current that was insensitive to these antagonists when the pipette contained SCN^-, which is highly permeant through glial glutamate transporters (Wadiche et al., 1995)(Fig. 2*A*); in the absence of a permeant anion, the current remaining in AMPA receptor antagonists was very small (<5 pA; n = 7). Glutamate transporters, unlike ionotropic receptors, show a near absolute dependence on external Na^+ (Robinson and Dowd, 1997). When external Na^+ was replaced by Li^+ (135 mM) the NBQX/GYKI-insensitive current was blocked (n = 5; Fig. 2*B*). These data suggest that both AMPA receptors and glutamate transporters are present in patches removed from the somata of Bergmann glial cells.

The current-to-voltage relation of L-glutamate evoked transporter currents rectified inwardly and did not reverse at positive potentials (n = 6; Figs 2*C* and *D*), as would be expected under these ionic conditions (Wadiche et al., 1995). Transporter currents in patches were blocked by the broad spectrum substrate/antagonist THA (300 μM; 92 ± 4% inhibition; n = 11; Fig. 2*E*), but were not substantially affected by DHK (300 μM; 9 ± 3% inhibition; n = 6), a selective inhibitor for the GLT-1 transporter (Arriza et al., 1994). These data are consistent with the predominant expression of GLAST in Bergmann glia at this age (Shibata et al., 1996). L-glutamate reduced the standing inward current produced by THA (Fig. 2*E*). This may reflect the replacement of THA with L-glutamate as THA unbinds or is transported, causing the larger steady-state response produced by THA to decay to the steady-state level produced by L-glutamate. L-aspartate (2 mM) also evoked a current in patches which was insensitive to NBQX/GYKI and remained inward at potentials up to 70 mV (n = 6; Fig. 2*F*), consistent with the

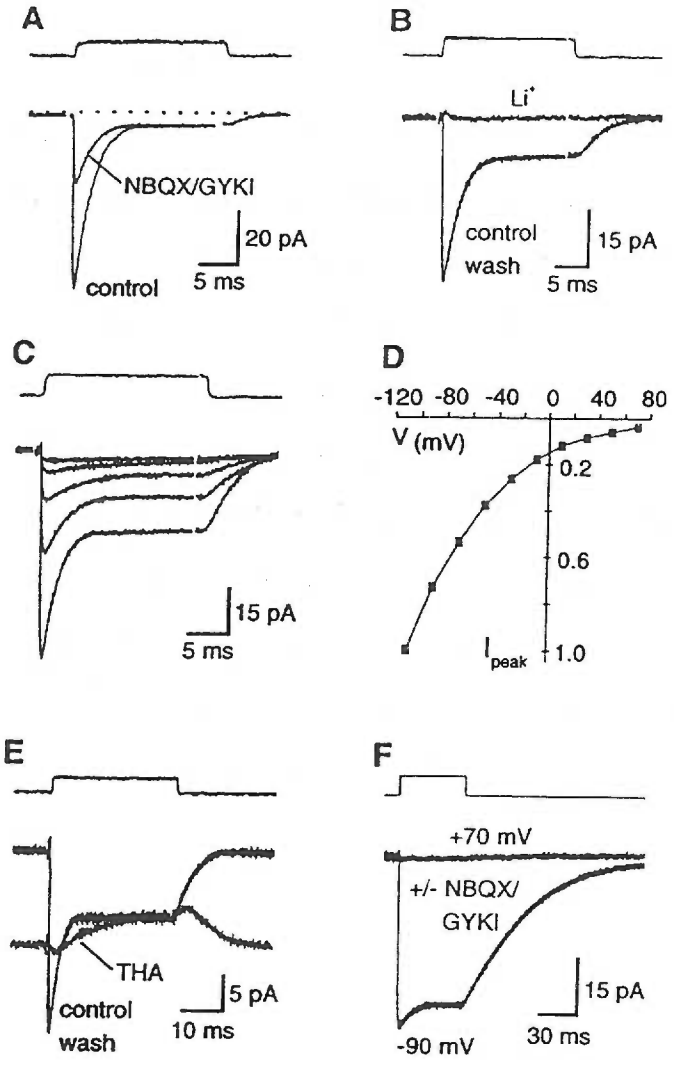

Figure 2. ***Glutamate transporter currents and AMPA receptor currents can be evoked in outside-out patches from Bergmann glia.***

(A) L-glutamate (10 mM) activates a transient current that is blocked by NBQX and GYKI-52466 and a smaller biphasic current in patches using a KSCN internal solution. Vm = -90 mV. *(B)* Complete substitution of extracellular Li^+ for Na^+ blocks the transporter current evoked by 10 mM L-glutamate. Vm = -90 mV. *(C)* and *(D)* The current-voltage relationship of the transporter current is inwardly rectifying and does not reverse. Responses in *(C)* were recorded at membrane potentials of -10, -30, -50, -70, and -90 mV. In *(D)*, each point is an average of responses from 6 patches normalized to

the peak response at -110 mV. *(E)* In the presence of NBQX (10 μM) and GYKI (25 μM), THA (300 μM) produces a steady-state inward current and occludes the response to a pulse of 10 mM L-glutamate. The outward current elicited by L-glutamate in the presence of THA probably reflects the replacement of THA with L-glutamate, which is likely to occur at these concentrations. Vm = -110 mV. *(F)* L-aspartate (2 mM) activates a transporter current but not an AMPA receptor current. The control responses and those recorded in NBQX (10 μM) and GYKI (25 μM) are superimposed. This result also indicates that at these concentrations neither NBQX nor GYKI alter transporter function. KSCN-based internal solutions were used in all recordings. The uppermost traces in each figure are the "open-tip" response obtained by solution exchanges after disrupting the membrane patch. In each case the concentrations listed were used, except in *F*, where ACSF was diluted with 50% dH_2O to increase the size of the junction current. Traces are averages of 5 - 15 responses.

selective activation of glutamate transporters (Klockner et al., 1994) and not AMPA receptors (Patneau and Mayer, 1990).

Transporter currents decayed to steady-state levels in the continued presence of L-glutamate. The explanation for the transient transporter current is not known, in part, because it comprises both the charge movement coupled to glutamate translocation and the uncoupled anion current. The decay to a steady-state level may reflect desynchronization of the transporters (Bruns et al., 1993) as they reach non-conducting states in the transport cycle. L-aspartate evoked transporter currents with slower kinetics and larger steady-state components than those elicited by L-glutamate, possibly reflecting the higher apparent affinity and slower transport rate of L-aspartate (Arriza et al., 1994; Klockner et al., 1994).

A recent study demonstrated that glutamate released following parallel fiber stimulation in cerebellar slices activates both AMPA receptors and transporters in glial membranes (Clark and Barbour, 1997). In addition to parallel fibers, Purkinje cells receive powerful excitatory input from single CFs which form contacts with 1,000 or more spines on individual Purkinje cells (Palay and Chan-Palay, 1974). These terminals are also ensheathed by Bergmann glial processes (Palay and Chan-Palay, 1974; Spacek, 1985), suggesting that glutamate released from CF synapses might also reach receptors and transporters in Bergmann glial membranes. Focal stimulation in the granule cell layer elicited all-or-none, inward currents in Bergmann glia voltage-clamped at their resting potential, consistent with activation of a single CF (Ito, 1984)(Fig. 3*A* and *B*). Unlike parallel fiber responses in Bergmann glia (Clark and Barbour, 1997), CF currents were not contaminated by the detection of the extracellular field response through the low resistance glial membrane, a consequence of the activation of many parallel fibers. CF-evoked currents had two kinetically distinct components that could be separated pharmacologically. Addition of antagonists of ionotropic receptors, NBQX, D-CPP, and SR-95531 (gabazine), inhibited a component that had rapid kinetics (n = 24; Fig. 3*C*).

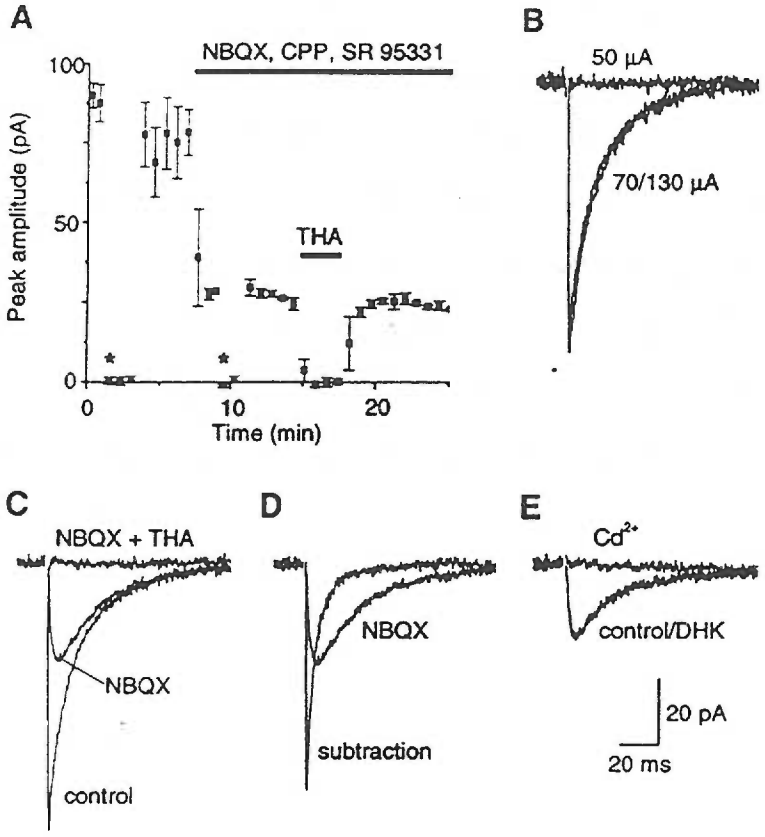

Figure 3. ***CF stimulation elicits both glutamate transporter currents and AMPA receptor currents in Bergmann glia.***

(A) Bergmann glia response to CF stimulation is all-or-none and blocked by a combination of NBQX (10 µM), D-CPP (5 µM), gabazine (5 µM) and THA (300 µM). Each point represents the average of three consecutive responses. Holding potential was -90 mV. At the first asterisk the stimulation intensity was decreased to 10 µA, below the threshold for the response. The stimulation intensity was then increased in steps of 20 µA, with three responses recorded at each intensity. The response returned at 70 µA and did not increase in amplitude when the intensity was increased further (up to 130 µA). A

stimulation intensity of 110 μA was maintained during the rest of the experiment except at the second asterisk where the threshold was again tested. *(B)* Averaged Bergmann glial responses to stimuli of different intensities. *(C)* The rapid component of the current is blocked by NBQX (10 μM) and the remaining slow component is blocked by THA (300 μM). *(D)* Comparison of the kinetics of the AMPA receptor and transporter currents evoked by CF stimulation. The AMPA receptor current was obtained by subtracting the response in the presence of NBQX from the control response. *(E)* $CdCl_2$ (30 μM), but not DHK (300 μM), blocked the NBQX-insensitive current. All traces are averages of 3 - 5 consecutive responses and were recorded from the same Bergmann glial cell. KNO_3-based internal solution.

The time course of this component, determined by subtraction of the remaining slower current (Fig. 3*D*), had a 20 - 80% rise time of 1.0 ± 0.5 ms and a half decay time of 4.2 ± 2.0 ms (n = 24). This fast response was also blocked by NBQX alone (10 μM; n = 3) indicating that it was mediated by AMPA receptors. The component remaining in NBQX had a 20 - 80% rise time of 1.9 ± 0.7 ms, and a half decay of 17.3 ± 4.2 ms (n = 24). This slow response was blocked by Cd^{+2} (30 μM, n = 5; Fig. 3*E*), and was inhibited by 93 ± 8% by THA (300 μM, n = 15; Fig. 3*C*), suggesting that it reflects the activation of glutamate transporters by glutamate released following Ca^{+2}-dependent exocytosis at CF terminals. Consistent with the results obtained from patch experiments, DHK (300 μM) inhibited this current by only 3.8 ± 4.9% (n = 6; Fig. 3*E*) indicating that it is mediated primarily by GLAST, the predominant glutamate transporter expressed in Bergmann glia (Rothstein et al., 1994; Lehre et al., 1995).

In recordings where NO_3^- was the primary anion in the pipette solution (e.g., Fig. 3), which is also highly permeant through GLAST (Wadiche et al., 1995), the amplitude of the CF-evoked transporter current was 44 ± 13% of the control response in the absence of ionotropic receptor antagonists (n = 17), whereas when the impermeant anion gluconate was the primary anion, the transporter current was 30 ± 16% of control (n = 7; p = 0.017). These results are consistent with a potentiation of the transporter current by an associated anion conductance. However, the time course of the synaptic transporter currents were not different in cells with the two internal solutions (20 - 80% rise times: 1.8 ± 0.5 vs. 2.2 ± 0.9; half decay times: 17.2 ± 4.4 vs. 17.7 ± 4.0; for NO_3^- and gluconate-filled cells, respectively). It was not possible to use a SCN^--based internal solution to record CF responses, because a large SCN^- leakage current was induced upon breaking-in and cells tended to visibly shrink over time, confounding recordings of evoked responses.

To estimate the glutamate concentration profile at Bergmann glial membranes following CF stimulation, the concentration dependence of the kinetics of AMPA receptor and transporter currents in patches was compared to that of the synaptically activated currents. The rise (20 - 80%) and half decay times of AMPA receptor currents in patches to steps of L-glutamate were 130 ± 40 μs and 1.6 ± 0.2 ms at 10 mM, 260 ± 120 μs and 1.7 ± 0.1 ms at 1 mM, 320 ± 60 μs and 2.0 ± 0.5 ms at 500 μM, and 600 ± 190 μs and 2.4 ± 0.6 ms at 250 μM (n = 5; Fig. 4*A*). If the AMPA receptors expressed in Bergmann glia were to sense an instantaneous rise of glutamate following CF stimulation, the rise and half-decay times of the synaptic current (1.0 and 4.2 ms) would be consistent with an average peak concentration of less than 250 μM.

