Distribution, Density, and Clustering of Functional Glutamate Receptors Before and After Synaptogenesis in Hippocampal Neurons

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Cottrell, Jeffrey R., Gilles R. Dubé, Christophe Egles, and Guosong Liu. Distribution, density, and clustering of functional glutamate receptors before and after synaptogenesis in hippocampal neurons. J Neurophysiol 84: 1573–1587, 2000. Postsynaptic differentiation during glutamatergic synapse formation is poorly understood. Using a novel biophysical approach, we have investigated the distribution and density of functional glutamate receptors and characterized their clustering during synaptogenesis in cultured hippocampal neurons. We found that functional α-amino-3-hydroxy-5-methyl-4-isoxazolpropionate (AMPA) and N-methyl-D-aspartate (NMDA) receptors are evenly distributed in the dendritic membrane before synaptogenesis with an estimated density of 3 receptors/μm². Following synaptogenesis, functional AMPA and NMDA receptors are clustered at synapses with a density estimated to be on the order of 10⁴ receptors/μm², which corresponds to ~400 receptors/synapse. Meanwhile there is no reduction in the extrasynaptic receptor density, which indicates that the aggregation of the existing pool of receptors is not the primary mechanism of glutamate receptor clustering. Furthermore our data suggest that the ratio of AMPA to NMDA receptor density may be regulated to be close to one in all dendritic locations. We also demonstrate that synaptic AMPA and NMDA receptor clusters form with a similar time course during synaptogenesis and that functional AMPA receptors cluster independently of activity and glutamate receptor activation, including following the deletion of the NMDA receptor NR1 subunit. Thus glutamate receptor activation is not necessary for the insertion, clustering, and activation of functional AMPA receptors during synapse formation, and this process is likely controlled by an activity-independent signal.

INTRODUCTION

Glutamatergic synapses are the primary source of excitatory synaptic transmission in the CNS. Their formation is critical in the establishment of synaptic connections and the refinement of these connections that occur during development (Jessell and Kandel 1993) and likely during learning and memory (Milner et al. 1998). Despite the importance of these synapses, the details of their formation, especially regarding postsynaptic differentiation, remain to be determined. A model system for understanding postsynaptic differentiation during synaptogenesis is the cholinergic vertebrate neuromuscular junction (NMJ) (Hall and Sanes 1993; Sanes and Lichtman 1999), where acetylcholine receptors (AChRs) are inserted at a high-density (~1,000/μm²) along the muscle membrane before synapse formation. Following axonal contact and the activation of a receptor tyrosine kinase on the muscle (Glass et al. 1996), these AChRs aggregate independently of activity to the synaptic site. Subsequently, the subjunctional nuclei increase, and extrajunctional nuclei decrease, expression of AChRs, resulting in a dramatic difference in AChR density between junctional (~10⁴/μm²) and extrajunctional (~10/μm²) regions (Hall and Sanes 1993; Sanes and Lichtman 1999). Because of similarities in their structure and function, there may be similarities between the formation of the postsynaptic site of the NMJ and glutamatergic synapses.

To understand postsynaptic differentiation during glutamatergic synapse formation, we need to determine the distribution and density of glutamate receptors during synapse formation, the mechanism of glutamate receptor cluster formation, and the signals that regulate postsynaptic differentiation and glutamate receptor clustering. These processes in developing glutamatergic synapses are poorly understood. A number of studies, based primarily on antibody-labeling experiments, have shown that the ionotropic glutamate receptor subtypes α-amino-3-hydroxy-5-methyl-4-isoxazolpropionate (AMPA) and N-methyl-D-aspartate (NMDA) receptors and the metabotropic glutamate receptors (mGluRs) are clustered at synaptic sites (Baude et al. 1995; Craig et al. 1993; Richmond et al. 1996). However, the exact receptor densities before and after synapse formation have not been determined primarily because the antibody approach does not have adequate sensitivity or quantitative power. Furthermore although a number of hypotheses have been proposed (Craig et al. 1994; Nusser et al. 1998; Richmond et al. 1996; Steward 1995; Tovar and Westbrook 1999), there is little information regarding the exact mechanism of receptor clustering. Last, the signals regulating glutamate receptor clustering during synapse formation remain to be determined. Antibody-labeling data indicate that glutamate receptor clustering occurs independently of neuronal activity (Craig et al. 1994; Mammen et al. 1997). In contrast, electrophysiological data suggest that NMDA receptor activation is required for AMPA receptor insertion, clustering, and/or activation during synaptogenesis (Durand et al. 1996; Isaac et al. 1997; Liao and Malinow 1996; Wu et al. 1996). This hypothesis has been supported by recent morphological studies (Gomperts et al. 1998).
In this study, we have addressed these issues regarding postsynaptic differentiation during glutamatergic synapse formation in cultured hippocampal neurons. Using a novel glutamate receptor map technique, we determined the distribution and density of functional glutamate receptors before and after synapse formation. Furthermore we assessed the time course of functional AMPA and NMDA receptor clustering and determined the role of neuronal activity and glutamate receptor activation in the insertion and clustering of functional glutamate receptors during synapse formation.

