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Synaptic NMDA Receptor Channels Have a Low Open Probability

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Realistic estimates of channel-gating parameters of synaptic receptors are essential to an understanding of synaptic transmission and modulation. However, the gating of *N*-methyl-D-aspartate (NMDA) channels appears to differ, depending on recording conditions; thus, it remains unclear what measurements are most relevant to synaptic receptors. To further explore this discrepancy, we examined the open probability (P_o) of NMDA channels in whole-cell and outside-out patch recording from cultured hippocampal neurons. Currents were evoked by rapid application of saturating concentrations of NMDA in the presence or absence of the "irreversible" open channel blocker, MK-801 ((+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine). The reduction of the peak amplitude and the acceleration of the decay of the current in MK-801 were used to derive P_o by fitting the current traces to a multistate kinetic model. The P_o in whole cell was low (0.04), similar to that previously measured for synaptically activated NMDA channels.

In contrast, ensemble average currents from outside-out patches were much more rapidly blocked in the presence of MK-801, indicative of a significantly higher P_o. The P_o also gradually increased with the duration of recording in both whole-cell and outside-out configurations, suggesting that channel gating is sensitive to mechanical alterations of the patch or that washout of cytoplasmic factors leads to an increase in channel open probability. To test whether the disparity in P_o could be attributed to different gating of synaptic and extrasynaptic channels, the P_o of whole-cell currents was measured before and after all synaptic NMDA channels (> 90%) were blocked by evoking EPSCs in the presence of MK-801. In microisland cultures containing a single excitatory neuron, 81 ± 4% of NMDA channels were synaptic. However, the P_o of synaptic and extrasynaptic channels was equivalent, suggesting that P_o recorded in the whole-cell configuration is the same as at the synapse. The low open probability for synaptic channels implies that with each presynaptic stimulus only 50% of channels that bind glutamate actually open; thus, the NMDA receptor-mediated EPSC has a significant functional 'reserve.'

[Key words: synaptic transmission, glutamate receptors, patch clamp, hippocampus, MK-801, NMDA receptors]

The modulation of transmitter-gated receptor channels can involve different components of channel function. Kinetic studies using rapid application of defined concentrations of agonist to whole-cell and outside-out patch preparations are useful in separating the binding steps from intrinsic gating of ligand-gated ion channels. For the NMDA channel, this approach has been used to examine the mechanisms of glycine regulation and desensitization, the number of agonist binding sites, and the behavior of the channel that underlies shape of the synaptic response (Mayer et al., 1989; Lester et al., 1990; Sather et al., 1990; Clements and Westbrook, 1991; Hestrin 1992; Lester and Jahr, 1992). Recently, the open channel blocker MK-801 has been employed to determine the open probability (P_o) of NMDA channels. However, this technique has resulted in a wide range of reported open probabilities (Huettner and Bean, 1988; Jahr, 1992; Hessler et al., 1993; Rosenmund et al., 1993), albeit using somewhat different analytical approaches and preparations. The discrepancy could be due to alteration of NMDA channels in outside-out patches. For example, desensitization and the calcium-sensitivity of NMDA channels are affected by patch recording (Benveniste et al., 1990; Sather et al., 1990, 1992; Lester and Jahr, 1992; Lester et al., 1993; Rosenmund and Westbrook 1993a,b). Alternatively, synaptic NMDA channels may behave differently than extrasynaptic channels that may dominate in outside-out patches pulled from the cell soma.

To discriminate between these possibilities, we compared the P_o of NMDA channels in whole-cell recording and outside-out patches from cultured hippocampal neurons. We also tested for differences in gating between synaptic and extrasynaptic channels by analyzing currents on neurons that received inputs from only a single presynaptic cell. Our results suggest that the P_o of synaptic and extrasynaptic receptors recorded in the whole-cell configuration is low, similar to that of synaptically activated NMDA receptors (Rosenmund et al., 1993; but see Hessler et al., 1993). However, the P_o in patches increased with the duration of recording reaching a value approximately eight times that of the whole-cell P_o. The time dependence implies that channel gating is sensitive to mechanical alterations during pulling of the patch or to the loss of soluble cytoplasmic constituents during patch dialysis. The low open probability of synaptic NMDA channels has important implications for the expected number and behavior of NMDA receptors at synaptic sites.

Materials and Methods

Cell culture. Cell cultures were prepared as described previously (Legendre and Westbrook, 1990). Briefly, cultured neurons were prepared

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from the hippocampi of neonatal rats (Sprague–Dawley). Rat pups were anesthetized with halothane and killed by decapitation. The hippocampi were dissected free of meninges and incubated for 1 hr in low Ca^{2+} medium containing papain (20 U/ml, Worthington). The tissue was then triturated using fire-polished pipettes, and plated at a density of 5–20 $\times$ 10^3/cm^2 on a confluent glial feeder layer. Growth medium contained 5% horse serum, 95% minimal essential medium (MEM; GIBCO), and a growth supplement including insulin, transferrin, selenium, triiodothyronine, progesterone, and corticosterone. Microisland cultures were prepared as described in Bekkers and Stevens (1991).