The rise time (20 - 80%) of transporter currents in patches to steps of L-glutamate were also very fast relative to the synaptic transporter currents, except at 10 μM: 206 ± 39 μs at 10 mM, 287 ± 30 μs at 1 mM, 780 ± 75 μs at 100 μM, and 3.12 ± 0.68 ms at 10 μM (n = 6; Fig. 4*B*). As the rise time of the synaptic transporter current was 1.9 ms, the patch data imply that glutamate reaches a peak concentration of 10 to 100 μM at these extrasynaptic sites. In addition, the decay time of synaptic transporter currents (17.3 ms) was slow relative to the decay of patch responses during steps of glutamate (half-decays of 2.8 and 3.9 ms for steps of 10 mM and 100 μM L-glutamate, respectively) as well as at the end of glutamate applications (1 ms pulse, 1.7 ± 0.2 ms, n = 3; 30 ms pulse, 2.7 ± 0.3 ms, n = 7). These data suggest that glutamate can remain elevated for more than ten milliseconds at Bergmann glial membranes following exocytosis.

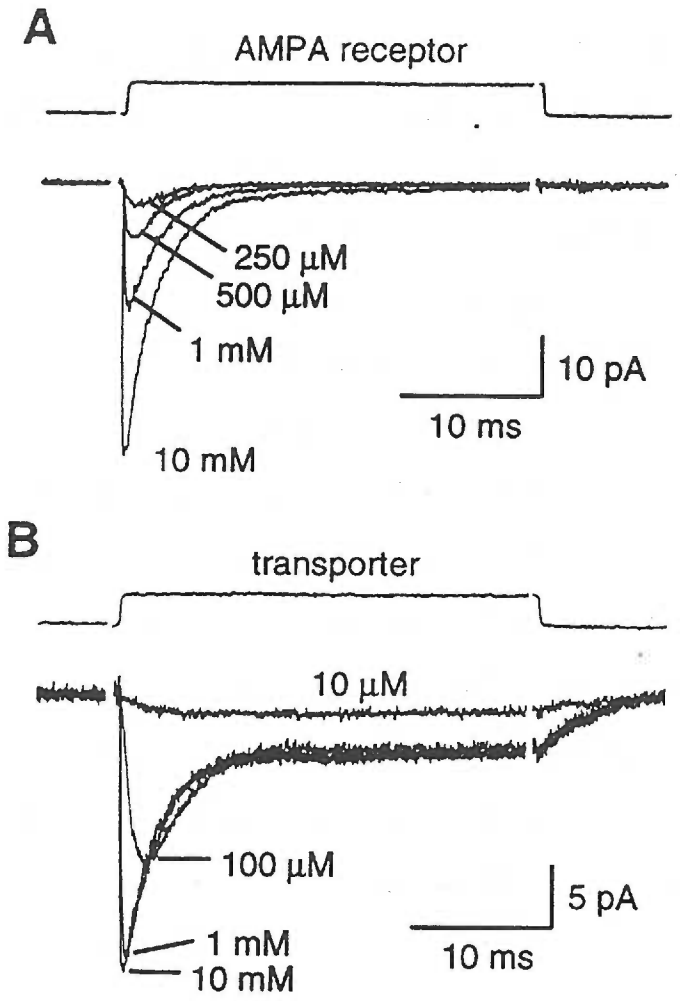

Figure 4. ***Concentration-dependence of the kinetics of glutamate transporter and AMPA receptor currents in patches from Bergmann glial cells.***

(A) Currents evoked in an outside-out patch by 30 ms applications of L-glutamate at 250 µM, 500 µM, 1 mM and 10 mM. Cs_2SO_4-based internal solution. *(B)* Currents evoked in an outside-out patch in the presence of NBQX (10 µM) and GYKI (25 µM) by applications of L-glutamate at 10 µM, 100 µM, 1 mM and 10 mM. KSCN internal solution. Traces are averages of 15 - 50 responses. Vm = -90 mV.

Discussion

Our results demonstrate that glutamate transporters expressed in Bergmann glial cells have rapid kinetics and are capable of binding glutamate on a submillisecond time scale, similar to the binding rates estimated for ionotropic glutamate receptors (Jonas and Sakmann, 1992). This rapid binding of glutamate may provide an efficient mechanism for capturing glutamate which escapes the synaptic cleft, reducing the time that glutamate is elevated in the extracellular space following release. The rapid charge movement described here contrasts with the time required to complete a transport cycle, estimated at 70 milliseconds for the glial glutamate transporter GLT-1 (Wadiche et al., 1995). The exact steps in the transport cycle which permit the flow of anions are not yet known, however the rapid onset of the anionic current and the demonstration that mutant transporters which are deficient in K^+ binding have an anion conductance which is indistinguishable from wild-type (Kavanaugh et al., 1997), suggest that it reflects early steps in the transport cycle and may parallel the transit of glutamate.

In hippocampal cultures the concentration of glutamate in the synaptic cleft following release decays from 1 - 3 mM to tens of micromolar within several milliseconds after release, due to rapid diffusion out of the cleft and buffering by transporters (Clements et al., 1992; Tong and Jahr, 1994; Diamond and Jahr, 1997). Stimulation of single CFs elicited both AMPA receptor and glutamate transporter currents in Bergmann glia which had rapid onsets, suggesting that substantial glutamate must diffuse out of the synaptic cleft shortly after release. This is consistent with the binding and perhaps transport of only 22% of the glutamate released by postsynaptic transporters (Otis et al., 1997), though it is not known how much is taken up into CF terminals. Comparison of the time course of this synaptic current and the rapid kinetics of AMPA receptors measured in patches suggests that the glutamate transient at the Bergmann glial membranes nearby synapses peaks within a millisecond, though at a significantly lower concentration than is thought to occur in the synaptic cleft (Clements

et al., 1992; Diamond and Jahr, 1997). The slow decay of the synaptic transporter current indicates that clearance of glutamate from extrasynaptic sites requires more than ten milliseconds. The differences in the clearance times of synaptic and extrasynaptic spaces could be explained by a clustering of transporters near release sites, although clearance at CF synaptic clefts may be slower than those in hippocampal cultures (Barbour et al., 1994; Otis et al., 1997). These estimates assume that the receptors and transporters are not altered by patch excision and that those expressed at the soma are representative of those detecting glutamate released from synapses.

The response to brief applications of a high concentration of L-glutamate to ionotropic receptors in patches often mimic the time course of miniature AMPA EPSCs, demonstrating that the kinetics of the receptors are the primary determinants of the response time course in neurons (Jonas and Spruston, 1994). In contrast, the kinetics of AMPA receptors and transporters in patches from Bergmann glia were faster than responses elicited through CF stimulation. A number of factors may account for the slower time course of these synaptic responses including electrotonic filtering, release asynchrony, and diffusion of transmitter to distant sites. The degree of slowing of the synaptic responses by filtering will depend on the location of the receptors and transporters, the active and passive properties of Bergmann glial membranes, the location of conductances, and the internal resistance of the processes, none of which are known. However, the membrane time constant of Bergmann glia is fast (see Fig. 1*A*) suggesting that charging of the membrane should occur rapidly, minimizing the effects of electrotonic filtering (Zhang and Trussell, 1994). If AMPA receptors and transporters have similar locations, the degree of slowing of the transporter current should be less because of its slower time course.

Release asynchrony may slow the time course of the synaptic response as exocytosis does not occur at all synapses simultaneously following Ca^{2+} influx into presynaptic terminals. Although release asynchrony has not been measured at CF inputs,

at auditory nerve inputs to the anteroventral cochlear nucleus which have similarly high release probabilities, release is complete in 1 - 2 milliseconds (Isaacson and Walmsley, 1995), too fast to account for the slow time course of the synaptic responses described here. In addition, CF-evoked AMPA responses in Bergmann glia had a faster time course than the evoked transporter current, even though the degree of slowing due to asynchrony should be the same, suggesting that the slower time course of the transporter current does not result from release asynchrony.

Unlike postsynaptic ionotropic receptors which are clustered opposite release sites, the receptors and transporters expressed by Bergmann glia may be spread over several micrometers of membrane surrounding synaptic clefts (Baude et al., 1994; Chaudhry et al., 1995). This spread may slow the rise time of the responses, as activation would be staggered in time as glutamate diffuses to distant receptors. In addition, receptors and transporters located further away from the synaptic clefts would experience a lower concentration of transmitter, slowing activation due to the concentration dependence of binding. As the decay of the AMPA receptor and transporter responses in patches were slower in response to lower concentrations of L-glutamate, this dilution of glutamate could also contribute to the slow decay of synaptic responses. The higher apparent affinity of the glutamate transporters, estimated from the dose-response relationship (EC_{50} (transporter) = 58 μM; n = 8 vs. EC_{50} (AMPA receptor) = 1.15 mM; n = 5; see Fig. 4), suggests that they will be activated further away from release sites where the concentration of glutamate would be too low for significant AMPA receptor activation.

A number of studies have suggested that aspartate is released from CFs, although the more than 10 fold greater affinity of vesicular transporters for glutamate has cast doubt on this hypothesis (Nicholls and Attwell, 1990). The data presented here suggest that aspartate cannot be the sole CF transmitter, as substantial glutamate must be released to account for the activation of AMPA receptors (Patneau and Mayer, 1990). However,

these results do not exclude the possibility that some aspartate is released along with glutamate. The kinetics of transporter currents in patches elicited by L-aspartate were dramatically slower than those elicited by L-glutamate (compare Fig. 2*E* and 2*F*), possibly reflecting the higher affinity of this agonist for these transporters (K_m = 6.5 ± 3 μM, L-aspartate; K_m = 12 ± 4 μM, L-glutamate)(Klockner et al., 1994). If aspartate were released with glutamate the time course of the synaptically activated transport current would be slower, with the degree of slowing dependent on the relative proportion of aspartate to glutamate released.

Although micromolar levels of ambient glutamate have been measured in the extracellular space using microdialysis techniques (Rothstein et al., 1996) and theoretical predictions based on the stoichiometry of transport suggest that transporters may be able to lower extracellular glutamate to submicromolar levels at equilibrium (Zerangue and Kavanaugh, 1996), the data presented here suggest that the concentration of glutamate outside the synaptic cleft is not static but fluctuates in the vicinity of synapses where release has occurred. The fact that glutamate escapes the cleft and remains elevated extrasynaptically for several milliseconds suggests that glial transporters may determine the degree to which receptors located outside the cleft are activated following each release event.

References

Arriza, JL, Fairman, WA, Wadiche, JI, Murdoch, GH, Kavanaugh, MP, and Amara, SG (1994) Functional comparisons of three glutamate transporter subtypes cloned from human motor cortex. J Neurosci 14: 5559-69.

Barbour, B, Keller, BU, Llano, I, and Marty, A (1994) Prolonged presence of glutamate during excitatory synaptic transmission to cerebellar Purkinje cells. Neuron 12: 1331-43.

Baude, A, Molnar, E, Latawiec, D, McIlhinney, RA, and Somogyi, P (1994) Synaptic and nonsynaptic localization of the GluR1 subunit of the AMPA- type excitatory amino acid receptor in the rat cerebellum. J Neurosci 14: 2830-43.

Bruns, D, Engert, F, and Lux, HD (1993) A fast activating presynaptic reuptake current during serotonergic transmission in identified neurons of Hirudo. Neuron 10: 559-72.

Chaudhry, FA, Lehre, KP, van Lookeren Campagne, M, Ottersen, OP, Danbolt, NC, and Storm-Mathisen, J (1995) Glutamate transporters in glial plasma membranes: highly differentiated localizations revealed by quantitative ultrastructural immunocytochemistry. Neuron 15: 711-20.

Clark, BA, and Barbour, B (1997) Currents evoked in Bergmann glial cells by parallel fibre stimulation in rat cerebellar slices. J Physiol (Lond) 502: 335-50.

Clements, JD, Lester, RA, Tong, G, Jahr, CE, and Westbrook, GL (1992) The time course of glutamate in the synaptic cleft. Science 258: 1498-501.

de Barry, J, Langley, OK, Vincendon, G, and Gombos, G (1982) L-glutamate and L-glutamine uptake in adult rat cerebellum: an autoradiographic study. Neuroscience 7: 1289-97.

Diamond, JS, and Jahr, CE (1997) Transporters Buffer Synaptically Released Glutamate on a Submillisecond Time Scale. J Neuroscience 17: 4672-4687.

Hestrin, S, Sah, P, and Nicoll, RA (1990) Mechanisms generating the time course of dual component excitatory synaptic currents recorded in hippocampal slices. Neuron 5: 247-53.

Isaacson, JS, and Nicoll, RA (1993) The uptake inhibitor L-trans-PDC enhances responses to glutamate but fails to alter the kinetics of excitatory synaptic currents in the hippocampus. J Neurophysiol 70: 2187-91.

Isaacson, JS, and Walmsley, B (1995) Counting quanta: direct measurements of transmitter release at a central synapse. Neuron 15: 875-84.

Ito, M (1984) The Cerebellum and Neuronal Control. New York: Raven.

Jonas, P, and Sakmann, B (1992) Glutamate receptor channels in isolated patches from CA1 and CA3 pyramidal cells of rat hippocampal slices. J Physiol (Lond) 455: 143-71.

Jonas, P, and Spruston, N (1994) Mechanisms shaping glutamate-mediated excitatory postsynaptic currents in the CNS. Curr Opin Neurobiol 4: 366-72.

Kavanaugh, MP, Bendahan, A, Zerangue, N, Zhang, Y, and Kanner, BI (1997) Mutation of an amino acid residue influencing potassium coupling in the glutamate transporter GLT-1 induces obligate exchange. J Biol Chem 272: 1703-8.

Klockner, U, Storck, T, Conradt, M, and Stoffel, W (1994) Functional properties and substrate specificity of the cloned L- glutamate/L-aspartate transporter GLAST-1 from rat brain expressed in Xenopus oocytes. J Neurosci 14: 5759-65.

Lehre, KP, Levy, LM, Ottersen, OP, Storm-Mathisen, J, and Danbolt, NC (1995) Differential expression of two glial glutamate transporters in the rat brain: quantitative and immunocytochemical observations. J Neurosci 15: 1835-53.