**Methods**

**Cell Culture**

Procedures for culturing hippocampal neurons from neonatal rats and mice were as previously described (Liu and Tsien 1995). All pharmacological treatments were added to the culture medium at 0 days in vitro (DIV) and refreshed every 3 days. The glutamate receptor antagonist cocktail included 10 μM 6,7-dinitroquinoxaline-2,3-dione (DNQX; RBI), 5 μM 3-((R)-2-carboxy-4-yl)-propyl-1-phosphonic acid (D-CPP; RBI), 500 μM RS-1-aminoindan-1,5-dicarboxylic acid (AIDA; Tocris), 500 μM 2S-alpha-ethylglutamatic acid (EGLU; Tocris), and 500 μM RS-alpha-cyclpropyl-4-phosphonophenylglycine (CPPC; Tocris) to block AMPA receptors, NMDA receptors, and group I–III mGluRs, respectively (Jane et al. 1996; Pellicciani et al. 1995).

**FM1-43 Labeling and Synaptic Density Measurements**

Cultures were stained with 10 μM N-(3-triethylammoniumpropyl)-4-(4-dibutylamino)styryl) pyridinium dibromide (FM1-43; Molecular Probes) in (in mM): 39 NaCl, 90 KCl, 30 glucose, and 25 HEPES, pH adjusted to 7.4 with NaOH for 1 min. They were then washed for >5 min in Tyrode solution (in mM): 128 NaCl, 5 KCl, 30 glucose, 25 HEPES, 1 MgCl₂, 2 CaCl₂, plus 0.5 μM tetrodotoxin (TTX; Oretek), 5 μM glycine (Sigma), and 50 μM picrotoxin (Sigma), pH adjusted to 7.4 with NaOH. During electrophysiological experiments, the FM1-43 solution contained 5 μM D-CPP and 10 μM DNQX to block NMDA and AMPA receptors, respectively. Therefore we would expect no significant changes in the number and properties of these receptors due to acute receptor activation during FM1-43 loading. Although possible, we know of no cases in which simple depolarization without simultaneous receptor activation changed the function of synaptic receptors. Imaging was performed with an inverted Olympus Fluoview Personal Confocal Microscope, using an Olympus 40× planapochromat water immersion lens (1.15 NA). The number of FM1-43 punctae per 20 μm was determined by manually counting on 400 dendritic segments taken from 10 different regions from at least two different cultures. Values are the mean of 10 regions ± the standard error of the mean (SE).

**Electrophysiology**

Whole cell patch-clamp recordings (V_m = −60 mV) were made from presumptive pyramidal cells at room temperature using an Axon Instruments model 200B Integrating Patch Clamp amplifier with a 1-kHz (8-pole Bessel) low-pass filter. Data were digitized at 10 kHz by a Digidata 1200B A/D converter. Patch pipettes (2–4 MΩ) contained (in mM): 125 CsMeSO₃, 10 HEPES, 8 NaCl, 1 CaCl₂, 10 EGTA, 2 Mg-ATP, 0.3 GTP, pH adjusted to 7.25 with CsOH. Extracellular solution was Tyrode solution as described above. Access resistance was monitored on-line and was typically <10 MΩ. Glutamate iontophoresis (MVCS 02C microiontophoresis controller, NPI Electronics) was performed as previously described (Liu et al. 1999).