Drug delivery. The extracellular medium contained (mM) NaCl, 167; KCl, 2.4; HEPES, 10; glucose, 10; $CaCl_2$, 0.2–2; and glycine 0.01. The agonist solution contained a saturating concentration of NMDA (1 mM) in 0–5 mM Ca^{2+}. The osmolarity was 325 mOsm and the pH 7.2. TTX (1 μM), picrotoxin (100 μM), and strychnine (2 μM) were added to inhibit spontaneous synaptic activity and GABA/glycine chloride channels. For whole-cell experiments, control and agonist solution were applied using an array of quartz flow pipes (Polymicro Tech.; 430 μm i.d.) positioned within 100–200 μm of the neuron, and connected to gravity-fed reservoirs. Each flow pipe was controlled by solenoid valves and was moved with a piezoelectric bimorph (Vernitron). The solution exchange time constant was estimated using a method described in Vyklicky et al. (1990) and ranged from 15–22 msec (mean, 16.4 ± 2.1 msec; n = 4). Solutions were applied to outside-out patches using a gravity fed square flowpipes (four-square pattern) with individual tip diameters of 80 μm. The solution was exchanged by moving the patch into the stream of control solution and then moving the flowpipe with a two-dimensional piezoelectric bimorph (Corey and Hudspeth, 1980). The flow rate from flowpipes was 2 ml/min for whole-cell recording and 0.1 ml/min for outside-out patches, corresponding to a rate of 0.2–0.3 m/sec and 0.26 m/sec, respectively. In addition to local perfusion with flowpipes, the whole bath (volume, 300–500 μl) was perfused at 2 ml/min to prevent accumulation of drugs. Adequate positioning and solution exchange was examined in open-tip control at the end of each experiment. Solution exchange time constant for outside-out patches ranged from 200 to 500 μsec (mean, 365 ± 55 μsec, n = 16).

Experimental protocols and data analysis. Experiments were performed in hippocampal neurons after 5–14 d in culture. To examine synaptically activated NMDA channels, experiments were performed using recurrent excitatory synapses (autapses) on microisland cultures 7–9 d after plating. Currents were recorded using an Axopatch 1C amplifier (Axon Instruments). Patch pipettes were fabricated from borosilicate glass (TWF 150, World Precision Instruments) pulled with a two-step puller (Narishige). Pipettes had "bubble numbers" ranging from 7.2 to 8.0. After fire polishing, the inner pipette tip diameter was 2 to 3 μm and the resistance was 1–2.5 MΩ Pipette solutions included (mM) Cs-gluconate, 135; HEPES, 10; Cs_4-BAPTA, 10; $MgCl_2$, 2; Mg-ATP, 5. The pH was 7.3 and the osmolarity 310 mOsm. For P_o measurements on NMDA channels during "rundown," EGTA was substituted for BAPTA and ATP was excluded from the pipette solution. For synaptic experiments, potassium salts were substituted for cesium salts. In some experiments, ultrapure salts with added Mg^{2+} (0–100 μM) were used to determine the effect of Mg^{2+} on the current in the presence of MK-801. The access resistance was 60–90% compensated; only recordings with an uncompensated access resistance below 8 MΩ were included in the analysis. Cell capacitance (5–25 pF) was compensated. Data were acquired on a IBM 386 clone using pCLAMP version 5.5, (Axon Instruments) software and analyzed on a Macintosh using AXOGRAPH software (Axon Instruments). Acquisition rate was 0.4–2 kHz, and data were filtered at half the acquisition rate with an eight-pole Bessel filter (Frequency Devices). Data are expressed as percentage of control ± SE. Significance was tested using one-way analysis of variance with the Bonferroni–Dunn procedure for multiple comparisons (STATVIEW 4.0). Significance levels are given for $p < 5\%$.