Linden, DJ (1997) Long-term potentiation of glial synaptic currents in cerebellar culture. Neuron 18: 983-94.

Mennerick, S, and Zorumski, CF (1994) Glial contributions to excitatory neurotransmission in cultured hippocampal cells. Nature 368: 59-62.

Nicholls, D, and Attwell, D (1990) The release and uptake of excitatory amino acids [see comments]. Trends Pharmacol Sci 11: 462-8.

Otis, TS, Kavanaugh, MP, and Jahr, CE (1997) Postsynaptic glutamate transport at the climbing fiber-Purkinje cell synapse. Science 277: 1515-8.

Otis, TS, Wu, YC, and Trussell, LO (1996) Delayed clearance of transmitter and the role of glutamate transporters at synapses with multiple release sites. J Neurosci 16: 1634-44.

Palay, SL, and Chan-Palay, V. (1974). The Neuroglial Cells of the Cerebellar Cortex and The Climbing Fiber. In Cerebellar Cortex Cytology and Organization, S. L. Palay and V. Chan-Plalay, eds. (New York, Heidelberg, Berlin: Springer-Verlag), pp. 288-321.

Patneau, DK, and Mayer, ML (1990) Structure-activity relationships for amino acid transmitter candidates acting at N-methyl-d-aspartate and quisqualate receptors. J. Neurosci. 10: 2385-2399.

Robinson, MB, and Dowd, LA (1997) Heterogeneity and functional properties of subtypes of sodium-dependent glutamate transporters in the mammalian central nervous system. Adv Pharmacol 37: 69-115.

Rothstein, JD, Dykes-Hoberg, M, Pardo, CA, Bristol, LA, Jin, L, Kuncl, RW, Kanai, Y, Hediger, MA, Wang, Y, Schielke, JP, and Welty, DF (1996) Knockout of glutamate transporters reveals a major role for astroglial transport in excitotoxicity and clearance of glutamate. Neuron 16: 675-86.

Rothstein, JD, Martin, L, Levey, AI, Dykes-Hoberg, M, Jin, L, Wu, D, Nash, N, and Kuncl, RW (1994) Localization of neuronal and glial glutamate transporters. Neuron 13: 713-725.

Sarantis, M, Ballerini, L, Miller, B, Silver, RA, Edwards, M, and Attwell, D (1993) Glutamate uptake from the synaptic cleft does not shape the decay of the non-NMDA component of the synaptic current. Neuron 11: 541-9.

Shibata, T, Watanabe, M, Tanaka, K, Wada, K, and Inoue, Y (1996) Dynamic changes in expression of glutamate transporter mRNAs in developing brain. Neuroreport 7: 705-9.

Spacek, J (1985) Three-dimensional analysis of dendritic spines. III. Glial sheath. Anat Embryol 171: 245-52.

Takahashi, M, Sarantis, M, and Attwell, D (1996) Postsynaptic glutamate uptake in rat cerebellar Purkinje cells. J Physiol. 497: 523-530.

Tanaka, K, Watase, K, Manabe, T, Yamada, K, Watanabe, M, Takahashi, K, Iwama, H, Nishikawa, T, Ichihara, N, Kikuchi, T, Okuyama, S, Kawashima, N, Hori, S, Takimoto, M, and Wada, K (1997) Epilepsy and exacerbation of brain injury in mice lacking the glutamate transporter GLT-1. Science 276: 1699-702.

Tong, G, and Jahr, CE (1994) Block of glutamate transporters potentiates postsynaptic excitation. Neuron 13: 1195-203.

Wadiche, JI, Amara, SG, and Kavanaugh, MP (1995) Ion fluxes associated with excitatory amino acid transport. Neuron 15: 721-8.

Wadiche, JI, Arriza, JL, Amara, SG, and Kavanaugh, MP (1995) Kinetics of a human glutamate transporter. Neuron 14: 1019-27.

Wahl, LM, Pouzat, C, and Stratford, KJ (1996) Monte Carlo simulation of fast excitatory synaptic transmission at a hippocampal synapse. J Neurophysiol 75: 597-608.

Zerangue, N, and Kavanaugh, MP (1996) Flux coupling in a neuronal glutamate transporter. Nature 383: 634-7.

Zhang, S, and Trussell, LO (1994) A characterization of excitatory postsynaptic potentials in the avian nucleus magnocellularis. J Neurophysiol 72: 705-18.

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The Concentration of Synaptically Released Glutamate Outside of the Climbing Fiber-Purkinje Cell Synaptic Cleft

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Abstract

AMPA receptors and glutamate transporters expressed by cerebellar Bergmann glial cells are activated by neurotransmitter released from climbing fibers (Bergles et al., 1997). Based on anatomical evidence, this is most likely the result of glutamate diffusing out of the climbing fiber - Purkinje cell synaptic clefts (Palay and Chan-Palay, 1974). We used the change in the EC_{50} of the Bergmann glia AMPA receptors produced by cyclothiazide (CTZ) to estimate the concentration of glutamate reached at the glial membrane. The decrease of the EC_{50} gives rise to a concentration-dependent potentiation of the AMPA receptor-mediated responses (Patneau et al., 1993). By comparing the increase in amplitude of the AMPA response in the Bergmann glia (840% ± 240%, n=8) to the shift in the glutamate dose response curve measured in excised patches (EC_{50}: 1810 μM in control vs. 304 μM in CTZ), we estimate that the extrasynaptic transmitter concentration reaches 160 - 190 μM. This contrasts with the concentration in the synaptic cleft, thought to rapidly rise above 1 mM, but is still high enough to activate glutamate receptors. These results indicate that the sphere of influence of synaptically released glutamate can extend beyond the synaptic cleft.

(Key Words: ion channels, AMPA receptors, glutamate transporters, glutamate, extrasynaptic, glia)

Vesicular release of the neurotransmitter glutamate from presynaptic terminals results in millimolar levels of glutamate in the synaptic cleft (Clements et al., 1992; Clements, 1996). The close apposition of the pre- and postsynaptic membranes limits the dilution of neurotransmitter before reaching the postsynaptic receptors and thereby ensures a high

concentration of glutamate activates these receptors. The actions of the glutamate that diffuses out of the synaptic cleft are less clear. Eventually, high affinity Na^+ dependent transporters in the surrounding membranes take up the glutamate (Nicholls and Attwell, 1990; Kanai et al., 1993). Although glutamate transporters are capable of reducing extracellular glutamate to submicromolar levels, attaining such low concentrations is not instantaneous (Zerangue and Kavanaugh, 1996). For example, release of glutamate at excitatory synapses in both cerebellum and hippocampus results in transporter activation in surrounding glia for over 10 ms (Bergles et al., 1997; Bergles and Jahr, 1997; Clark and Barbour, 1997). The concentration reached outside of parallel and climbing fiber synapses on cerebellar Purkinje cells is sufficient to activate low affinity AMPA receptors on the surrounding Bergmann glia (Bergles et al., 1997; Clark and Barbour, 1997).

The emerging picture of the perisynaptic space is that of a region where the concentration of transmitter can be transiently elevated with synaptic activity. These increases in extrasynaptic glutamate can result in modulation of synaptic transmission via activation of both metabotropic (Scanziani et al., 1997) and ionotropic (Clarke et al., 1997) receptors. The concentration of glutamate reached outside of the cleft after synaptic release is critical in determining the extent of extrasynaptic receptor activation. Recent estimates based on the concentration dependence of the kinetics of AMPA receptor responses in outside-out patches indicated that the glutamate concentration peaks at < 250 μM at the Bergmann glia membranes (Bergles et al., 1997). However, the responses below this concentration were too small to analyze, limiting the measurement. By using CTZ to alter the dose-response relation of glutamate at Bergmann glia AMPA

receptors, we report that the glutamate concentration transiently peaks at 160 - 190 μM in the extrasynaptic space. This concentration is sufficient for activation of perisynaptic glutamate receptors.

Materials and Methods

Slices and Solutions. Sagittal slices of 13-15 day old rat cerebella were cut at a thickness of 300 μm using a vibratome (Ted Pella Intruments, St Louis, MO) in an ice cold external solution containing (in mM) 119 NaCl, 2.5 KCl, 2.5 $CaCl_2$, 1.3 $MgCl_2$, 1 NaH_2PO_4, 26.2 $NaHCO_3$, 11 glucose, and bubbled with 95% O_2 / 5% CO_2. The slices were placed in the same solution warmed to 34° C for 15-30 min and then stored at room temperature. During recordings the slices were perfused with the above solution with the addition of 100 μM picrotoxin and 5 μM (DL)-3-(2-Carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP). Patch experiments were carried out in the presence of 200 μM DL-threo-β-hydroxy-aspartic acid (THA) to isolate the α-Amino-3-hydroxy-5-methyl-4 isoxazolepropionic acid (AMPA) receptor response, with an extracellular solution containing (in mM) 135 NaCl, 5.4 KCl, 5 HEPES, 1.8 $CaCl_2$, 1.3 $MgCl_2$, at pH 7.4. THA has previously been shown to have no direct effect on AMPA receptors (Tong and Jahr, 1994). For the synaptic and dose response experiments in Bergmann glia the pipette solutions contained (in mM) 130 KNO_3, 20 HEPES, 10 EGTA, and 1 $MgCl_2$, pH 7.2. For the voltage jump experiments the internal solution had (in mM) 100 Cs-methanesulfonate, 20 TEA-Cl, 20 HEPES, 10 EGTA , 1 $MgCl_2$, 3.1 Mg-ATP, 0.3 Na_2-GTP, and 4 phosphocreatine, pH 7.2; this solution approximately doubled the input resistance (28 ± 14 MΩ with KNO_3 to 58 ± 18 MΩ with Cs-methanesulfonate). For the

synaptic and patch experiments in Purkinje cells the pipette solution contained (in mM) 107.5 Cs-gluconate, 10 TEA-Cl, 20 HEPES, 10 EGTA, 0.3 Na2-GTP, 4 Mg-ATP, and 8 NaCl, pH 7.2. Sources of chemicals: L-glutamate, HEPES, EGTA, Mg-ATP, phosphocreatine, THA, $CaCl_2$, and $MgCl_2$, (SIGMA, St. Louis, MO); NaCl, KCl, NaH_2PO_4, $NaHCO_3$, and glucose, (Mallinckrodt, Paris, Kentucky); Na_2-GTP, (Boehringer Mannheim, Germany), 6-Nitro-7-sulphamoylbenzo(f)quinoxaline-2,3-dione (NBQX), (Tocris, Ballwin, MO); ± CPP, CTZ, and picrotoxin, (RBI, Natick, MA).

Recording and Perfusion Techniques. Bergmann glia were identified by their size (~10 μm soma diameter) and location (near the Purkinje cell bodies) using a 40x water-immersion objective on an upright microscope (Zeiss Axioskop, Germany) equipped with IR/DIC optics. Further confirmation of their identity was provided by their large negative membrane potential (-90 to -80 mV), low input resistance (28 ± 14 MΩ with KNO_3), and lack of action potentials. Bergmann glia currents were recorded at their resting potential. Purkinje cell bodies were identified by their large size (20 to 30 μm), layered arrangement, and large dendritic tree. Purkinje cell EPSCs were recorded near – 10 mV to minimize the current amplitude. Patch pipettes were pulled from WPI (Sarasota, FL) glass number PG10165-4 and had resistances of 1.5 - 3 MΩ. Climbing fibers were stimulated with a theta glass pipette (Warner Instruments Cat. No. TGC150-10, Hamden, CT) pulled to a 4 to 6 μm tip and filled with external solution. The stimulating electrode was placed in the granule cell layer between 10 and 50 μm from the Purkinje cell layer. A constant current stimulator (Warner Instruments, Hamden, CT) was used to supply a 100 μs pulse of 10 – 100 μA. The pipette was repositioned and stimulus intensity adjusted until the current required to elicit an all or none response was

minimized. This was done to eliminate any significant parallel fiber activation. We tested for parallel fiber activation by stimulating at an intensity near threshold that produced some climbing fiber failures, and only proceeded when the failures were complete, that is, when no residual response was evident. Synaptic currents were filtered at 1 kHz and sampled at 10 kHz and outside-out patch currents were filtered at 2-5 kHz and sampled at 50kHz. A theta glass flowpipe mounted on a piezoelectric bimorph (Vernitron, Bedford, OH) was used for agonist applications to outside-out patches as described previously (Lester and Jahr, 1992; Tong and Jahr, 1994). The ability to change the solution flowing through both sides of the flowpipe was added via miniature manifolds (Warner Instruments, Hamden, CT) (Bergles et al., 1997). The solutions were allowed to flow for at least 2 minutes between conditions to allow complete exchange through the manifold and tubing. Two to three concentrations with and with-out CTZ present were tested in each patch, and the amplitudes were normalized to the response to 3 mM glutamate with-out CTZ. Data was acquired and some analysis done using AXOBASIC software (Axon Instruments, Foster City, CA), and further analysis was preformed using Origin 5.0 (Microcal Software, Northampton, MA), Igor Pro (Wavemetrics, Lake Oswego, OR), and InStat (Graph Pad Software, SanDiego, CA). Experiments were performed at room temperature (22 - 24° C) unless otherwise noted. Values are given as mean ± standard deviation, and p values are for paired Student's t-tests unless noted otherwise.