**Glutamate Receptor Map**

Patch-clamp recordings were performed from the cell body, and the glutamate iontophoresis electrode was visually guided to the dendritic location where the map was to begin. A confocal image of the dendrite and iontophoresis electrode was taken and transferred into home-designed software. The area to be mapped was selected by indicating to the software the starting and ending dendritic locations and the desired interval between each point, typically 0.5–1 μm. During each step, glutamate was applied to the dendrite via −100 nA iontophoretic current, and the resulting glutamate-evoked response was recorded at the cell body. This iontophoretic current saturates the glutamate receptors of single synapses (see Fig. 1E). Each successive glutamate application was delayed by 1.5 s. Microelectrode movements were controlled by micromanipulators (MP-285; Sutter Instrument). Custom-written software was used to control glutamate iontophoresis, acquisition of current signals with whole cell recordings, and off-line data analysis.

**Receptor Density Measurements**

Receptor density was estimated from the glutamate-evoked responses according to the following relationship (assuming a homogenous population of channels with a constant γ, V_m, and p_o):

\[ R = \frac{\gamma(V_m - V_i) \cdot p_o \cdot N}{D \cdot A} \]

where R was recorded response, γ was single channel conductance, V_m was membrane potential, V_i was reversal potential, p_o was probability of opening, N was number of receptors, D was density of receptors, and A was surface area of stimulated region. Synaptic receptor number and density were estimated according to this relationship, using the values reported in the following text and the experimentally measured R. For extrasynaptic receptor density, since the relationship between evoked current and surface area was linear (see Figs. 2D and 4C) and γ, V_m, and p_o are assumed constant, then D can be estimated from the following relationship:

\[ D = \frac{slope}{\gamma(V_m - V_i) \cdot p_o} \]

where slope = R/A = γ(V_m - V_i) * p_o * D

or

\[ D = \frac{slope}{\gamma(V_m - V_i) \cdot p_o} \]

where the y intercept is forced to be 0, since we assumed that a dendrite with 0 surface area will have 0 evoked current. For AMPA receptor density estimates, we assumed that V_m = −60 mV, AMPA receptor V_i ~ 3 mV (see Fig. 1C), and p_o = 0.52 with a −100-nA iontophoretic pulse (Diamond and Jahr 1997) (see Appendix). Measurements of AMPA receptor γ have varied from 7 to 12 pS (Benke et al. 1998; Hauser and Roth 1997; Jonas et al. 1993; Spruston et al. 1995; Traynelis et al. 1993; Vodyanoy et al. 1993). Therefore we used the intermediate value of 10 pS. For NMDA receptor density estimates, we assumed that γ = 50 pS (Clark et al. 1997; Silver et al. 1992), V_m = −40 mV for synaptic measurements and −60 mV for extrasynaptic measurements, NMDA receptor V_i ~ 3 mV (see Fig. 1C), and p_o = 0.034 (see Fig. 3). A for extrasynaptic measurements was calculated using the equation for the surface area of a cylinder: A = 2πrl, where r is the dendritic radius, determined by the DIC image, and l is the length of the dendrite affected by glutamate, estimated to
be 3 μm for AMPA receptor and 8 μm for NMDA receptor measurements (see Fig. 1 and Appendix). The published value for postsynaptic density surface area in cultured hippocampal neurons (0.027 μm²) as determined from serial electronmicroscopy sectioning was used for A in synaptic measurements (Schikorski and Stevens 1997).

The surface area of neurons at 8 DIV was roughly estimated by determining the surface area of a model cell based on confocal images of cells filled with the intracellular dye Alexa Flour 568 hydrazide (5 A length of 30 μm). First, presynaptic boutons were labeled with FM1-43.

**RESULTS**

To reduce glutamate spillover to extrasynaptic locations (see results), a suction electrode (tip diameter ~ 2 μm) was placed opposite to the iontophoresis electrode. Multiple pulses of glutamate were applied to the synapse in the absence of extracellular Mg²⁺ or at +40 mV and in the presence of 5 or 20 μM (SR,105) (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine (MK-801; Tocris), and suction (2 PSI; Picospritzer ID, General Valve) was applied for 200 ms at 1 s following the iontophoretic pulse.