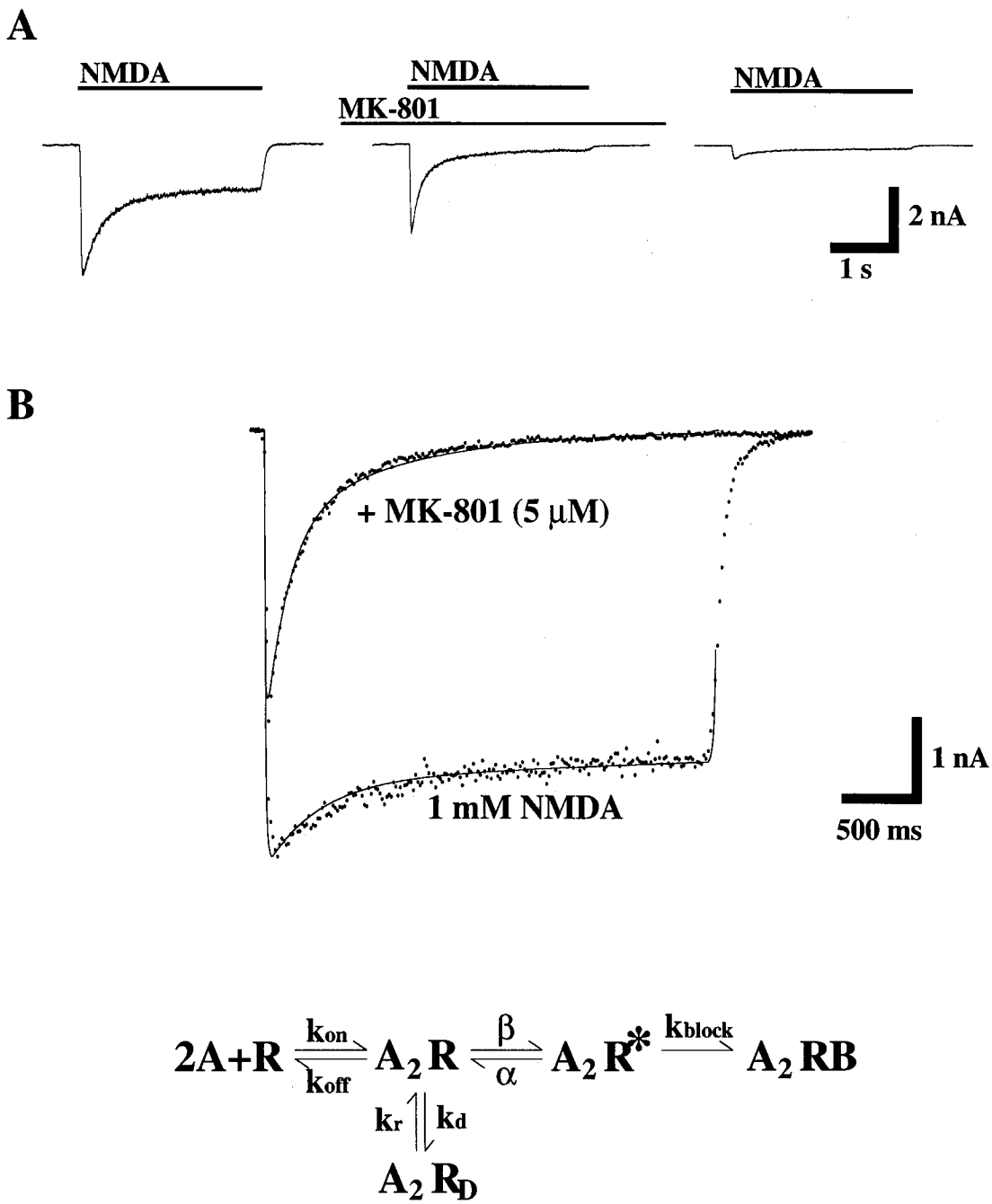
Fitting procedures and simulation. For each cell/patch, currents evoked by NMDA (1 mM, 1–3 sec) in low extracellular calcium (0.2–0.5 mM) were normalized to the peak amplitude. They were then fitted using SCoP (Simulation Resources, Berrien Springs, MI) to a standard kinetic model (Fig. 1*B*) by a least squares procedure. Several rates were fixed based on prior determinations: (1) binding (k_{on} = 5 μM^{-1}sec^{-1}) and unbinding (k_{off} = 150 sec^{-1}) rates were taken from Clements and Westbrook (1991) such that the K_d for NMDA was 30 μM; (2) The closing rate (α) was fixed to 250 sec^{-1}, as estimated by the rising phase of the response in patches. This value is in general agreement with previous measurements of mean open times in patches and by whole-cell fluctuation analysis (Jahr and Stevens, 1987; Cull-Candy et al., 1988; Mayer et al., 1988; Rosenmund and Westbrook, 1993b); and (3) The MK-801 binding rate (k_{block}) was fixed to 25 μM^{-1}sec^{-1} (Huettner and Bean, 1988; Jahr, 1992). The reduction in the peak whole-cell current by MK-801 is primarily dependent on the MK-801 binding rate (Rosenmund and Westbrook, 1993b); this was used to confirm that k_{block} was ≈ 25 μM^{-1}sec^{-1} in our experiments. The currents in the absence of MK-801 were first fitted allowing the desensitization, resensitization, and channel number to vary; the opening rate was set to an arbitrary value. The values for the desensitization (k_d) and resensitization (k_r) rates were then used to fit the current transient in the presence of MK-801, while allowing the opening rate and channel number to vary. The product of channel number and opening rate was conserved to preserve the amplitude information of the control response. The whole-cell solution exchange time constant (17 msec) was incorporated into the model, improving the fitting to the peak response for whole-cell currents. Simulated first latency distribution and synaptic single channels were generated using a kinetic simulation program developed by Dr. John Clements.

Results

P_o of NMDA channels in whole-cell recordings

Whole-cell currents were evoked by 3 sec applications of saturating concentrations of NMDA (1 mM) in the presence and absence of MK-801 (5–20 μM). After a 10 sec preincubation in MK-801, coapplication of agonist with MK-801 reduced the peak amplitude of the current and greatly accelerated the decay such that there was little or no steady-state current at the end of the drug application (Fig. 1*A*, middle trace). An NMDA application 30 sec later evoked only a small residual current; thus, the inhibition was "irreversible" within the time frame of the experiment (Fig. 1*A*, right trace). To avoid the slow Ca-dependent inactivation apparent in the control record of Figure 1*A* (left trace), all experiments, unless otherwise indicated, were performed in low extracellular calcium (0.2–0.5 mM). Typical responses in low calcium solution, in the presence and absence of MK-801, are superimposed in Figure 1*B* along with the kinetic model used to fit the current traces. The control traces shows only the 10–20% calcium- and glycine-independent desensitization observed with saturating NMDA concentrations. The model incorporates two identical agonist binding steps, an open state (A_2R^*) and a desensitized state (A_2R_D); currents in the presence of MK-801 were fitted with a model containing an additional blocked state (A_2R_B). Because MK-801 block was irreversible, only an entry rate (k_{block}) was included in the model. As the channel must enter the open state to be blocked by MK-801, the decay of the current when NMDA and MK-801 are coapplied reflects the sum of rates leading into the open state, forming the basis for measurement of the opening rate, Values for k_{on}, k_{off}, k_{block} have been previously measured (see Materials and Methods). Thus, the opening rate was determined from optimized fits to the current transients after the desensitization and resensitization rates, k_d and k_r, were determined for each control response (see Materials and Methods). The opening rate (β), as determined by the model fit, was 9.6 ± 1.4 sec^{-1} (n = 12) for low calcium solutions. In the presence of normal extracellular calcium, NMDA currents run down due to a progressive reduction in open probability (Rosenmund and Westbrook, 1993b). Consistent with this, the opening rate after rundown as estimated by this approach was 5.3 ± 0.9 sec^{-1} (n = 5).