Voltage Jump Protocol. A novel method to estimate the time course of synaptic conductances introduced by Pearce (1993) has been formalized by Häusser and Roth (1997), and the experiments illustrated in figure 6 were based on that method. Each trial

consisted of a single climbing fiber stimulation combined with a voltage step from –50 mV to –80 mV. There were 32 trials in a single run through the experiment and the voltage was stepped at a different time in each trial, 1 ms increments were used at times near the climbing fiber stimulus and 4 ms increments were used at earlier and later times. The process was repeated several times (5-14) for averaging purposes, and 200 μM CTZ was used to increase the size of the currents. Interleaved voltage jump trials without synaptic stimulation were subtracted, and the charge transfer was calculated by integration. The recovered charge was plotted versus time relative to the stimulation. The resulting curve was fitted with the following equation, where s is the time of the jump relative to the stimulus, $\tau_{epscrise}$ and $\tau_{epscdecay}$ are the time constants of the conductance approximated by the sum of two exponentials, τ_v is the time constant of the voltage change at the conductance, A is a constant, and Q is the recovered charge:

For $s \leq 0$

$$Q = A\left(\tau_{epscrise} - \tau_{epscdecay} + e^{s/\tau_v}\left(\frac{1}{1/\tau_{epscdecay} + 1/\tau_v} - \frac{1}{1/\tau_{epscrise} + 1/\tau_v}\right)\right)$$

For $s > 0$

$$Q = A\left(\tau_{epscrise} e^{-s/\tau_{epscrise}} - \tau_{epscdecay} e^{-s/\tau_{epscdecay}} + \frac{e^{-s/\tau_{epscdecay}}}{1/\tau_{epscdecay} + 1/\tau_v} - \frac{e^{-s/\tau_{epscrise}}}{1/\tau_{epscrise} + 1/\tau_v}\right)$$

The fits were carried out using custom Igor macros written by Dr. Michael Häusser. Two controls were performed to confirm the linearity of the membrane between –50 and –80 mV. Holding at –70 mV and stepping ± 5, 10, 15 and 20 mV resulted in currents which overlapped exactly when scaled by the command voltage (data not shown). In addition,

Bergmann glia AMPA receptor responses recorded at –50 mV had the same time course as those recorded at –80 mV (Figure 6c).

NEURON simulation. Using the Windows version of the program NEURON (Hines, 1993), a template for a Bergmann glial cell was constructed with the following parameters: soma, 10 μm long, 10 μm diameter, 10 segments; 12 processes, 300 μm long, 0.5 μm diameter, 100 segments; and end feet, 5 μm long, 5 μm diameter, 10 segments (Palay and Chan-Palay, 1974; de Blas, 1984; Bergles et al., 1997). Two identical neighboring cells were connected to the recorded cell via cylinders 1 μm long and 1 μm in diameter. The electrical parameters were: R_a = 150 Ω-cm, C_m = 1 μF/cm^2, g_m = 100 μS/cm^2 in the soma and processes, and g_m = 500 μS/cm^2 in the end feet for simulations of recordings using the Cs-methanesulfonate based internal solution. For the KNO_3 based internal solution the passive membrane conductances were made 4 times higher. The reversal potential for the passive conductances was set at – 90 mV.

The model parameters above were determined by an iterative process. In addition to the general morphology of the Bergmann glial cell (Palay and Chan-Palay, 1974; de Blas, 1984; Bergles et al., 1997), the passive response of the Bergmann glia to a square 1 nA current injection was reproduced along with the amount of filtering estimated by the Häusser voltage jump analysis. The simulated passive response was constrained to an input resistance of 28 MΩ and a single exponential rise of 1.4 ms using the KNO_3 internal solution. With the Cs-methanesulfonate solution, the rise was 5.8 ms and the input resistance was 59 MΩ. The simulation was further constrained to filter a synaptic conductance that decays with a tau of 5.9 ms such that the EPSC recorded at the soma

decays with a tau of 8.4 ms, using the Cs-methanesulfonate internal solution. The synaptic conductance was simulated with a point process consisting of a sum of one rising and one decaying exponential placed on each process. The location of the synapse was one of the parameters altered in the development of the model and in the final model each synapse was located 210 μm from the soma.

This model was used to estimate the error in the peak amplitude of the Bergmann glia AMPA receptor response to climbing fiber stimulation in the presence and absence of CTZ. To reproduce the experimental data the synaptic conductance parameters were set as follows: in CTZ, τ_{rise} = 1.22 ms, τ_{decay} = 8.34 ms, and g_{max} = 570 pS; and in control, τ_{rise} = 0.4 and 0.7 ms, τ_{decay} = 3.85 ms, and g_{max} = 67 and 64 pS. A range of values were used for the rise time in control because it was not uniquely defined by the system; values down to 100 μs were slowed by cable filtering to near the 0.83 ± 0.27 ms 20-80% rise time measured at the soma. In patches from Bergmann glia somata the 20-80% rise time of a response to 250 μM glutamate was 0.6 ms (Bergles et al., 1997). Thus, a range around this value was used for the synaptic conductance to account for differences in concentration and speed of agonist application.

Results

CTZ increases the Bergmann glia AMPA receptor response

CTZ increases the apparent affinity of AMPA receptors (Patneau et al., 1993; Yamada and Tang, 1993; Partin et al., 1994; Partin et al., 1996). We used this property of CTZ to measure the concentration of glutamate reaching the AMPA receptors in the Bergmann glial cell membrane.

As reported previously (Bergles et al., 1997), climbing fiber stimulation in cerebellar slices activates currents in Bergmann glia mediated by both AMPA receptors and glutamate transporters. After establishing a stable baseline of the combined AMPA receptor / transporter current for five minutes (stimulating every 15-20 seconds), a saturating concentration of CTZ (200 μM) was applied while continuing to stimulate. Over a period of minutes, the amplitude of the response increased dramatically (Figure 1a and b). After the response stabilized, the AMPA receptor antagonist NBQX (10 μM) was applied to isolate the transporter current (Figure 1a and 1b) (Bergles et al., 1997; Clark and Barbour, 1997). Separation of the AMPA receptor-mediated response in the presence and absence of CTZ was achieved by subtracting the transporter current from the combined responses (Figure 1c). For this subtraction to be valid the transporter current must be unaffected by CTZ, as will be shown below.

CTZ increased the peak amplitude of the AMPA receptor component 840 ± 240% ($p<0.0001$, $n=8$) and slowed the time course. The 20-80% rise-time of the response in CTZ was 1.6 ± 0.5 ms compared to 0.8 ± 0.3 ms for the control response and the half-decay time was 9.4 ± 2.8 ms in CTZ versus 4.3 ± 2.9 ms in control ($n = 8$). This slowing is due at least in part to the relief of desensitization, that under control conditions is likely to truncate the rising phase and speed the decay of the response. An increased affinity would also prolong the response, as receptors may remain occupied longer and be sensitive to lower concentrations of glutamate diffusing to more remote receptors.

There is evidence that the effects of extrasynaptic glutamate are reduced at physiological temperatures (Kullmann and Asztely, 1998; Asztely et al., 1997). The strong temperature dependence of transport (Wadiche et al., 1995) and the expression of

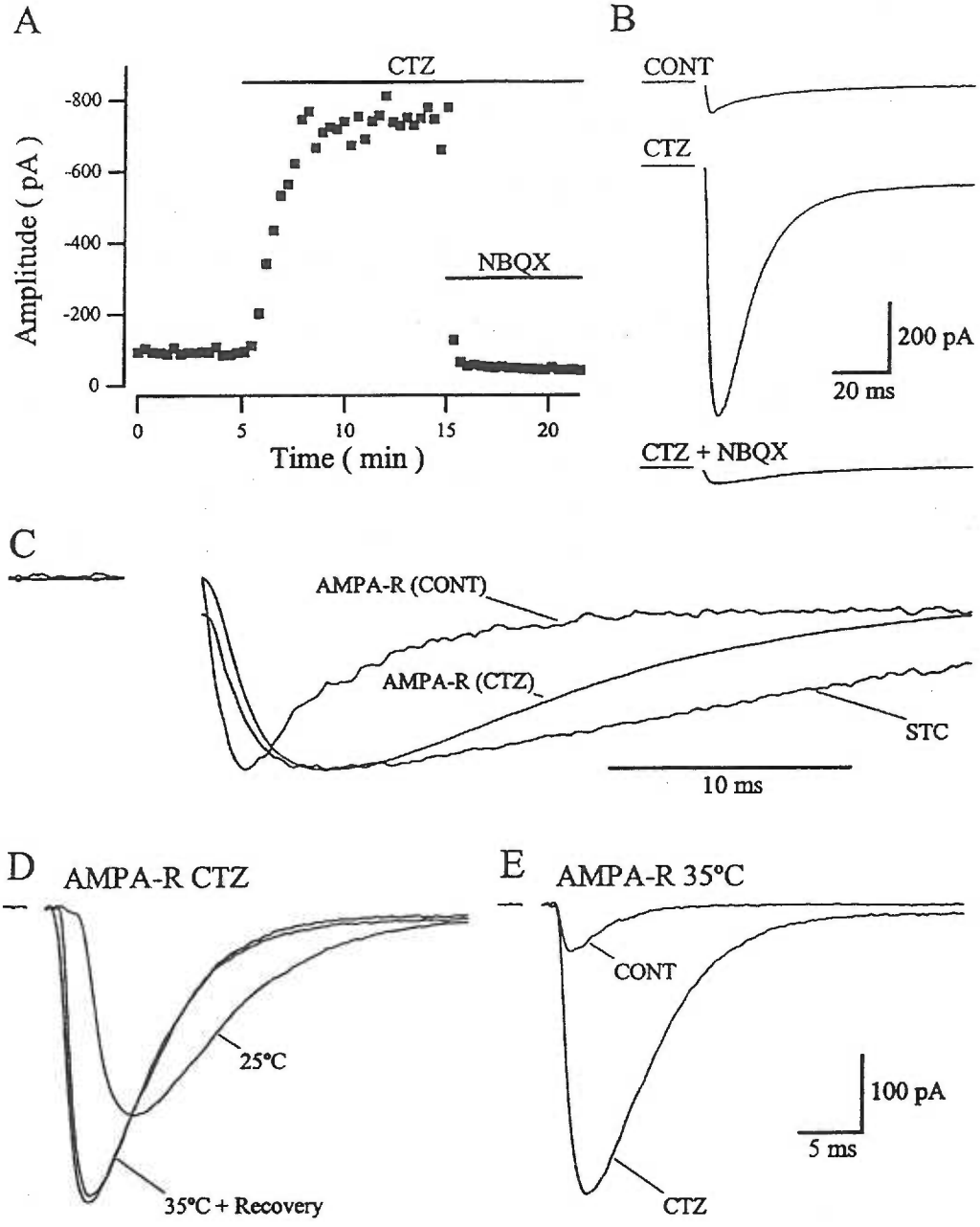

Figure 1. ***The effects of CTZ and temperature on the Bergmann glial cell's response to climbing fiber activation.***

(A) A plot of the peak amplitude of the Bergmann response to climbing fiber stimulation every 20 seconds. After five minutes of stable baseline, 200 μM CTZ was applied in the perfusate followed ten minutes later by 10 μM NBQX. The cell's resting/holding potential was –88 mV. (B) Averages of five sweeps under control conditions, in CTZ, and in NBQX. (C) Comparison of the time course of the different components of the

response isolated by subtraction of the synaptically activated transporter current (STC), the current remaining in NBQX. Averages of five sweeps were scaled to their peaks. (D) The AMPA receptor-mediated response in CTZ at 35°C, at 25°C and again at 35°C. (E) The AMPA receptor-mediated response (STC subtracted) at 35°C under control (CONT) conditions and with 200 μM CTZ present (CTZ). The responses in (D) and (E) are from a different cell than in parts (A), (B), and (C), and are averages of five responses. This cell's resting/holding potential was –82 mV.

glutamate transporters in the postsynaptic Purkinje cells (Rothstein et al., 1994) may decrease the glutamate diffusing to the Bergmann glia membranes at physiological temperatures. However, consistent with the hippocampal astrocyte recordings of Bergles and Jahr (1998), the glial transporter and AMPA receptor-mediated currents were present in the Bergmann astrocytes at elevated temperatures. Raising the bath temperature from 25°C to 35°C slightly increased the amplitude and speeded the kinetics of the Bergmann glia responses (Figure 1d). At 35°C the 20-80% rise-time and half-decay time for the control AMPA receptor-mediated responses were 0.6 ± 0.2 ms and 3.0 ± 1.9 ms respectively, and in 200 μM CTZ they were 0.8 ± 0.1 ms and 5.5 ± 0.3 ms (n = 3). The effect of CTZ on the amplitude of the AMPA receptor-mediated response at 35°C was not significantly different from that seen at room temperature (Figure 1e) (740 ± 150% increase, p = 0.53, unpaired t-test). The remainder of the experiments were carried out at room temperature.

The Bergmann glia transporter current as a monitor of climbing fiber release probability

There is evidence that at some synapses CTZ can increase the probability of release (Barnes-Davies and Forsythe, 1995; Diamond and Jahr, 1995) which would complicate our analysis of transmitter concentration. Several findings indicate that CTZ alters climbing fiber release probability very little. First, the paired pulse ratio (30 ms interval) of the Bergmann glia AMPA receptor-mediated response to climbing fiber activation was unaffected by CTZ (0.15 ± 0.08 in control vs. 0.14 ± 0.06 in CTZ, p = 0.60, n = 7). Second, the transporter currents recorded at the end of experiments in which

CTZ was present were the same proportion of the initial control dual-component response as those recorded at the end of experiments in the absence CTZ (40.4 ± 18.3 %, n = 8, vs. 44.3 ± 13.4 %, n = 17, p = 0.55, unpaired t-test). Third, the transporter current isolated with NBQX was not affected by the addition of 200 μM CTZ (Figure 2a) (101 ± 10 % of control p=0.94, n = 3). These results suggest that the probability of release is not altered by CTZ at this synapse. However, it is possible that the transporter current is insensitive to changes in release due to saturation of the transporters. To demonstrate that they are sensitive to such changes, the release probability was altered while monitoring the transporter current (Figure 2b). Transporter currents were reduced to 63 ± 12 % of control ($p < 0.0001$, n = 8) by adding 5 μM $CdCl_2$ and increased to 116 ± 3 % of control ($p < 0.003$, n = 4) by raising external Ca^{+2} to 7.5 mM. A large increase was not expected because the probability of release at this synapse is already very high (Dittman and Regehr, 1998; Silver et al., 1998).