The NMDA receptor density calculation

To determine the distribution and density of functional glutamate receptors at single postsynaptic sites with the glutamate applications. Therefore we determined the dose-response relationship between iontophoretic current and evoked current at a single synapse in the absence of extracellular Mg²⁺. As shown in the dose-response curve of Fig. 1E, the strength of the iontophoretic pulse used in the glutamate receptor map (~100 nA) is sufficient to saturate both AMPA and NMDA receptors at single postsynaptic sites. The similar apparent affinities of AMPA and NMDA receptors for glutamate evident in Fig. 1E (EC₅₀ ~ ~50 nA for both) are due to the rapid time course of the glutamate application (Dube and Liu 1999). Furthermore the addition of cyclothiazide to block AMPA receptor desensitization did not significantly affect the peak evoked AMPA receptor response, suggesting that the glutamate iontophoresis results in little desensitization. Therefore the glutamate-evoked response can provide a good estimation of the number of receptors at the glutamate application site (data not shown).

The estimation of the number and density of receptors at a
synaptic site depends on the assumption that an FM1-43 fluorescent puncta represents a single synaptic release site as opposed to a cluster of synapses or a synapse with multiple active zones. Analysis of serial electron microscopy sections in hippocampal cultures has shown that the majority of synaptic boutons contain single active zones, with estimates ranging from 69% (Schikorski and Stevens 1997) to 97% (Forti et al. 1997). Furthermore, morphological analysis from our lab suggests that ~80% of FM1-43 puncta represent single synapses (Liu et al. 1999). Here, we provide physiological evidence that each FM1-43 puncta spot contains only one functional synapse. When we mapped multiple synapses from single cells, we found that there was little variability in the peak AMPA receptor response from synapse to synapse within individual neurons (Fig. 1F). In fact, the variation within the neurons (25 ± 5% of mean) was much smaller than the variation between neurons (190%). This suggests that most of the mapped synapses within individual neurons contained similar numbers of AMPA receptors and, therefore, that each FM1-43 puncta contains a similar number of postsynaptic sites. Since it is unlikely that all of these FM1-43 puncta contained multiple synapses, we propose that the vast majority of FM1-43 punctae from which we collected data contained only one active zone and one single synapse. However, in one case, the recorded AMPA receptor response was approximately double the size of the response from other synapses on the same neuron (Fig. 1F). A logical interpretation of this response is that this synapse was one of the minority of FM1-43 puncta that contained either multiple active zones or clusters of multiple synapses.

Distribution and density of functional AMPA receptors during synaptogenesis

Using the glutamate receptor map, we characterized the distribution of functional AMPA receptors in the dendritic membrane before and after synaptogenesis. Following plating, hippocampal neurons develop neurites and begin to form synapses after 4 DIV (Basarsky et al. 1994; Fletcher et al. 1994). However, until after 7 DIV, the number of synapses as labeled by FM1-43 remains very low. Between 8 and 10 DIV, there is a dramatic increase in the density of FM1-43 labeled boutons (Fig. 2C), reflecting a rapid rate of synapse formation during these days in culture. When we mapped the dendrites of neurons from 6 to 7 DIV that had no FM1-43 labeled boutons, we found a small but consistent level of evoked AMPA receptor current along every mapped dendritic region (Fig. 2A). When dendrites were mapped after synapse formation (>8 DIV), functional AMPA receptor clusters were found exclusively colocalized with presynaptic terminals, while a small AMPA receptor current was evoked in all mapped extrasynaptic regions (Fig. 2B). These data indicate that, prior to synaptogenesis, there is a pool of functional AMPA receptors in the dendrite and that, following synapse formation, functional AMPA receptors cluster at synaptic sites and a receptor pool remains in the extrasynaptic membrane.

To estimate the density of AMPA receptors prior to synapse formation, we plotted the glutamate-evoked responses from dendrites that contained no synapses (<8 DIV) against the surface area of the dendritic segment affected by the glutamate applications, which varies only with the diameter of the dendrite (Fig. 2D, see METHODS). These data could be fit linearly (Fig. 2D: slope = 0.88, 95% C.I. = 0.81–0.95, R = 0.72, P < 0.0001), indicating that the pool of AMPA receptors before synapse formation is evenly distributed throughout the dendrite. The slope of the linear fit is equal to the AMPA receptor-mediated current density from these dendritic regions (0.88 ± 0.04 pA/µm²; V_m = −60 mV). From this value, we estimated the density of this AMPA receptor pool to be 2.7 ± 0.1 receptors/µm². (See METHODS for details of all density estimates.) Current densities were converted to conductance densities by normalizing them by the holding potential at which the recordings were made. All conductance densities and estimated receptor densities are reported in Table 1.