The closing rate of NMDA channels, the reciprocal of the channel mean open time, has been measured by many investigators. Although open time histograms of single NMDA channels in steady-state recording are fitted by more than one time constant (e.g., Jahr and Stevens, 1987; Cull-Candy et al., 1988),



$$2A+R \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} A_2R \underset{\alpha}{\overset{\beta}{\rightleftharpoons}} A_2R^* \xrightarrow{k_{block}} A_2RB$$

$$A_2R \underset{k_d}{\overset{k_r}{\rightleftharpoons}} A_2R_D$$

Figure 1. The open probability (P_o) of NMDA channels is low in whole-cell recording. *A*, Whole-cell currents were evoked by rapid step application (3 sec duration) of saturating NMDA concentration (1 mM) in the presence (*middle trace*) and absence (*left and right traces*) of MK-801 (5 μM). MK-801 accelerated the decay of the current until nearly complete block occurred. The extracellular medium contained 3 mM Ca. Holding potential −60 mV. Cells were preequilibrated with MK-801 before application of MK-801 and NMDA. *B*, Responses in presence (*single trace*) and absence of MK-801 were superimposed and are represented as *dots*. The transients were fitted to a five-state kinetic model (*below*) to determine the opening rate (β) using values for the rate constants k_{on}, k_{off}, k_r, k_d, and α, as described in Materials and Methods. The optimally fitted transients from the kinetic model are superimposed (*lines*). The P_o for this neuron was 0.038; the extracellular calcium was 0.2 mM. *A*, molecule of transmitter; *R*, NMDA receptor; *R**, open state; R_D, desensitized state; *RB*, irreversibly blocked state.

fluctuation analysis provides a weighted average in the range of 4–5 msec (e.g., Nowak et al., 1984; Mayer et al., 1988; Rosenmund and Westbrook, 1993b), corresponding to a closing rate of 200–250 sec^{-1}. Thus, we used an average closing rate (a) of 250 sec^{-1} to calculate the open probability (P_o) where

$$P_o = \beta/(\beta + \alpha). \quad (1)$$

The estimated P_o was 0.037 in low calcium solutions. No evidence was found for a subpopulation of channels with higher P_o, as this would be expected to produce an initial fast decaying component in the presence of MK-801. This was not observed.

Magnesium ions can inhibit MK-801 binding in the channel (Huettner and Bean, 1988). Thus, small amounts of contaminating magnesium due to relatively poor perfusion of whole cells compared to outside-out patches could slow the decay of the current by MK-801, and give a falsely low value of the opening