Purkinje cell AMPA receptor response as a monitor of climbing fiber release probability

CTZ was also ineffective in altering the climbing fiber release probability as measured by Purkinje cell responses. The amplitude of the Purkinje cell EPSC resulting from climbing fiber stimulation was unchanged by application of 200 μM CTZ (100 ± 7 % of control, p = 0.96, n = 5) (Figure 3). The paired pulse ratio of the climbing fiber – Purkinje cell EPSC was also not altered by CTZ (0.44 ± 0.16 in control vs. 0.38 ± 0.06 in CTZ; p = 0.46, n = 4) (Figure 3c). Consistent with the lack of a CTZ effect on the amplitude of the Purkinje cell EPSCs, the amplitude of patch responses to a saturating

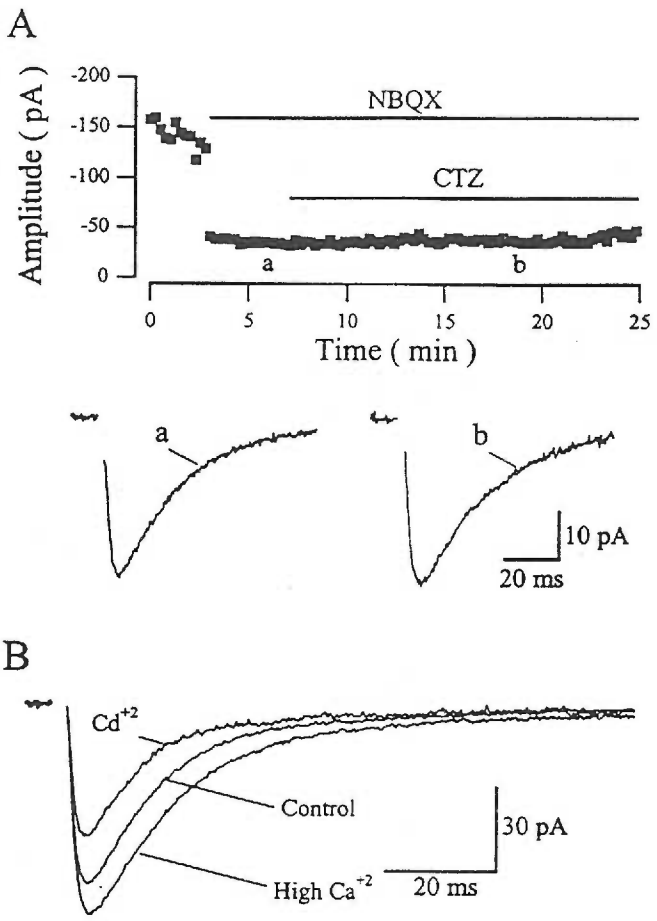

Figure 2. ***The Bergmann glia transporter current indicates no effect of CTZ on climbing fiber release.***

(A) *top:* Peak amplitude plot of the Bergmann glia response. The AMPA response was blocked with 10 μM NBQX and no increase in amplitude of the remaining transport current was seen upon application of 200 μM CTZ. *bottom:* Averages of ten traces with the last trace located at the time points indicated in the amplitude plot. The cell's membrane potential was –80 mV. (B) The transporter current (isolated with 10 μM NBQX) of a different cell showing the effects of adding 5 μM Cd^{+2} or 5 mM Ca^{+2} to the external solution. Averages of 5 to 15 traces. This cell's membrane potential was –78 mV.

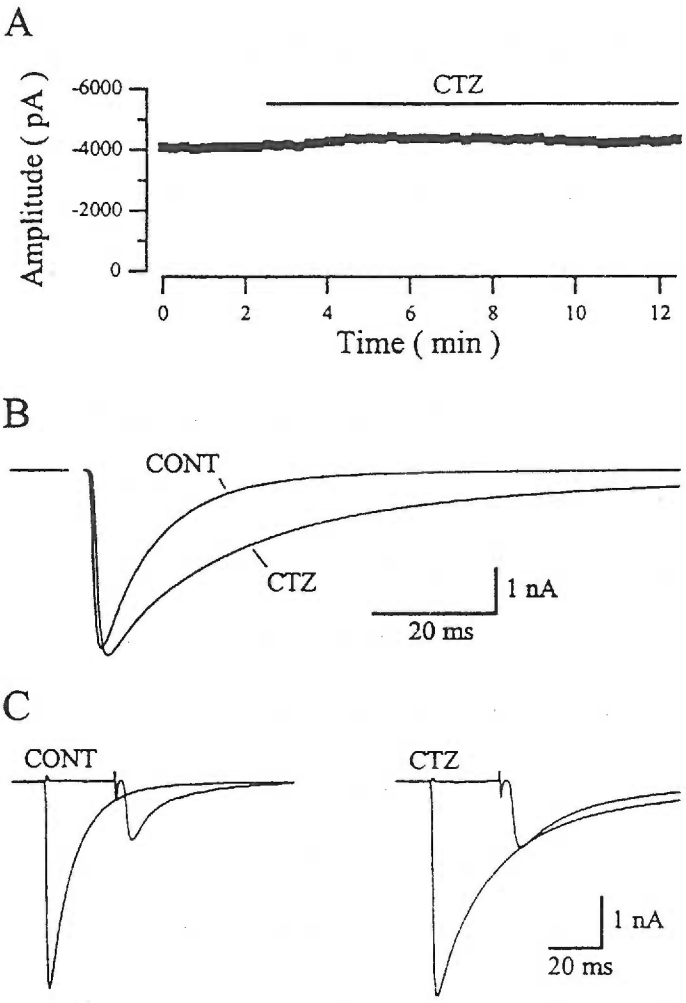

Figure 3. ***The Purkinje cell EPSC shows no increase of climbing fiber release in CTZ.*** (A) Peak amplitude plot of the Purkinje cell EPSC evoked by climbing fiber stimulation. After a stable baseline period, 200 μM CTZ was added to the external solution. The cell was held at –6 mV. (B) Averages of five responses under control conditions and in the presence of 200 μM CTZ. (C) The above averages along with the second of a pair of responses isolated by subtraction of the single responses. The paired stimuli were delivered 30 ms apart.

dose (3 mM) of glutamate were also unaffected by CTZ (109 ± 10 % of control, p = 0.55, n = 4). These results argue that the large increase in amplitude of the Bergmann glia AMPA receptor response caused by CTZ is not the result of an increase in climbing fiber release probability but rather is due to a direct effect of CTZ on the apparent affinity of the AMPA receptors.

Estimation of transmitter concentration at the Bergmann glia membrane

To estimate the peak transmitter concentration at Bergmann glia AMPA receptors, we compared the CTZ-induced increase in peak amplitude of the climbing fiber response to the ratio of AMPA receptor dose-response relationships in the presence and absence of CTZ. Dose-response relationships were determined with rapid applications of glutamate to outside-out patches taken from Bergmann glia somata. CTZ caused a dose-dependent potentiation of the glutamate response, concomitant with a relief of receptor desensitization. As an example of the concentration dependence, the average peak amplitude of the response to 300 μM glutamate was increased 7.0-fold whereas the 3 mM response increased by only 2.3-fold (Figure 4).

As shown in figure 5a, there was an increase in both the apparent affinity for glutamate (control, EC_{50} = 1810 μM; CTZ, EC_{50} = 304 μM) and the maximal response (1.5-fold) in CTZ. Measurements were made at the peak of the responses. Using the ratio of the two dose-response curves we generated a curve which indicates the expected fold increase in the receptor response for concentrations raging from 3 μM to 30 mM (Figure 5b). The climbing fiber-evoked Bergmann glia response increased 8.4-fold which corresponds to 192 μM on the ratio curve. The 95% confidence interval for the fold increase is 10.4 to 6.4, indicating a range of 80 to 372 μM glutamate. This

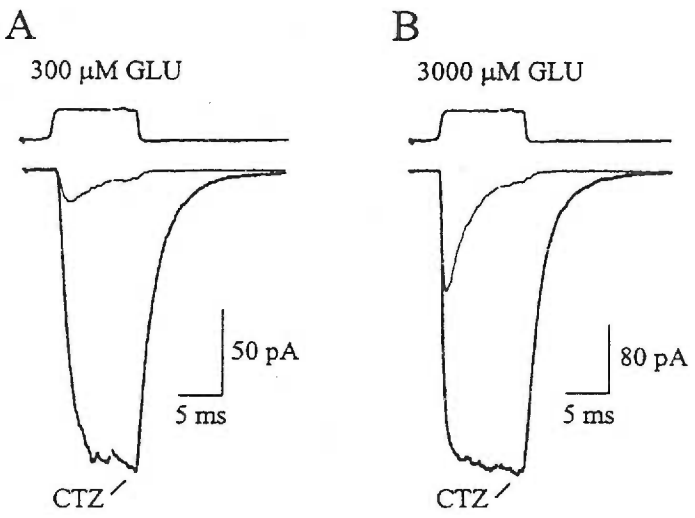

Figure 4. ***CTZ increased Bergmann glia patch responses to glutamate in a dose dependent manner.***

(A) Responses of an outside-out patch to 300 and 3000 μM glutamate (GLU) under control conditions (thin lines) and in the presence of 200 μM CTZ (thick lines). The 300 μM response was increased 9.6 fold by CTZ while the 3000 μM response was only increased 2.5 fold. The responses are averages of 20 – 40 traces. To account for a small amount of rundown, the conditions were repeated 2-3 times interleaving the various conditions. The patch was held at –80 mV. (B) The same four responses superimposed and at the same scale. The applications were 10 ms long and separated by 10 seconds.

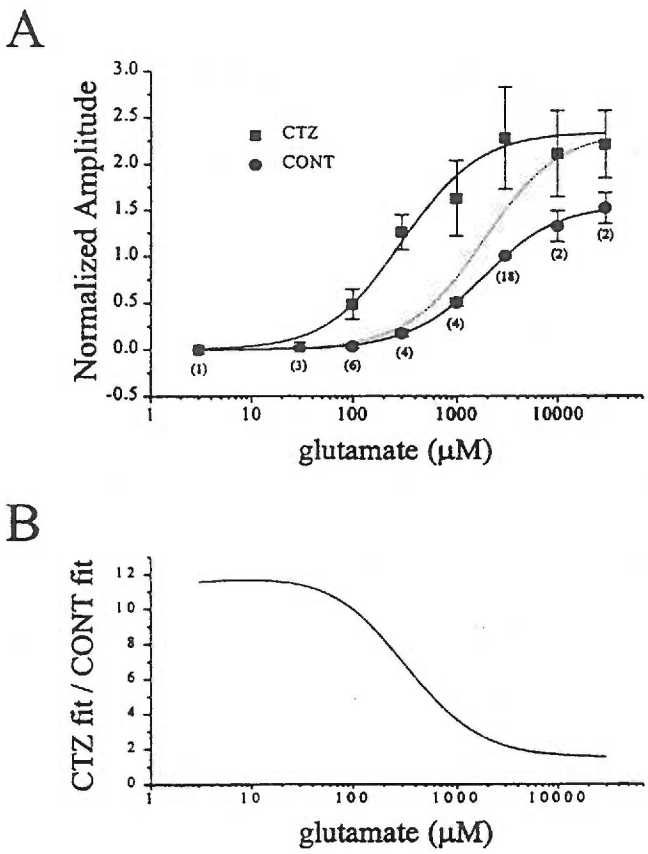

Figure 5. ***Quantification of the glutamate reaching the Bergmann glia AMPA receptors.***

(A) Dose-response curves were constructed using data from a total of 18 patches. Two or three concentrations ± CTZ were tested in each patch. The patch responses, including those in CTZ, were all normalized to 3 mM glutamate under control conditions. The control EC_{50} for glutamate was 1810 μM and in the presence of CTZ it was 304 μM. The dotted line is the fit of the control data multiplied by the increase in maximal amplitude (1.5 fold), illustrating what the data would look like with no change in affinity. (B) The ratio of the CTZ fit over the control fit plotted against concentration. The average fold increase of the Bergmann glia AMPA response (8.4 fold) falls at 192 μM on the x-axis.

estimation of the concentration of glutamate at the Bergmann glia AMPA receptors (190 μM) is consistent with our earlier estimates (<250 μM) based on a kinetic analysis (Bergles et al., 1997).

Voltage jump analysis of somatic recordings

In the presence of CTZ, AMPA receptor currents in patches do not desensitize (Figure 4). If CTZ affects synaptically-activated AMPA receptors on Bergmann glia similarly, the time course of the AMPA mediated conductance should reflect the period over which clearance of glutamate from the extracellular space occurs. This is because the intrinsic kinetics of the receptors, as demonstrated by patch recordings (Figure 4) are rapid relative to the synaptic responses (Bergles et al., 1997). To determine if the current recorded at the soma accurately reflects the kinetics of the conductance, a voltage jump analysis introduced by Pearce (1993) and formalized by Häusser and Roth (Häusser and Roth, 1997) was employed in the presence of CTZ. This method not only extracts information about the amount of filtering of the recorded signals, but also gives an indication of the average electrotonic distance of the conductances from the soma.