To determine if this pool of AMPA receptors aggregates to form synaptic AMPA receptor clusters, we estimated the number and density of AMPA receptors per synapse and predicted the resultant decrease in the density of the AMPA receptor pool following synaptogenesis. Using the mean peak evoked AMPA receptor-mediated current from synapses at 8–10 DIV (125 ± 14 pA, n = 11), we calculated the synaptic AMPA receptor conductance and receptor density (Table 1) and found that each synapse contains ~370 ± 40/synapse. Since neurons at 8 DIV have an estimated surface area of ~5,000 µm² (see METHODS), each neuron contains on the order of 14,000 functional AMPA receptors in the membrane prior to synapse formation given a baseline receptor density of ~3/µm². If clusters formed by the aggregation of existing inserted receptors, the formation of as few as 10 synapses would decrease the extrasynaptic receptor density.

FIG. 1. Glutamate receptor map technique. A: schematic of the glutamate receptor map. See METHODS for details. B: α-amino-3-hydroxy-5-methyl-4-isoxazolpropionate (AMPA) receptor map (V_m = −60 mV). White dots indicate the locations at which glutamate was applied to the dendrite. The glutamate-evoked currents from 4 locations are displayed with the peak current aligned with the location of the glutamate application. The distance between the white and yellow dots represents the peak amplitude of the glutamate-evoked current at the corresponding white dots. This convention is used for all map figures. C: voltage dependency of the glutamate-evoked response at a single synapse. Glutamate was applied (~100 nA) during 20 mV incremental steps of the holding potential from −80 to +40 mV. Inset: evoked current traces of I-V paradigm. AMPA and N-methyl-D-aspartate (NMDA) receptor-mediated components of the glutamate-evoked response were resolved by their kinetics and voltage dependency. Peak AMPA receptor current was defined as the maximum current amplitude measured between 1 and 4 ms following glutamate application. Peak NMDA receptor current was measured as the average of the evoked current over 20 ms at 10 ms following glutamate application. D: NMDA receptor map (V_m = +40 mV; 10 µM 6,7-dinitroquinoxaline-2,3-dione (DNQX)). Representative glutamate-evoked current traces (white lines) are shown for 3 dendritic locations. E: dose-response relation of glutamate-evoked response at a single synapse. Inset: glutamate-evoked responses, recorded from the cell body, resulting from increasing iontophoretic applications from −12 to −150 nA to a single FM1-43-labeled synapse in the absence of extracellular Mg²⁺. Main: resulting dose-response curve. AMPA and NMDA receptor-mediated components of the glutamate-evoked response were resolved by their kinetics as described in the preceding text. F: evoked AMPA receptor responses from multiple synapses on single neurons. The AMPA receptor-mediated iontophoretic response was recorded from multiple synapses (2–5) in 17 neurons and plotted against the number of the sampled neuron. The evoked currents were plotted in ascending order according to smallest amplitude.
density by 25%, assuming that the receptors are freely diffusible.

We analyzed the AMPA receptor current from extrasynaptic regions of cells that contained functional AMPA receptor clusters by plotting the glutamate-evoked response from these areas against the stimulated dendritic surface area. These data could also be fit linearly with the same line as the data from before synaptogenesis (Fig. 2D; slope = 0.88, 95% C.I. = 0.73–1.01, R = 0.84, P < 0.0001). Since the AMPA receptor current density (slope = 0.88 ± 0.08 pA/μm²; V_m = −60 mV) was exactly the same as that for before synapse formation, the estimated density of extrasynaptic AMPA receptors remained constant following synapse formation (Table 1). These density estimates highlight the tight clustering of functional AMPA receptors at synaptic sites and suggest that synaptic AMPA receptor clusters do not form solely by the lateral aggregation of receptors present in the dendritic membrane prior to synapse formation (see Discussion).