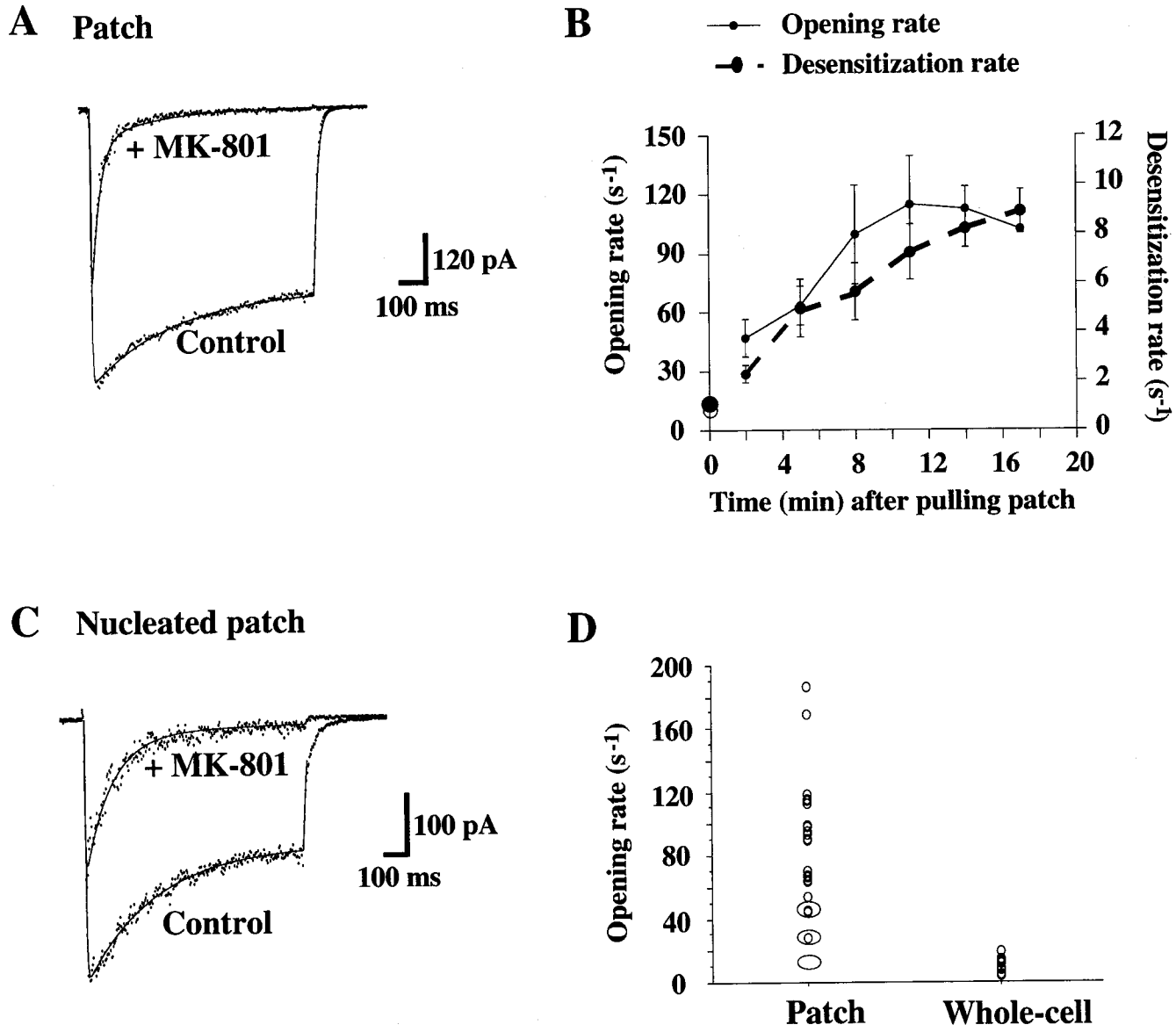


Figure 2. Time-dependent increase in P_o in outside-out patches. *A,* Current transients (*dots*) and model fits (*smooth line*) for an outside-out patch. Experimental protocol was the same as in Figure 1, except that the duration of drug application was 1 sec. The rapid decay of the current in the presence of MK-801 reflects the faster opening rate in outside-out patches compared to whole-cell recording. The opening rate was 97 sec^{-1}, corresponding to a P_o of 0.28 for this patch. *B,* Time-dependent increase of opening and desensitization rates. The *dots* at $t = 0$ indicate the average value of opening rate and desensitization rate for whole-cell recording. Data were obtained from four to six repetitive measurements on seven patches. Between applications of MK-801, patches were unblocked with repeated pulses of NMDA (1 mM) at +60 mV until the initial peak amplitude recovered. *C,* Current transients and model fits from a representative nucleated patch. The opening rate was 25 sec^{-1}, corresponding to a P_o of 0.09. *D,* Summary of opening rates in outside-out patches and in whole-cell recording at the first application of MK-801. *Large ovals* represent results obtained in nucleated patches.

rate for the whole-cell experiments. To test this possibility, additional whole-cell experiments were performed in an extracellular solution of ultrapure salts ± 100 μM Mg^{2+}. Use of ultrapure salts did not alter the estimate of opening rate. Mg^{2+} (100 μM) reduced the initial block of the current by MK-801, consistent with reduced effectiveness of MK-801 in blocking the channel, but caused only a twofold decrease in the apparent P_o estimate. Therefore, Mg^{2+} contamination cannot account for the difference in P_o between whole-cell and outside-out patches.

Open probability in outside-out patches

The value for NMDA channel open probability obtained in our whole-cell experiments is in a similar range as P_o estimated for synaptically activated NMDA channels (Rosenmund et al., 1993), but much lower than has been reported in outside-out patches (Jahr, 1992). These differences could result from different assumptions or technical factors, but might also be due to true gating differences between the whole-cell and patch preparations. Thus, we directly compared our whole-cell results with ensemble average currents on outside-out patches (Fig. 2*A*). The opening rate in patches was obtained in a similar manner as outlined in Figure 1. The opening rate as determined in the fitting procedure averaged 82.4 ± 7.7 sec^{-1}, corresponding to a P_o of 0.24 ($n = 27$). A slow decay component in the presence of MK-801 was sometimes observed ($n = 12$, not shown) and could not be fitted with the simple model. When present, this

component contributed 8.3 ± 5.3% of the total current. This component could represent a small number of channels that emerge from a long-lived desensitized state not included in the model, or to a subpopulation of channels with a lower MK-801 binding rate. In these cases, the opening rate for the fast component was estimated by fitting only the first 400 msec of the response in presence of MK-801. In contrast to NMDA channels in whole-cell recordings, the concentration of extracellular calcium (0.2–2 mM) had no significant effect on opening rate in the patch (n = 12).

Desensitization of NMDA channels in outside-out patches and in whole-cell recordings of acutely isolated cells increases with time (Benveniste et al., 1990; Sather et al., 1990; Lester et al., 1993). Ascher and colleagues (Sather et al., 1992) attributed this to a "washout" of a necessary cellular factor(s) because the rate of change in desensitization significantly decreased for large "nucleated" patches. Similar factors could also modulate open probability, thus leading to the discrepancy between the results of whole-cell and patch experiments. Consistent with this possibility, the opening rate was significantly lower in nucleated patches (28.3 ± 5.3 sec^{-1}; n = 3; Fig. 2*C,D*) than in conventional outside-out patches. Furthermore, the opening rate increased with time during whole-cell recording (data not shown). We examined the time dependence of P_o by repeated measurements of the opening rate in outside-out patches. The opening rate increased with aging of the patch (Fig. 2*B*). Two minutes after the start of recording, the opening rate was 45.3 ± 12.2 sec^{-1} (n = 6), but had increased to 106.4 ± 14.2 sec^{-1} ($p > 0.01$) after 15 min. Given that the number of channels in the membrane is unlikely to change over such a short period, a time-dependent increase in P_o should result in an increase in the peak ensemble average current. However, this did not occur. Also, the charge transfer in the presence of MK-801, an indicator of the number of active NMDA channels in the patch, decreased over time. The decrease in active channels may represent accumulation in desensitized states.