The method is based on the observation that a change in the driving force will affect the amount of charge transfer only if it occurs during the active conductance. This is illustrated in Figure 6a, which shows the voltage jump procedure at three separate time points (relative to the synaptic stimulation) applied to the Bergmann glia AMPA receptor-mediated response in the presence of 200 μM CTZ. In Figure 6b, the time integral of the AMPA receptor response (the charge transfer) is plotted versus the time at which the voltage was jumped. The charge recovery curves were made up of thirty-two time points; each point was an average of 5 to 14 measurements in a single cell. The

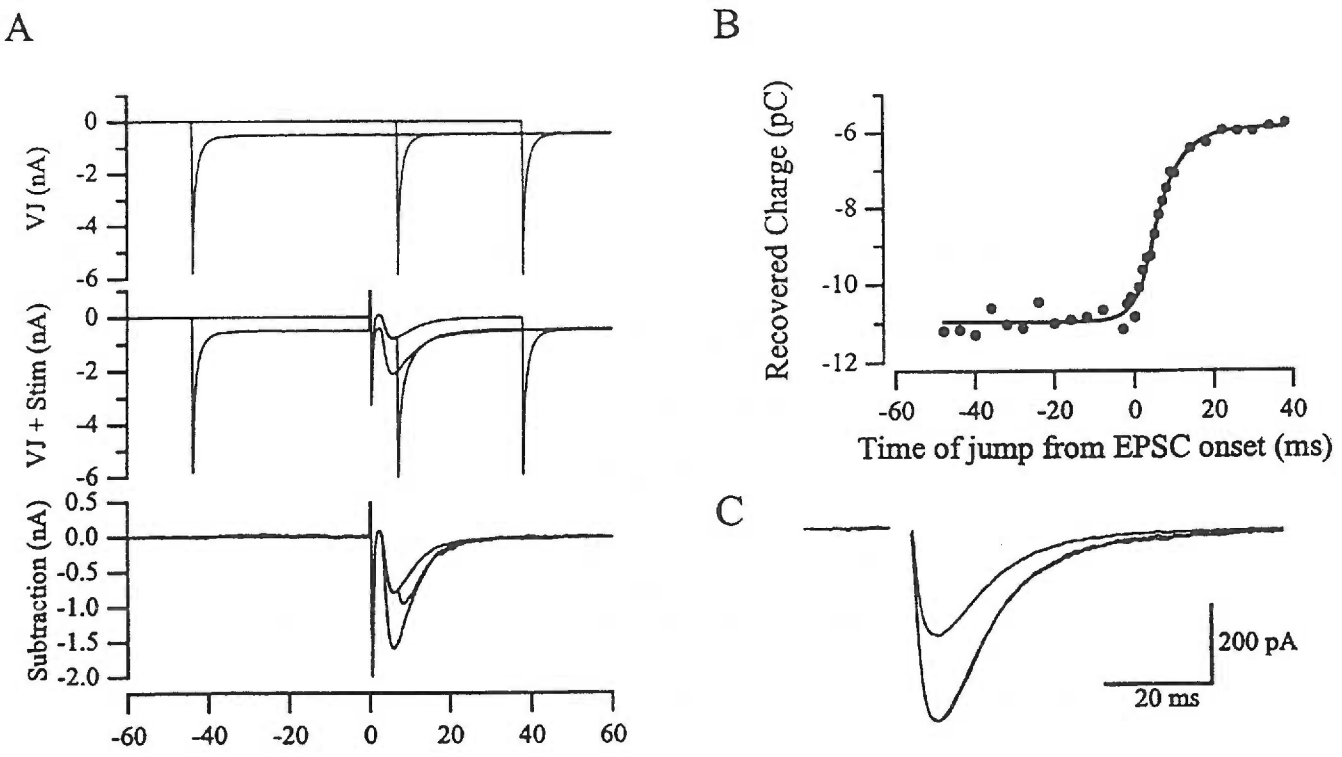

Figure 6. ***Voltage jump analysis of the Bergmann glia response.***

(A) Three example time points superimposed to illustrate the voltage jump protocol. *top:* Currents resulting from jumping the holding voltage from –50 mV to –80 mV at –44, 8, and 38 ms relative to the stimulus time point. *middle:* The command voltages are the same and climbing fiber stimulation is added at t = 0. *bottom:* Subtractions of the currents in the top panel from the middle panel. These are integrated to calculate the charge transferred. These experiments were carried out in the presence of 200 μM CTZ to maximize the amount of charge recovered. (B) The resulting charge recovery curve fit with the full analytical function (see methods) and with a single exponential fit to the data to the right of 3 ms. The $\tau_{voltage}$ = 3.2 ms, the τ_{decay} = 6.0 ms, and the single exponential fit gave a τ = 5.5 ms. A single exponential fit to the average response recorded at the soma had a time constant of 6.6 ms. (C) Averages of five responses at –50 mV and at –80 mV. The response at –50 mV is also scaled up to the peak of the –80 mV response to compare their time course.

curve was fitted with an analytical function (see methods; Häusser and Roth, 1997) in which the exponential time constant for the charging of the membrane (where the conductance occurs) and the decay of the synaptic conductance were allowed to vary. The smallest interval measured was 1 ms so the time constant for the rise time was fixed at 1 ms (a rise time of 0.5 or 1.5 ms did not affect the results of the fitting).

The average time constant of the decay of the conductance calculated from the full analytical fit of the charge recovery curves was 5.9 ± 1.6 ms (n = 7). This is not significantly different from single exponential fits to the charge recovery curves starting 4 ms after the synaptic stimulation (5.8 ± 1.4 ms, p = .657, n = 7). This was significantly faster than the decay of the Bergmann glia AMPA receptor response recorded with a somatic recording electrode (8.4 ± 1.0 ms, p = .004, n = 7), demonstrating that there is some filtering of the conductance (a 42% slowing of the decay). The average time constant for the charging of the membrane at the active conductance was 4.4 ± 1.9 ms, indicating the average conductance is electrotonically distant from the soma. This is consistent with the observation that most of the climbing fiber synaptic contacts are out in the dendritic tree of the Purkinje cell (Palay and Chan-Palay, 1974) and with the filtering of the decay time course.

Our estimation of the transmitter concentration reaching the Bergmann glial membranes depends on our somatic measurement of the amplitude ratio of the receptor responses in the presence and absence of CTZ. While taking a ratio eliminates concerns of a uniform error in the amplitude measurement, the slowing and large increase in amplitude of the AMPA receptor response in CTZ may lead to differential amounts of

filtering and voltage escape. We were unable to apply the voltage jump analysis to the Bergmann responses under control conditions because of their small size and variability. Therefore, we used the information on filtering gained from the voltage jump analysis in CTZ to construct a simple NEURON simulation addressing these concerns.

The model was constructed based on the general morphology of Bergmann glial cells (Palay and Chan-Palay, 1974; de Blas, 1984; Bergles et al., 1997), the passive response of the cells to a square current injection, and the extent of filtering indicated by the voltage jump analysis. The inclusion of end feet with a conductance five times higher than the rest of the cell (Newman, 1986) was critical in reproducing the electrical properties of the Bergmann glia. Holding the other model parameters constant, the synaptic conductance kinetics and maximum value were altered so that the simulated AMPA receptor responses monitored at the soma matched the experimental data in the presence and absence of CTZ (Figure 7). The unfiltered current flowing at the conductance under perfect voltage clamp can by calculated by the NEURON simulation. The perfectly clamped synaptic current peaked at 207 – 217 pA (see methods) in control (experimental value recorded at soma: 34 pA, Figure 7a) and 1850 pA in CTZ (experimental value recorded at soma: 286 pA, Figure 7b). Accounting for cable filtering and voltage escape, the fold increase in the amplitude of the AMPA receptor response with CTZ should be adjusted from 8.4 fold (corresponding to 192 μM) to 8.5 – 8.9 fold indicating a range of 160 – 186 μM glutamate on the ratio curve.

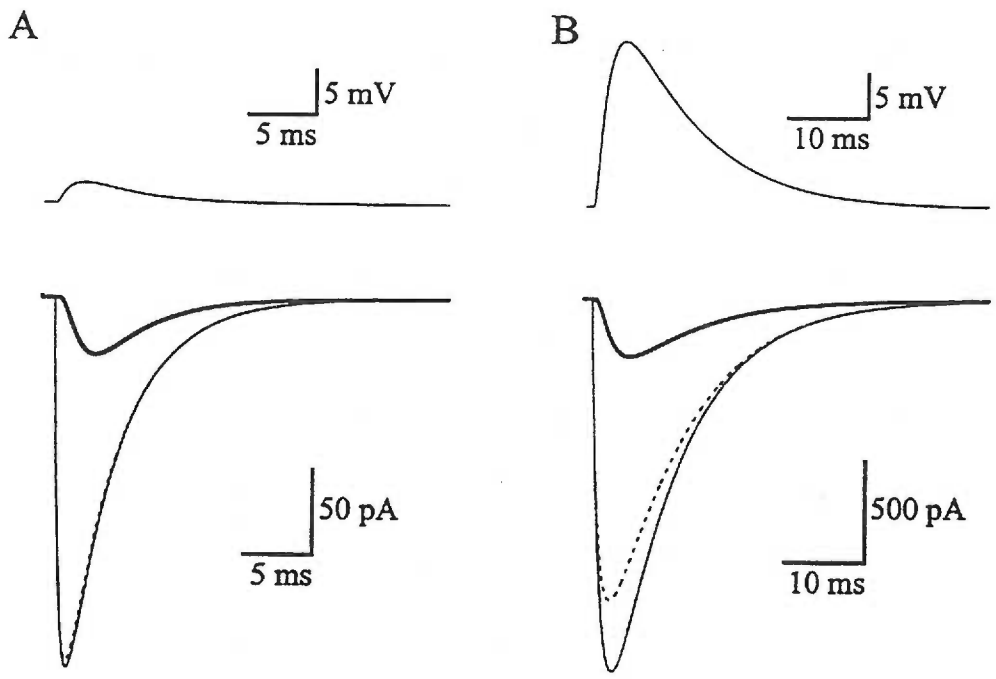

Figure 7. ***Simulation of somatic recordings of the Bergmann glia AMPA receptor responses.***

(A) Simulated recordings under control conditions. *Top:* Membrane voltage at the synapse. *bottom:* Current measured at the soma (bold), flowing at the synapse (dotted), and flowing at the synapse under perfect voltage clamp (thin). (B) Simulated recordings in the presence of CTZ. *Top:* Membrane voltage at the synapse. *bottom:* Current measured at the soma (bold), flowing at the synapse (dotted), and flowing at the synapse under perfect voltage clamp (thin).

Discussion

Our results, indicating that 160 - 190 μM glutamate reaches the Bergmann glia membrane after climbing fiber stimulation, depend on the modulation of the Bergmann glia AMPA receptor response by CTZ. CTZ has been shown to greatly reduce desensitization and to increase the apparent affinity of the AMPA receptors (Patneau et al., 1993; Yamada and Tang, 1993; Partin et al., 1994; Partin et al., 1996). The mechanism of its action is not completely understood, but is thought to occur via a destabilization of the desensitized state or a stabilization of the closed nondesensitized state (Trussell et al., 1993; Yamada and Tang, 1993; Partin et al., 1994; Trussell et al., 1994; Partin et al., 1996). The important property of CTZ's action for this study is that it produced a concentration-dependent increase in the response of the receptors. This allowed the quantification of the glutamate concentration at the Bergmann glia membrane by comparing the CTZ-induced increase of the synaptic response to that of the patch responses to a range of glutamate concentrations.

In contrast to Bergmann glia responses, the amplitudes of Purkinje cell EPSC and patch responses were not altered by CTZ. This result is similar to that of Dittman and Regehr (1998) who found that climbing fiber-EPSC peak amplitudes measured in Purkinje cells were slightly reduced by CTZ. The high concentrations of glutamate in the Purkinje cell experiments (>1mM in the cleft, and 3 mM for patches) can partially explain this result, as an increase in affinity would have little effect near saturation. However, even at the top of the dose response curve there was a fifty percent increase in the Bergmann glia patch response. This difference between Purkinje cell patch and Bergmann glia patch responses may be caused by the faster kinetics of the Bergmann

AMPA receptors leading to a significant amount of desensitization at the peak of the response in control conditions. *In situ* hybridization and single cell PCR studies indicate that Purkinje cells and Bergmann glial cells express AMPA receptors of different subunit compositions and this may explain the difference in modulation by CTZ. GluR A flip/flop, B flip/flop, and C flip are expressed in Purkinje cells whereas GluR A and D, primarily in the flip form, are present in the Bergmann glia (Burnashev et al., 1992; Lambolez et al., 1992; Baude et al., 1994; Muller and Kettenmann, 1995). The dominance of the flip splice variants in the Bergmann glia cells is consistent with a larger effect of CTZ on these cells (Partin et al., 1994). Additionally, while there is not a predominance of flip subunits in the Purkinje cells, GluR B is the most abundant subunit present and heteromeric receptors that contain the GluR B flip subunit desensitize relatively slowly (Partin et al., 1994). This may explain the lack of any increase in the Purkinje cell AMPA receptor response. If the Purkinje cell AMPA receptors desensitize much more slowly than they activate, very few receptors would be desensitized at the peak of the response and CTZ would not alter the amplitude.

The observation that CTZ increased the peak amplitude of the Bergmann glia climbing fiber response to the same degree at 35°C and 25°C (740 ± 150% vs. 840 ± 240 %, $p = 0.53$) suggests that a similar peak concentration of glutamate reaches the glial AMPA receptors at the two temperatures. Despite the strong temperature dependence of transport (Wadiche et al., 1995), it is possible that the initial bolus of glutamate that quickly reaches the nearby Bergmann glia is relatively unaffected by the faster transport at physiological temperatures. The turnover time for glutamate transporters at elevated temperatures may be as fast as 12 ms (Bergles and Jahr, 1998), slower than the time to

peak of the Bergmann glia AMPA receptor response (2-5 ms). Although the faster decay of the Bergmann responses at 35°C is consistent with a more rapid clearance of glutamate and more efficient synapse sequestration, it appears the initial wave of glutamate reaching the Bergmann glia AMPA receptors is too quick to be altered significantly by an increased transport rate.

The variability in the degree of potentiation by CTZ of the Bergmann glia AMPA response (840 ± 240 %, n=8) may indicate some biological variability in the concentration of glutamate seen by the Bergmann glia. The spatial arrangement of the receptors around the cleft, the degree of synapse envelopment, the maturity of the cell being recorded from, and other factors may vary from cell to cell and give rise to different average concentrations seen by a given Bergmann glia. Interestingly, in searching for the primary all-or-none climbing fiber response in the Bergmann glia, there was occasionally a secondary all-or-none input. Since Bergmann glia processes are not planar like Purkinje cell dendrites (de Blas, 1984), each Bergmann glial cell may interact with more than one climbing fiber.