Estimation of the NMDA receptor probability of opening

As with AMPA receptors, we wanted to assess the density of functional NMDA receptors in the dendritic membrane. However, this calculation depends critically on the NMDA receptor p_o, a value whose estimates have varied by a factor of 100 and been derived by relatively indirect methods (Hessler et al.)
GLUTAMATE RECEPTOR DISTRIBUTION, DENSITY, AND CLUSTERING

TABLE 1. AMPA and NMDA receptor conductance density measurements and receptor density estimates

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<th>AMPA</th>
<th>NMDA</th>
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<td>Conductance Density, pS/μm²</td>
<td>Receptor Density, R/μm²</td>
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<tr>
<td>Before</td>
<td>14 ± 0.6 (36)</td>
<td>2.7 ± 0.1</td>
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<tr>
<td>After</td>
<td>Extrasympatic</td>
<td>14 ± 1.2 (11)</td>
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<td></td>
<td>Synaptic</td>
<td>7.1 × 10⁴ ± 0.8 × 10⁴ (11)</td>
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<td>Receptors/Synapse</td>
<td>370 ± 40</td>
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All values are the means ± SE. Parentheses enclose number of recordings.

1993; Huettner and Bean 1988; Jahr 1992; Rosenmund et al. 1993, 1995). Because of this wide discrepancy, we estimated the $p_o$ of NMDA receptors in our system by perfusing the NMDA receptor open channel blocker MK-801 (5 or 20 μM), directly activating synaptic NMDA receptors with iontophoretically applied glutamate, and using the rate of NMDA receptor blockade to calculate the $p_o$ (Huettner and Bean 1988; Jahr 1992; Rosenmund et al. 1993, 1995). Since glutamate spillover to extrasynaptic receptors would complicate the interpretation of our data, we placed a suction pipette on the opposite side of the glutamate electrode, and suction (2 PSI) was applied for 200 ms beginning at 1 ms following the end of the glutamate application. This technique significantly reduces glutamate spillover to extrasynaptic NMDA receptors (Dubé and Liu 1999). At a holding potential of +40 mV, repeated applications of glutamate to a single synapse elicited constant AMPA and NMDA receptor-mediated currents (Fig. 3A, top).

In the presence of 20 μM MK-801, the NMDA receptor-mediated current decreased with each pulse of glutamate and approached baseline within 10 applications, while the AMPA receptor-mediated current remained relatively constant (Fig. 3A, bottom). Since the MK-801 blockade recovery is markedly faster at positive holding potentials ($τ$ = 2 min) (Huettner and Bean 1988), performing these experiments at +40 mV may have affected the data analysis. However, there were no differences in the results of these experiments ($n$ = 3) and those with negative holding potentials in zero Mg²⁺ ($n$ = 2).

Figure 3B shows the NMDA receptor-mediated charge transfer ($Q$) following sequential glutamate applications to a single synapse in 20 and 5 μM MK-801 normalized against the NMDA receptor-mediated $Q$ following the first glutamate application. This value decayed exponentially during the glutamate applications in both 20 μM ($R$ = 0.97) and 5 μM ($R$ = 0.99) MK-801, with the rate of decay markedly faster in 20 μM MK-801. These data indicate that equal proportions of NMDA receptors were blocked with each pulse of glutamate and that the rate of MK-801 block varied only according to the concentration of the drug. Using the methods described in Rosenmund et al. (1995) (see METHODS), we used the rate of MK-801 block to estimate the $p_o$ of NMDA receptors to be 0.034 ± 0.002 in MK-801 concentrations of 20 and 5 μM ($n$ = 5). Results in both MK-801 concentrations were not different and were therefore pooled.

Distribution and density of functional NMDA receptors during synaptogenesis

We assessed the distribution of NMDA receptors before and after synaptogenesis. All NMDA receptor recordings were performed at either +40 mV in 1 mM Mg²⁺ or at −60 mV in 0 Mg²⁺. When dendrites were mapped before synapse formation, there was a consistent level of NMDA receptor-mediated evoked current along the entire mapped dendritic tree (Fig. 4A). When the amplitude of the NMDA receptor-mediated evoked response from before synapse formation was plotted against the surface area of the stimulated dendritic region, the

![Fig. 3](http://jn.physiology.org/)