Estimating P_o *by analyzing the charge transfer in MK-801*

The P_o of the channel can also be estimated by analyzing the charge transfer in the presence of MK-801, a measurement that can be accurately determined for each cell. This provides an alternative method that does not rely on fitting the decay of the current in the presence of MK-801 or on the validity of the kinetic model. This requires the following derivation. Based on the MK-801 blocking rate, each channel has a mean open time before it is blocked (t_m) equal to

$$t_m = 1/([\text{MK-801}]*k_{\text{block}}) = 1/(5\ \mu\text{M}*25\ \mu\text{M}^{-1}\text{sec}^{-1}) \approx 8\ \text{msec}. \quad (2)$$

The charge transfer (Q) in the presence of MK-801 is

$$Q = N*t_m*i, \quad (3)$$

where N is the number of channels on the cell that open during the agonist application and i is the single channel current. Because the agonist application was continued until all the current was blocked, N represents the total number of functional channels on the cell. For experiments using 5 μM MK-801, the number of channels was 78,900 ± 13,900, with a peak current of 5.17 ± 1.04 nA (n = 7) for whole-cell recording, and 775 ± 528 (n = 4), with a peak amplitude of 291 ± 94 pA for outside-out patches.

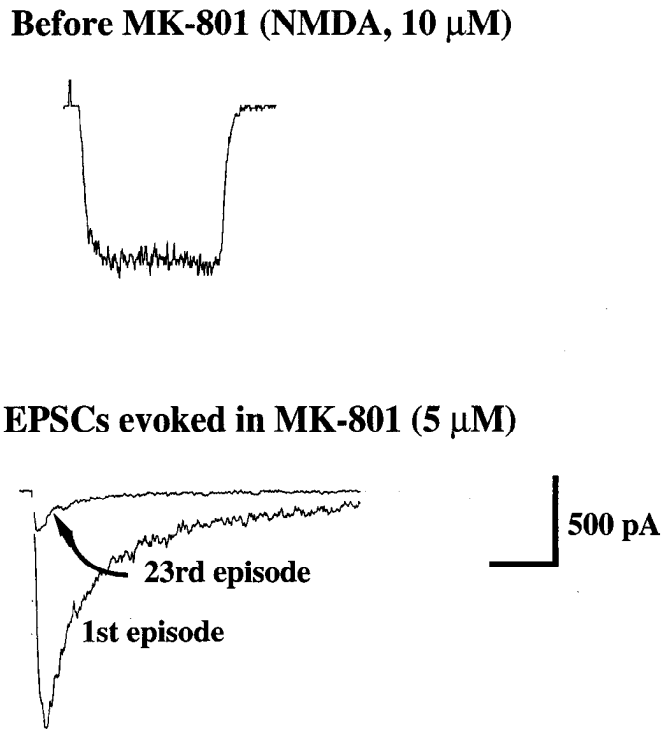


Figure 3. A high percentage of the whole-cell current was due to synaptic receptors. In microislands containing a single autaptic neuron, the fraction of synaptic channels was determined by measuring NMDA currents evoked by application of 10 μM NMDA before (*top trace*) and after (*bottom trace*) the EPSC was blocked. Synaptic receptors were blocked by evoking a train of NMDA EPSCs in the presence of MK-801 (*middle trace*). For this neuron, 35% of the NMDA whole-cell response remained after the EPSC was reduced to 18% of control, suggesting that 80% of the receptors on this cell were synaptic. Time calibration: 1 sec for the whole-cell currents and 100 msec for the EPSC.

Similar values (62,503 ± 11,000 channels, n = 14) were obtained with 20 μM MK-801, providing evidence that the charge transfer measurements and MK-801 blocking rates were accurate.

The whole-cell current (I) at any time t in the presence of MK-801 is

$$I(t) = N(t)*i, \quad (4)$$

and the open probability at any time t is

$$P_o(t) = N(t)/N, \quad (5)$$

where $N(t)$ is the number of channels open at time t. Combining Equations 3 and 4 allows $P_o(t)$ to be calculated from the charge transfer and the amplitude of the current:

$$P_o(t) = I(t)*t_m/Q \quad (6)$$

In the presence of saturating concentrations of agonist such that

the channels rapidly reach the fully bound, but closed state (A_2R), and before channels enter desensitized states (i.e., binding and/or closing rate ≫ desensitization rate), the value of $P_o(t)$ at the peak of the current thus approaches $\beta/(\beta + \alpha)$, which is P_o as defined in Equation 1. The P_o calculated in this manner was 0.025 ± 0.002 (n = 18) for whole-cell recording and 0.37 ± 0.13 (n = 4) for outside-out patches, consistent with the values generated by the kinetic analysis.

Synaptic and extrasynaptic NMDA channels

In order to compare whole-cell studies to synaptically activated channels, one usually is forced to assume that synaptic channels behave identically to extrasynaptic channels, or that synaptic channels represent the vast majority of channels on the cell. To examine the possibility that somatic NMDA channels, the source of outside-out patches in our experiments, behave differently than dendritic synaptically activated channels, we developed a simple assay to measure the ratio of synaptic channels contributing to the whole-cell response. The current amplitude evoked by whole-cell NMDA application was measured before and after complete, irreversible blockade of the NMDA-receptor mediated EPSC. This assay was made possible by using microislands containing a single neuron with an autaptic EPSC. NMDA receptor-mediated EPSCs were evoked by brief (1–2 msec) depolarization to 0 mV in the presence of MK-801 (5–20 μM). Stimuli were repeated until >90% of the NMDA receptor-mediated EPSC was blocked. This concentration of MK-801 had no effect on transmitter release as measured by the AMPA receptor-mediated EPSC (not shown). After the EPSC was blocked, the remaining NMDA current was also subtracted from the initial whole-cell response and revealed that 81.4 ± 4.1% (n = 9) of the NMDA channels were synaptically located. The opening rate for the channels before and after block of the EPSC was then determined as in Figure 1. The opening rate of the extrasynaptic fraction was 5.7 ± 0.44 sec^{-1} (n = 5), which was not significantly different to opening rates measured from the entire cell. In four neurons that had synaptic currents that were not blocked by CNQX (6-cyano-7-nitroquinoxaline-2,3-dione; 5 μM) or AP-5 (2-amino-5-phosphonovalerate; 100 μM), that is, presumably GABAergic interneurons, similar opening rates (6.2 ± 1.5 sec^{-1}) were measured. In these cells, all NMDA receptors must be extrasynaptic. These experiments provide further evidence that the opening rate is similar for synaptic and nonsynaptic channels.