Previously CTZ has been linked to an increase the probability of release at some synapses (Barnes-Davies and Forsythe, 1995; Diamond and Jahr, 1995, but see Isaacson and Walmsley 1996, and Mennerick and Zorumski, 1995). In contrast, we show here that the climbing fiber release probability was not significantly altered by CTZ. This difference might be explained by the high release probability of the climbing fiber terminal (Dittman and Regehr, 1998; Silver et al., 1998). The strong paired-pulse depression seen at this synapse along with little increase in synaptic strength with elevated external calcium indicate that release probability is near maximal (Dittman and

Regehr, 1998; Silver et al., 1998). Consistent with our results, Dittman and Regehr (1998) recently showed little effect of CTZ on the paired pulse ratio of climbing fiber evoked EPSCs recorded in Purkinje cells. The presynaptic action of CTZ on terminals with a low probability of release was confirmed in one of our Purkinje cell recordings in which we monitored the effects of CTZ on a parallel fiber input. These inputs have a low release probability, as demonstrated by their paired pulse facilitation (Atluri and Regehr, 1996). In contrast to the climbing fiber-evoked response in Purkinje cells, the parallel fiber EPSCs increased about 6-fold and the paired-pulse ratio switched from facilitation to depression in CTZ. The variability in the presynaptic effects of CTZ with different pathways may also reflect differential expression of CTZ receptivity in the terminals.

In the presence of CTZ, we estimate that the Bergmann glia AMPA receptor conductance decays with a time constant of 6 ms. This should reflect the decay of the glutamate concentration at these receptors since there is no desensitization in CTZ and the intrinsic kinetics of the receptors are rapid. This is faster but not inconsistent with our previous report of a 17 ms decay of extrasynaptic glutamate based on the Bergmann glia transporter current (Bergles et al., 1997). Our current estimate is in part faster because it accounts for the cable filtering and voltage escape inherent in somatic voltage clamp recordings. In addition, the lower apparent affinity of the AMPA receptors would make them less sensitive than the transporters to reduced concentrations of glutamate diffusing to more distant areas. It is also possible that the AMPA receptors may be more localized to the membranes surrounding the synaptic clefts (Baude et al., 1994), while the transporters may cover more surface area of the Bergmann glia.

The main result of this paper is that glutamate escapes from the climbing fiber-Purkinje cell cleft and can reach perisynaptic membranes at concentrations of 160 -190 μM. This concentration is sufficient to activate all classes of glutamate receptors including metabotropic, NMDA, and non-NMDA receptors. There is a growing body of evidence indicating that synaptic transmission can be modulated by glutamate that escapes from the synaptic cleft (Clarke et al., 1997; Forsythe and Barnes-Davies, 1997; Scanziani et al., 1997). There is also evidence that the high affinity NMDA receptors at one synapse may sense glutamate released from neighboring synapses (Kullmann and Asztely, 1998; Rusakov and Kullmann, 1998). The results presented here demonstrate a basic requirement of both of these phenomena, that glutamate can escape the synaptic cleft and reach the surrounding membranes at a concentration sufficient to activate glutamate receptors. This alters the standard view of the actions of glutamate in the central nervous system, expanding the sphere of influence of synaptically released glutamate beyond the postsynaptic membrane.

References

Asztely, F, Erdemli, G, and Kullmann, DM (1997) Extrasynaptic glutamate spillover in the hippocampus: dependence on temperature and the role of active glutamate uptake. Neuron 18: 281-293.

Atluri, PP, and Regehr, WG, (1996) Determinants of the Time Course of Facilitation at the Granule Cell to Purkinje Cell Synapse. J Neurosci 16: 5661-5671.

Barnes-Davies, M, and Forsythe, ID (1995) Pre- and postsynaptic glutamate receptors at a giant excitatory synapse in rat auditory brainstem slices. J Physiol (Lond) 488: 387-406.

Baude, A, Molnar, E, Latawiec, D, McIlhinney, RA, and Somogyi, P (1994) Synaptic and nonsynaptic localization of the GluR1 subunit of the AMPA- type excitatory amino acid receptor in the rat cerebellum. J Neurosci 14: 2830-2843.

Bergles, DE, Dzubay, JA, and Jahr, CE (1997) Glutamate transporter currents in bergmann glial cells follow the time course of extrasynaptic glutamate. Proc Natl Acad Sci U S A 94: 14821-14825.

Bergles, DE, and Jahr, CE (1997) Synaptic activation of glutamate transporters in hippocampal astrocytes. Neuron 19: 1297-1308.

Bergles, DE, and Jahr, CE (1998) Glial contribution to glutamate uptake at Schaffer collateral- commissural synapses in the hippocampus. J Neurosci 18: 7709-7716.

Burnashev, N, Khodorova, A, Jonas, P, Helm, PJ, Wisden, W, Monyer, H, Seeburg, PH, and Sakmann, B (1992) Calcium-permeable AMPA-kainate receptors in fusiform cerebellar glial cells. Science 256: 1566-1570.

Clark, BA, and Barbour, B (1997) Currents evoked in Bergmann glial cells by parallel fibre stimulation in rat cerebellar slices. J Physiol (Lond) 502: 335-350.

Clarke, VR, Ballyk, BA, Hoo, KH, Mandelzys, A, Pellizzari, A, Bath, CP, Thomas, J, Sharpe, EF, Davies, CH, Ornstein, PL, Schoepp, DD, Kamboj, RK, Collingridge, GL, Lodge, D, and Bleakman, D (1997) A hippocampal GluR5 kainate receptor regulating inhibitory synaptic transmission. Nature 389: 599-603.

Clements, JD (1996) Transmitter timecourse in the synaptic cleft: its role in central synaptic function. Trends Neurosci. 19: 163-171.

Clements, JD, Lester, RA, Tong, G, Jahr, CE, and Westbrook, GL (1992) The time course of glutamate in the synaptic cleft. Science 258: 1498-1501.

de Blas, AL (1984) Monoclonal antibodies to specific astroglial and neuronal antigens reveal the cytoarchitecture of the Bergmann glia fibers in the cerebellum. J Neurosci 4: 265-273.

Diamond, JS, and Jahr, CE (1995) Asynchronous release of synaptic vesicles determines the time course of the AMPA receptor-mediated EPSC. Neuron 15: 1097-1107.

Dittman, JS, and Regehr, WG (1998) Calcium Dependence and Recovery Kinetics of Presynaptic Depression at the Climbing Fiber to Purkinje Cell Synapse. J Neuroscience 18: 6147-6162.

Forsythe, ID, and Barnes-Davies, M (1997) Synaptic transmission: well-placed modulators. Curr Biol 7: R362-365.

Häusser, M, and Roth, A (1997) Estimating the time course of the excitatory synaptic conductance in neocortical pyramidal cells using a novel voltage jump method. J Neurosci 17: 7606-7625.

Hines, M. (1993). NEURON - a program for simulation of nerve equations. In Neural systems: analysis and modeling, F. H. Eeckman, ed. (Boston: Kluwer), pp. 127 - 136.

Isaacson, JS, and Walmsley, B (1996) Amplitude and time course of spontaneous and evoked excitatory postsynaptic currents in bushy cells of the anteroventral cochlear nucleus. J Neurophysiol 76: 1566-71.

Kanai, Y, Smith, CP, and Hediger, MA (1993) The elusive transporters with a high affinity for glutamate. TINS 16: 365-370.

Kullmann, DM, and Asztely, F (1998) Extrasynaptic glutamate spillover in the hippocampus: evidence and implications. Trends Neurosci. 21: 8-14.

Lambolez, B, Audinat, E, Bochet, P, Crepel, F, and Rossier, J (1992) AMPA receptor subunits expressed by single Purkinje cells. Neuron 9: 247-258.

Lester, RA, and Jahr, CE (1992) NMDA channel behavior depends on agonist affinity. J Neurosci 12: 635-643.

Mennerick, S and Zorumski, CF (1995) Presynaptic influence on the time course of fast excitatory synaptic currents in cultured hippocampal cells. J. Neurosci. 15: 3178-3192.

Muller, T, and Kettenmann, H (1995) Physiology of Bergmann glial cells. Int Rev Neurobiol 38: 341-359.

Newman, EA (1986) High potassium conductance in astrocyte endfeet. Science 233: 453-454.

Nicholls, D, and Attwell, D (1990) The release and uptake of excitatory amino acids. Trends Pharmacol Sci 11: 462-468.

Palay, SL, and Chan-Palay, V. (1974). The Neuroglial Cells of the Cerebellar Cortex and The Climbing Fiber. In Cerebellar Cortex Cytology and Organization, S. L. Palay and V. Chan-Plalay, eds. (New York, Heidelberg, Berlin: Springer-Verlag), pp. 288-321.

Partin, KM, Fleck, MW, and Mayer, ML (1996) AMPA receptor flip/flop mutants affecting deactivation, desensitization, and modulation by cyclothiazide, aniracetam, and thiocyanate. J Neurosci 16: 6634-6647.

Partin, KM, Patneau, DK, and Mayer, ML (1994) Cyclothiazide differentially modulates desensitization of alpha-amino-3- hydroxy-5-methyl-4-isoxazolepropionic acid receptor splice variants. Mol Pharmacol 46: 129-138.

Patneau, DK, Vyklicky, L, Jr., and Mayer, ML (1993) Hippocampal neurons exhibit cyclothiazide-sensitive rapidly desensitizing responses to kainate. J Neurosci 13: 3496-3509.

Pearce, RA (1993) Physiological evidence for two distinct GABAA responses in rat hippocampus. Neuron 10: 189-200.

Rothstein, JD, Martin, L, Levey, AI, Dykes-Hoberg, M, Jin, L, Wu, D, Nash, N and Kuncl, RW (1994) Localization of neuronal and glial glutamate transporters. Neuron 13: 713-725.

Rusakov, DA, and Kullman, DM (1998) Extrasynaptic Glutamate Diffusion in the Hippocampus: Ultrastructural Constraints, Uptake, and Receptor Activation. J Neuroscience 18: 3158-3170.

Scanziani, M, Salin, PA, Vogt, KE, Malenka, RC, and Nicoll, RA (1997) Use-dependent increases in glutamate concentration activate presynaptic metabotropic glutamate receptors. Nature 385: 630-634.

Silver, RA, Momiyama, A, and Cull-Candy, SG (1998) Locus of frequency-dependent depression identified with multiple- probability fluctuation analysis at rat climbing fibre-Purkinje cell synapses. J Physiol (Lond) 510: 881-902.

Tong, G, and Jahr, CE (1994) Block of glutamate transporters potentiates postsynaptic excitation. Neuron 13: 1195-1203.

Trussell, LO, Raman, IM, and Zhang, S (1994) AMPA receptors and rapid synaptic transmission. Sem. Neurosci. 6: 71-79.

Trussell, LO, Zhang, S, and Raman, IM (1993) Desensitization of AMPA receptors upon multiquantal neurotransmitter release. Neuron 10: 1185-1196.

Yamada, KA, and Tang, CM (1993) Benzothiadiazides inhibit rapid glutamate receptor desensitization and enhance glutamatergic synaptic currents. J Neurosci 13: 3904-3915.

Wadiche, JI, Arriza, JL, Amara, SG and Kavanaugh, MP (1995) Kinetics of a human glutamate transporter. Neuron 14: 1019-1027

Zerangue, N, and Kavanaugh, MP (1996) Flux coupling in a neuronal glutamate transporter. Nature 383: 634-637.

Discussion

NMDA Channel Kinetics

In our investigation of the kinetics of NMDA channel opening, we found that most of the channels present in an excised patch that open before agonist dissociation do so for the first time within 10 milliseconds. This is based on the observation that limiting the block by MK-801 to early in the response of an excised patch to a rapid application of agonist results in the same amount of block as when it is present throughout the response. We used two experimental manipulations to limit the time over which the block by MK-801 could occur. First we used the lower affinity agonist cysteate, which unbinds from the receptors faster than glutamate and gives rise to a more rapid decay. The fact that the amount of block with cysteate was similar to that found using glutamate indicates that most of the channels that open do so during the brief cysteate response. In the second set of experiments MK-801 was present only during a 10 ms application of glutamate. This short co-application of glutamate and MK-801 led to an amount of block similar to that found when the MK-801 was always present. These results support a scheme in which the NMDA channels open with a short first latency, have a peak open probability of 0.3, and repeated openings give rise to the long decay of the response.

Low-concentration steady-state experiments have indicated that NMDA channels have a low probability of opening and that delayed openings underlie the slow NMDA receptor-mediated response (Edmonds and Colquhoun, 1992). One possible explanation for these contrasting schemes of NMDA channel behavior is that fast applications of

saturating glutamate may lead to channel activations with a higher open probability than that found with continous applications of low-concentrations of agonists (Edmonds and Colquhoun, 1992). A jump into a high concentration of glutamate, as occurs in the synapse, may increase the likelihood of the channels entering the "high-P_o" periods seen by many researchers (Jahr and Stevens, 1987; Howe et al., 1988; Gibb and Colquhoun, 1991; Gibb and Colquhoun, 1992). The ideal experiment to address this possibility would be to use fast applications of saturating glutamate on an excised patch that contained a single NMDA channel. One could then measure the average first latency directly. Unfortunately this has proven impossible in native cells because the receptors are either expressed in clusters or at too high a density to pull a single-channel patch. Recently such an experiment has been carried out by expressing recombinant NMDA NR1a/NR2A receptors at very low densities in *Xenopus* oocytes (Wyllie et al., 1997). The data from these single-channel patches indicated a mean first latency of 20-30 ms and a peak open probability of 0.3 (Wyllie et al., 1997).

With the recent report by Wyllie et al. (1997) it appears there is a general consensus as to the behavior of NMDA channels in excised patches when rapidly exposed to a saturating concentration of glutamate (Jahr, 1992; Jahr, 1994; Lin and Stevens, 1994; Rosenmund et al., 1995; Dzubay and Jahr, 1996; Wyllie et al., 1997). However, two studies using MK-801 on synaptic NMDA receptors have reported contrasting results regarding the channel open probability (Hessler et al., 1993; Rosenmund et al., 1993). Hessler et al. (1993) report an open probability of 0.31, similar to that found with excised patches (Jahr, 1992; Jahr, 1994; Lin and Stevens, 1994; Rosenmund et al., 1995; Dzubay and Jahr, 1996; Wyllie et al., 1997). Rosenmund et al.