**Fig. 3.** Estimation of NMDA receptor $p_o$. A: single synapse glutamate-evoked responses elicited at +40 mV in the absence of (top) and presence of 20 μM (5R,10S)-(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine (MK-801; bottom). The results of every 3rd glutamate application are depicted, and the number of the application is shown above each trace. B: NMDA receptor-mediated charge transfer ($Q$) following sequential pulses of glutamate ($Q_1$) applied to a single synapse in 20 μM (○) or 5 μM (●) MK-801 normalized against the NMDA receptor-mediated $Q$ following the first pulse ($Q_1$). Charge transfer was determined by integrating the NMDA receptor current from 15 to 100 ms following glutamate application when there is little contamination from the AMPA receptor-mediated current. Both sets of data were fit with a single exponential decay.
results could be fit linearly (Fig. 4C; slope = 0.29, 95% C.I. = 0.26–0.31, \( R = 0.88, P < 0.0001 \)), indicating that, like AMPA receptors, there is a homogeneously distributed pool of functional NMDA receptors in the dendritic membrane prior to synapse formation. From the slope of this fit (0.29 ± 0.04 pA/\( \mu \)m²), we estimated the density of this pool to be 2.7 ± 0.4/\( \mu \)m² (Table 1).

Following synapse formation, NMDA receptor clusters were resolved at synaptic sites, and there remained a baseline level of NMDA receptor-mediated evoked current in extrasynaptic locations (Fig. 4B). As with before synaptogenesis, the amplitude of the NMDA receptor-mediated current varied linearly with the stimulated dendritic surface area (Fig. 4C; slope = 0.31 ± 0.06 pA/\( \mu \)m²; 95% C.I. = 0.27–0.35, \( R = 0.88, P < 0.0001 \)), indicating that the pool of NMDA receptors in the extrasynaptic membrane remain evenly distributed with an estimated density of 2.9 ± 0.6/\( \mu \)m² (Table 1). Thus there was no significant change in the NMDA receptor density in extrasynaptic regions following synapse formation (\( P > 0.3 \)).

To estimate the number of NMDA receptors per synapse, it is necessary to subtract from the peak synaptic response the current resulting from spillover to extrasynaptic receptors by the time the peak NMDA receptor response was measured. We did not do this for AMPA receptor measurements because spillover is significantly smaller, and the total amount of spillover-induced current was normally <10% of the peak response. Baseline NMDA receptor current was calculated from the diameter of the dendrite from which the NMDA receptor current was sampled and from the slope in Fig. 4C. We subtracted this baseline NMDA receptor current from the peak synaptic NMDA receptor current. Using the corrected peak NMDA receptor amplitude values (28 ± 6 pA, \( n = 10 \),

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**Fig. 4.** Distribution of functional NMDA receptors before and after synapse formation. A and B: NMDA receptor maps (0 mM Mg²⁺; 10 \( \mu \)M DNQX; \( V_m = -60 \) mV) from dendrites before (7 DIV, A) and after (9 DIV, B) synapse formation. Inset: representative glutamate-evoked current traces. C: amplitude of extrasynaptic current prior to (black squares) and after (red circles) synaptogenesis plotted against the dendritic surface area affected by glutamate at the time the peak NMDA receptor response was measured (10 ms). Both sets of data were fit with linear regression analysis.
we calculated the synaptic NMDA receptor-mediated conductance density and estimated the number and density of synaptic NMDA receptors (Table 1).

Having estimated the densities of AMPA and NMDA receptors, we further calculated the ratio of AMPA to NMDA receptor densities in all dendritic regions. Interestingly, there were no significant differences in any of the densities measurements (Table 1; \( P > 0.5 \) for all cases). This suggests that the ratio of AMPA to NMDA receptor densities is close to one in all cases, including at synaptic sites.

**Time course of AMPA and NMDA receptor clustering during synaptogenesis**

Having established that functional AMPA and NMDA receptors are homogeneously distributed in the dendritic membrane prior to synaptogenesis and are clustered at individual synapses afterward, we attempted to determine the order in which these receptors cluster in developing synapses. If there is a significant discrepancy in the appearance of one type of receptor cluster, then we should observe synapses that contain only one or the other type of receptor in newly formed synapses between 8 and 10 DIV (see Fig. 2C). To determine the colocalization of clustered synaptic AMPA and NMDA receptors, we performed AMPA receptor maps at FM1-43 punctae and subsequently returned the glutamate electrode to the putative synapse to record the glutamate-evoked I-V response. The AMPA and NMDA receptor clusters were resolved in the I-V plot by their kinetics and voltage dependency. Like the example shown in Fig. 5A, the majority of FM1-43 punctae contained both AMPA and NMDA receptor clusters (68%, \( n = 43 \)). While four AMPA receptor-only clusters were detected (9%; Fig. 5B), we did not find any NMDA receptor-only synapses. Thus since most nascent glutamatergic synapses contained functional AMPA and NMDA receptor clusters, these receptor clusters likely form at a relatively similar time course during synapse formation. Additionally, a minority of FM1-43 punctae showed neither AMPA nor NMDA receptor clusters (23%; Fig. 5C). The glutamate-evoked currents from these locations were no different from the baseline-evoked currents. These synapses may have been GABAergic as a similar percentage of dendritic synapses in hippocampal cultures were found to be GABAergic (Benson and Cohen 1996).