A low P_o (slow opening rate) for synaptic channels dramatically influences the predicted behavior of an ensemble of synaptic channels. This can be seen by comparing the simulated behavior of glutamate-activated synaptic channels using the P_o estimates predicted by both the outside-out patch and whole-cell experiments (Fig. 4). For a low P_o value, the predicted first latency for synaptically activated channels is surprisingly long, e.g., an opening rate of 8 sec^{-1} predicts an average latency of 55 msec to the first channel opening. Also, about 50% of the channels that bind glutamate will not open (Fig. 4*B*), whereas a high P_o value as seen in patch experiments predicts that ≈90% of the channels will open. The mean channel lifetime estimated from the simulation was 12 msec, which is roughly similar to the time course of realigned openings at low steady-state agonist concentrations (Edmonds and Colquhoun, 1992). This suggests that unbinding and/or desensitization rather than extremely long openings contribute to the slow decay component of the NMDA-receptor mediated EPSC (for further discussion, see Jahr, 1994).

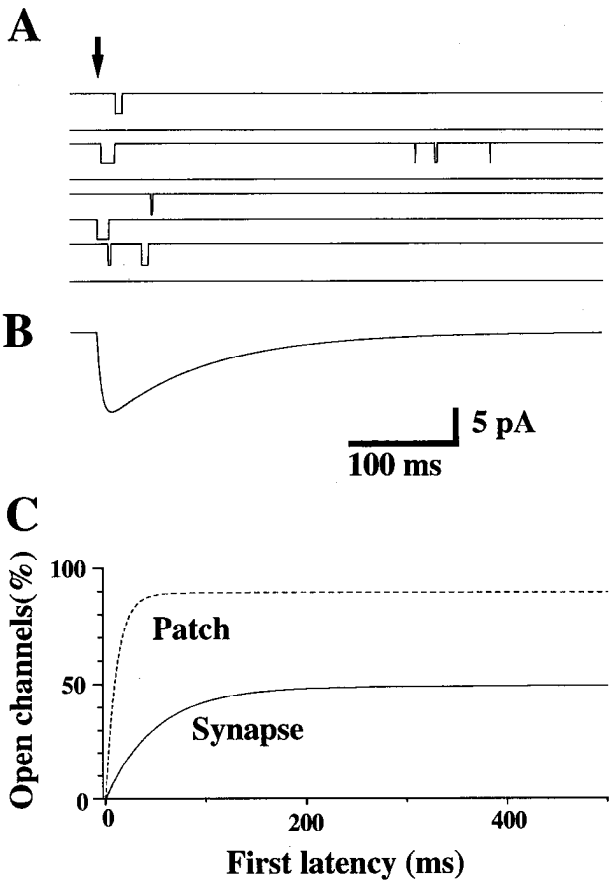


Figure 4. Simulation of single synaptic NMDA channels using a low value for P_o. *A*, Eight sample traces of a single NMDA channel, simulated using the low opening rate observed in whole-cell recording. Note that in some cases channel openings are delayed or do not occur during a 500 msec segment. The EPSC was simulated using a multistate kinetic model and values for the glutamate time course as described by Clements et al. (1992). *B*, Ensemble average current generated by 2000 sweeps containing 100 channels. *C*, First latency distribution of NMDA channel opening predicted using the opening rate either from whole-cell experiments (*"Synapse"*) or from outside-out patches (*"Patch"*). Note that a low opening rate results in a long first latency and a lower percentage of channels that open before the transmitter (glutamate) dissociates.

Of note, the low P_o has little effect on the rising phase of the NMDA-receptor mediated EPSC that is dominated by the much faster closing rate.

Discussion

Kinetic analysis of the block of NMDA channels by MK-801 was used to examine the open probability of channels in outside-out patches and whole-cell recording. Our main conclusion is that the open probability is low for synaptic and extrasynaptic NMDA channels compared to channels in outside-out patches. The block of NMDA currents by MK-801 accelerated with time in patch recordings, suggesting that loss of structural and/or cytosolic components results in an increase in the open probability. Our results also further emphasize the problems in accurately describing NMDA channel gating in its native state, as several phenomena including calcium sensitivity, desensitization, and the open probability are affected by the recording conditions.

Adequacy of kinetic analysis

Our conclusions depend on two main assumptions: the validity of the model and the mechanism of open channel block by MK-801. There are indications that a complete description of NMDA channel gating requires more than the five-state model that was used in this and other studies (Clements and Westbrook, 1991; Clements et al., 1992; Lester and Jahr, 1992; Rosenmund et al., 1993). The detailed analysis of single-channel records suggests multiple open and closed states as well as evidence for gating from the open into the desensitized state (e.g., Edmonds and Colquhoun, 1992; Gibb and Colquhoun, 1992; Lin and Stevens, 1994). Likewise, recent studies also suggest that the two binding sites are not equal and independent (Clements and Westbrook, 1994). The analytical approach we used does not have the resolution to distinguish models more complex than the five-state model. However, the experiments were specifically designed to allow measurement of rates leading into the open state. As a result, the determination of opening rate was relatively insensitive to many complexities of NMDA channel kinetics. For example, the use of high agonist concentrations makes the model insensitive to errors in the agonist binding and unbinding rates, as the channels are rapidly "forced" to the bound, but closed state. Likewise, an alternative method of determining P_o, based on the charge transfer of the current in the presence of MK-801, was consistent with the values determined from the kinetic analysis.