(1993) have found a much lower value of 0.053, closer to estimates made with continuous agonist applications (Huettner and Bean, 1988; Traynelis and Cull-Candy, 1990). Further study by Rosenmund et al. (1995) has indicated the P_o of NMDA channels is altered by patch excision, shifting from the low P_o (0.04) they find in whole cell and synaptic measurements to the relatively high P_o (0.3) generally found in outside-out patches. It is hypothesized that this change is due to a disruption of regulatory factors, perhaps involving an interaction with the cytoskeleton (Rosenmund and Westbrook, 1993; Rosenmund et al., 1995). Paradoxically, an increase in the current amplitude did not accompany the estimated increase in P_o. Also, using an alternative approach with another open channel blocker, 9-aminoacridine, Benviniste and Mayer (1995) have presented whole-cell evidence consistent with the higher P_o short first latency scheme.

Future directions. More work needs to be done on the issue of changes in NMDA channel properties associated with patch excision. Recently Clark et al. (1997) have shown, consistent with Rosenmund et al. (1995), that synaptic and extrasynaptic NMDA receptors have similar properties, but that channels in outside-out patches were altered. They found that the single channel conductance is reduced following formation of an outside-out patch (Clark et al., 1997). The possibility that synaptic NMDA channels behave differently than those in excised patches warrants further investigation of synaptic open probability and first latencies. Studies using less lipophillic blockers than MK-801 such as imipramine may help address this issue, ensuring accurate concentrations are attained in the synaptic cleft.

Bergmann glia transporter and AMPA receptor currents

We have demonstrated that fast application of glutamate to excised patches from cerebellar Bergmann glia gives rise to rapidly (<1 ms) activating glutamate transporter currents. The sub-millisecond binding of glutamate by the transporters suggests an efficient mechanism for the capture of extrasynaptic glutamate. The relatively slow 40 – 120 milliseconds estimated for a complete transport cycle of cloned and native transporters at room temperature (Wadiche et al., 1995; Bergles and Jahr, 1998; Otis and Jahr, 1998; Wadiche and Kavanaugh, 1998) indicate that the rapid binding of glutamate may be more important than the actual uptake in determining the time course of glutamate (Tong and Jahr, 1994; Diamond and Jahr, 1997). Climbing fiber activation resulted in rapidly rising AMPA receptor (1 ms) and glutamate transporter (2 ms) currents in the surrounding Bergmann glia. Based on the kinetics of these currents and those of the patch responses to a range of glutamate concentrations, the glutamate escaping from the cleft peaks within a millisecond at the Bergmann glia membrane at a concentration between 10 and 250 μM and remains elevated for several milliseconds. This transient increase of glutamate in the extrasynaptic space is higher than the ambient levels measured using microdialysis techniques (Rothstein et al., 1996), and much higher than theoretical equilibrium predictions based on the stoichiometry of transport (Zerangue and Kavanaugh, 1996).

In an effort to improve upon our initial measurements, we have used a novel method to estimate the peak concentration of glutamate reaching the Bergmann glial cell membrane outside the climbing fiber-Purkinje cell synapse. Based on the modulation of the AMPA receptor response by CTZ, we estimate that 160 – 190 uM glutamate reaches

the Bergmann receptors. This indicates that all classes of glutamate receptors could be activated by this escaping glutamate, supporting a developing hypothesis that transient elevations of extrasynaptic glutamate could act as a modulator of synaptic transmission (Clarke et al., 1997; Forsythe and Barnes-Davies, 1997; Scanziani et al., 1997). This relatively high concentration of glutamate reaching nearby membranes also has implications for the possibility of transmitter diffusing to receptors at adjacent synaptic contacts (Kullmann et al., 1996; Barbour and Hausser, 1997; Kullmann and Asztely, 1998; Rusakov and Kullmann, 1998). The morphology of the glial ensheathment and large quantal content at this synapse make it ideal for glial based measurement of extrasynaptic glutamate, but the ensheathment makes it less likely for spillover to occur. However, the estimate made here supports the possibility in general, especially at synapses were glial ensheathment is less complete (Palay and Chan-Palay, 1974; Spacek, 1985).

Glial recordings of the action of synaptically released glutamate are a relatively recent development in the study of the nervous system (Mennerick and Zorumski, 1994; Mennerick et al., 1996; Bergles et al., 1997; Bergles and Jahr, 1997; Clark and Barbour, 1997; Linden, 1997; Linden, 1998). The low input resistance and extended lamellar processes of glial cells distinguish them from neurons, and could prove detrimental to electrophysiological analysis. That is why the voltage jump method of Hausser and Roth and the NEURON simulation were useful in determining what effects the glial cell's electrical properties had on our results. Counter-intuitively the end feet with their high conductance for potassium may have actually been beneficial for the recordings. The lowering of the input resistance by the end feet tended to shorten the time needed to

charge the membrane and the large background potassium conductance acted as a biological clamp, helping to keep the cell's membrane at the reversal potential for potassium thereby minimizing voltage escape.

Future directions. The presence of calcium permeable AMPA receptors in the Bergmann glia, and their activation by synaptically released glutamate is intriguing. Elevations of cytosolic calcium has many possible downstream effects and has already been shown to reduce the conductance of both K^+ channels and gap junctions in the glial cell membrane (Muller and Kettenmann, 1995). It would be worth investigating a potential regulation of glutamate uptake by calcium influx through the Bergmann AMPA receptors, similar to what has been reported in the postsynaptic Purkinje cells (Kataoka et al., 1997).

Conclusions

Our results using the open channel blocker MK-801 indicate an average first latency of 10 ms for NMDA receptor channel opening in response to a rapid application of a saturating concentration of glutamate to an excised patch. This is consistent with the channels having a peak open probability of 0.3 and repeated openings underlying the long decay of the response. If synaptic NMDA channels have similar properties relatively few (5-30) channels would be required at individual synaptic sites to produce the NMDA receptor dependant calcium influx associated with synaptic plasticity and developmental regulation.

The binding kinetics of glutamate transporters measured in excised patches from Bergmann glia are rapid, suggesting an efficient mechanism for capturing extrasynaptic

glutamate. Rapidly activating AMPA receptor and glutamate transporter currents recorded in Bergmann glia indicate glutamate released from climbing fiber terminals quickly escapes synaptic clefts and reaches the surrounding glial membranes. The concentration dependence of the kinetics of patch responses as well as a pharmocological analysis using CTZ suggests that the glutamate transient at the Bergmann glial membranes reaches nearly 200 μM glutamate. This extrasynaptic wave of glutamate decays over several milliseconds.

References

Barbour, B, and Hausser, M (1997) Intersynaptic diffusion of neurotransmitter. Trends Neurosci 20: 377-384.

Bergles, DE, Dzubay, JA, and Jahr, CE (1997) Glutamate transporter currents in bergmann glial cells follow the time course of extrasynaptic glutamate. Proc Natl Acad Sci U S A 94: 14821-14825.

Bergles, DE, and Jahr, CE (1998) Glial Contribution to Glutamate Uptake at Schaffer Collateral-Commissural Synapses in the Hippocampus. J Neuroscience 18: 7709-7716.

Bergles, DE, and Jahr, CE (1997) Synaptic activation of glutamate transporters in hippocampal astrocytes. Neuron 19: 1297-1308.

Clark, BA, and Barbour, B (1997) Currents evoked in Bergmann glial cells by parallel fibre stimulation in rat cerebellar slices. J Physiol (Lond) 502: 335-350.

Clark, BA, Farrant, M, and Cull-Candy, SG (1997) A direct comparison of the single-channel properties of synaptic and extrasynaptic NMDA receptors. J Neurosci 17: 107-116.

Clarke, VR, Ballyk, BA, Hoo, KH, Mandelzys, A, Pellizzari, A, Bath, CP, Thomas, J, Sharpe, EF, Davies, CH, Ornstein, PL, Schoepp, DD, Kamboj, RK, Collingridge, GL, Lodge, D, and Bleakman, D (1997) A hippocampal GluR5 kainate receptor regulating inhibitory synaptic transmission. Nature 389: 599-603.

Diamond, JS, and Jahr, CE (1997) Transporters Buffer Synaptically Released Glutamate on a Submillisecond Time Scale. J Neuroscience 17: 4672-4687.

Dzubay, JA, and Jahr, CE (1996) Kinetics of NMDA channel opening. J Neurosci 16: 4129-4134.

Edmonds, B, and Colquhoun, D (1992) Rapid decay of averaged single-channel NMDA receptor activations recorded at low agonist concentration. Proc R Soc Lond B Biol Sci 250: 279-286.

Forsythe, ID, and Barnes-Davies, M (1997) Synaptic transmission: well-placed modulators. Curr Biol 7: R362-365.

Gibb, AJ, and Colquhoun, D (1992) Activation of *N*-methyl-d-aspartate receptors by l-glutamate in cells dissociated from adult rat hippocampus. J Physiol (Lond) 456: 143-179.

Gibb, AJ, and Colquhoun, D (1991) Glutamate activation of a single NMDA receptor-channel produces a cluster of channel openings. Proc. R. Soc. Lond. B. 243: 39-45.

Hessler, NA, Shirke, AM, and Malinow, R (1993) The probability of transmitter release at a mammalian central synapse. Nature 366: 569-572.

Howe, JR, Colquhoun, D, and Cull-Candy, SG (1988) On the kinetics of large-conductance glutamate-receptor ion channels in rat cerebellar granule neurons. Proc R Soc Lond B Biol Sci 233: 407-422.

Huettner, JE, and Bean, BP (1988) Block of *N*-methyl-D-aspartate-activated current by the anticonvulsant MK-801: Selective binding to open channels. Proc. Natl. Acad. Sci. USA 85: 1307-1311.

Jahr, CE (1992) High probability opening of NMDA receptor channels by L-glutamate. Science 255: 470-472.

Jahr, CE (1994) NMDA receptor kinetics and synaptic function. Semin Neurosci 6: 81-86.

Jahr, CE, and Stevens, CF (1987) Glutamate activates multiple single channel conductances in hippocampal neurons. Nature 325: 522-525.

Kataoka, Y, Morii, H, Watanabe, Y, and Ohmori, H (1997) A Postsynaptic Excitatory Amino Acid Transporter with Chloride Conductance Functionally Regulated by Neuronal Activity in Cerebellar Purkinje Cells. J Neuroscience 17: 7017-7024.

Kullmann, DM, and Asztely, F (1998) Extrasynaptic glutamate spillover in the hippocampus: evidence and implications. Trends Neurosci. 21: 8-14.

Kullmann, DM, Erdemli, G, and Asztely, F (1996) LTP of AMPA and NMDA receptor-mediated signals: Evidence for presynaptic expression and extrasynaptic glutamate spill over. Neuron 17: 461-474.

Lin, F, and Stevens, CF (1994) Both open and closed NMDA receptor channels desensitize. J Neurosci 14: 2153-2160.

Linden, DJ (1997) Long-term potentiation of glial synaptic currents in cerebellar culture. Neuron 18: 983-994.

Linden, DJ (1998) Synaptically evoked glutamate transport currents may be used to detect the expression of long-term potentiation in cerebellar culture. J Neurophysiol 79: 3151-3156.

Mennerick, S, Benz, A, and Zorumski, CF (1996) Components of glial responses to exogenous and synaptic glutamate in rat hippocampal microcultures. J Neurosci 16: 55-64.

Mennerick, S, and Zorumski, CF (1994) Glial contributions to excitatory neurotransmission in cultured hippocampal cells. Nature 368: 59-62.

Muller, T, and Kettenmann, H (1995) Physiology of Bergmann glial cells. Int Rev Neurobiol 38: 341-359.

Otis, TS, and Jahr, CE (1998) Anion Currents and Predicted Glutamate Flux through a Neuronal Glutamate Transporter. J Neuroscience 18: 7099-7110.

Palay, SL, and Chan-Palay, V. (1974). The Neuroglial Cells of the Cerebellar Cortex and The Climbing Fiber. In Cerebellar Cortex Cytology and Organization, S. L. Palay and V. Chan-Plalay, eds. (New York, Heidelberg, Berlin: Springer-Verlag), pp. 288-321.

Rosenmund, C, Clements, JD, and Westbrook, GL (1993) Nonuniform probability of glutamate release at a hippocampal synapse. Science 262: 754-757.

Rosenmund, C, Feltz, A, and Westbrook, GL (1995) Synaptic NMDA receptor channels have a low open probability. J Neurosci 15: 2788-2795.

Rosenmund, C, and Westbrook, GL (1993) Calcium-induced actin depolymerization reduces NMDA channel activity. Neuron 10: 805-814.

Rothstein, JD, Dykes-Hoberg, M, Pardo, CA, Bristol, LA, Jin, L, Kuncl, RW, Kanai, Y, Hediger, MA, Wang, Y, Schielke, JP, and Welty, DF (1996) Knockout of glutamate transporters reveals a major role for astroglial transport in excitotoxicity and clearance of glutamate. Neuron 16: 675-686.

Rusakov, DA, and Kullmann, DM (1998) Extrasynaptic Glutamate Diffusion in the Hippocampus: Ultrastructural Constraints, Uptake, and Receptor Activation. J Neuroscience 18: 3158-3170.

Scanziani, M, Salin, PA, Vogt, KE, Malenka, RC, and Nicoll, RA (1997) Use-dependent increases in glutamate concentration activate presynaptic metabotropic glutamate receptors. Nature 385: 630-634.

Spacek, J (1985) Three-dimensional analysis of dendritic spines. Anat Embrol 171: 245-252.

Tong, G, and Jahr, CE (1994) Block of glutamate transporters potentiates postsynaptic excitation. Neuron 13: 1195-1203.

Traynelis, SF, and Cull-Candy, SG (1990) Proton inhibition of *N*-methyl-D-aspartate receptors in cerebellar neurons. Nature 345: 347-350.

Wadiche, JI, Arriza, JL, Amara, SG, and Kavanaugh, MP (1995) Kinetics of a human glutamate transporter. Neuron 14: 1019-1027.

Wadiche, JI, and Kavanaugh, MP (1998) Macroscopic and microscopic properties of a cloned glutamate transporter/chloride channel. J Neuroscience 18: 7650-7661.

Wyllie, D, Edmonds, B, and Colquhoun, D (1997) Single activations of recombinant NMDA NR1a/NR2A receptors recorded in one-channel patches. J Physiology 501.P: 13P-14P.

Zerangue, N, and Kavanaugh, MP (1996) Flux coupling in a neuronal glutamate transporter. Nature 383: 634-637.