An accurate knowledge of the mechanism of action MK-801 is important to our result. The anticonvulsant MK-801 has been described as an open channel blocker, and binding and unbinding rates have been measured (Huettner and Bean, 1988; Jahr, 1992). In our experiments, 30 sec applications of MK-801 in the absence of agonist did not reduce subsequent NMDA responses in whole-cell or patch (Rosenmund, unpublished), suggesting that MK-801 blocks the channel only in presence of agonist. Although we did not directly measured MK-801 binding rates, the binding rate can be deduced from the percentage reduction of the peak response in the presence of MK-801 (Rosenmund et al., 1993). The percentage reduction seen in our experiment was consistent with the measured values of 23.7 and 25 μM^{-1} sec^{-1} (Huettner and Bean, 1988; Jahr, 1992).

An additional concern is whether the neurons we used have a homogeneous population of NMDA channels, as recombinant NMDA channels can differ with respect to channel kinetics and the effectiveness of channel blocking drugs (for review, see McBain and Mayer, 1994). Hippocampal neurons express primarily NR1, NR2A, and NR2B; thus, several combinations are possible. However, recent studies suggest that the expression of NR2 subunits in cultured cortical neurons follows a similar pattern to that *in vivo* (Sheng et al., 1994), with expression of NR2B being the primary NR2 subunit expressed at 5–14 d in culture (Zhong et al., 1994), the time of our experiments. Thus, it seems likely that our results reflect the behavior of an NR1/2B heteromer. A final caveat is that the P_o measurements we obtained were using NMDA as an agonist rather than the natural transmitter glutamate. As glutamate has a slightly higher efficacy (ca. 10%, see, e.g., Lester and Jahr, 1992), the P_o estimates for glutamate-activated channels in both whole-cell and outside-out patches will be proportionately greater.

Comparison to prior results

Our outside-out patch results confirm the measurements of open probability observed by Jahr (1992). In that study, the amount of current blocked during a single short pulse of 1 mM L-glutamate in the presence of MK-801 was used to determine the probability of opening. The determined open probability of 0.30 at peak is identical to our measurements in dialyzed outside-out patches, suggesting that the analysis method we used, the decay of current in presence of MK-801 during longer pulses of agonist, is not responsible for the low values of P_o observed in our whole-cell experiments. As Lin and Stevens (1994) have also obtained similar P_o values for outside-out patches without using MK-801, there would appear to be general consensus regarding the P_o in outside-out patches. However, Huettner and Bean (1988) obtained even lower values of P_o (≈ 0.002) from steady-state single-channel recording, possibly due to the increased desensitization and Ca-dependent rundown under those conditions. Thus, how does one explain the lower values of P_o predicted from our whole-cell experiments and the time-dependent increase in P_o we observed? The simplest explanation is that the outside-out patch disrupts regulatory factors influencing channel gating as has been observed with other channels (e.g., Trautmann and Siegelbaum, 1981). In the case of the NMDA receptor, this may include structural elements (e.g., Rosenmund and Westbrook, 1993a) or an unidentified second-messenger pathway.

One general problem in reconciling the data is that we did not observe a matching increase in the current amplitude response as P_o increased with time in the patches. This paradox cannot be explained by the model we have used, and may reflect a matching loss of active channels in the patch. Another possibility is that MK-801 can block the channel in a nonconducting (desensitized) but liganded state that could lead to an overestimate of P_o using the MK-801 method. Although there is no evidence for this with MK-801 at present, the channel blocker 9-aminoacridine can lock the channel in a nonconducting state with agonist bound (Benveniste and Mayer, 1993; Costa and Albuquerque, 1994).

Synaptic versus extrasynaptic channels

A surprising result of these studies to us was the high percentage of channels on cultured hippocampal neurons that are blocked by MK-801 following synaptic stimulation. This implies that the cues necessary for the formation and maintenance of receptor clusters are relatively well preserved in this preparation. This is consistent with the electrophysiological demonstration of glutamate receptor "hot spots" at areas of presumed synaptic contacts (e.g., Trussell et al., 1988; Jones and Baughman, 1991) and clustering of glutamate receptor subunits on dendritic spines in cultured neurons (Craig et al., 1993). Our experiments were conducted on cells after 1–2 weeks in culture, a time at which clusters in the preparation used by Craig and colleagues are just beginning to show morphologically defined receptor clusters. It is possible that this represents different maturational states of the two culture systems, but could also indicated that physiological evidence for clusters precedes well-defined morphologically identified clusters. Our results also indicate that at least one parameter in gating, the opening rate, is the same in synaptic and extrasynaptic receptors. However, more detailed studies will be necessary to decide if synaptic and extrasynaptic NMDA receptors are identical. For example, maturational changes in subunit composition are well know to affect the gating of acetylcholine receptors at the neuromuscular junction (e.g., Sakmann and Brenner, 1978; Brehm, 1989), although junctional and extrajunctional receptors appear to have the same properties except in denervated muscle (Brehm, 1989). The use of autaptic neu-

rons (Segal and Furshpan, 1990; Bekkers and Stevens, 1991) may thus prove valuable for studies of central synapse development and receptor clustering.

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