Neither activity nor glutamate receptor activation is necessary for AMPA receptor insertion, clustering, and activation

The fact that functional glutamate receptor cluster formation and presynaptic terminal formation correlate both spatially and temporally during synaptogenesis raises the possibility that vesicle release from the newly formed terminal may trigger AMPA receptor insertion, clustering, and/or activation. In fact, electrophysiological data have suggested that glutamate release and specifically, NMDA receptor activation, may be necessary for this process during synaptogenesis (Durand et al. 1996; Isaac et al. 1997; Liao and Malinow 1996; Wu et al. 1996). Therefore we used the glutamate receptor map technique to directly test the role of neuronal activity and glutamate receptor activation in AMPA receptor insertion, clustering, and activation during synaptogenesis.

We first examined whether NMDA receptor activation is necessary for this process by blocking NMDA receptors during synaptic development with the NMDA receptor antagonist d-CPP (5 \( \mu M \)) beginning at 0 DIV. Between 8 and 10 DIV following NMDA receptor blockade, functional AMPA receptor clusters were observed colocalized with presynaptic terminals (Fig. 6A; 7 synapses, 3 cells, 3 cultures), suggesting that NMDA receptor activation is not required for AMPA receptor insertion, clustering, and activation during synapse formation.

To control for nonspecific pharmacological effects of the d-CPP treatment, we cultured hippocampal neurons from mice in which the NMDA receptor NR1 subunit has been deleted (Li et al. 1994). This subunit is critical for the formation of functional NMDA receptors (Monyer et al. 1992). Indeed, the synapses from neurons derived from NR1 knockout mice did not contain any functional NMDA receptors, as demonstrated by the voltage dependency of the glutamate-evoked response (Fig. 6C). Consistent with the results of the pharmacological NMDA receptor blockade, functional AMPA receptor clusters were observed colocalized with presynaptic terminals at 10 DIV in NR1 deficient neurons (Fig. 6B; 12 synapses, 5 cells, 2 cultures). Together these data show that NMDA receptor activation is not required for AMPA receptor insertion, clustering, and activation during synaptogenesis.

We next attempted to determine if any type of neuronal activity or glutamate receptor activation is required for the insertion and clustering of functional AMPA receptors during synaptogenesis. We added TTX (1 \( \mu M \)) to the culture medium.
FIG. 6. Blockade of neither activity nor glutamate receptor activation prevents AMPA receptor insertion, clustering, and activation. A: AMPA receptor map performed at 9 DIV following treatment with d-CPP beginning at 0 DIV. B: AMPA receptor map performed at 8 DIV on a dendrite of an NR1(--)/neuron. C: voltage-dependency of glutamate-evoked response at NR1(--/--) synapse. Left: evoked current traces of I-V paradigm. Right: resulting I-V curve. D and E: AMPA receptor maps at 8–10 DIV following pharmacological treatments with 1 μM TTX (D) and glutamate receptor antagonist cocktail (E; 5 μM d-CPP, 10 μM DNQX, 500 μM AIDA, 500 μM EGLU, and 500 μM CPPG to block NMDA, AMPA/kainate, and group I–III metabotropic glutamate receptors, respectively). All map scale bars, vertical: 40 pA; horizontal: 2 μm.
at the time of plating to block activity-dependent vesicle release. Between 8 and 10 DIV following TTX treatment, functional AMPA receptor clusters were resolved colocalized with FM1-43-stained terminals (Fig. 6D; 8 synapses, 4 cells, 3 cultures), indicating that activity-dependent vesicle release is not necessary for AMPA receptor insertion, clustering, and activation. These data do not rule out the possibility that activity-independent vesicle release may trigger this process. Therefore we added a cocktail of antagonists to every known glutamate receptor subtype to the culture medium beginning at 0 DIV. The cocktail included 10 μM DNQX, 1 μM t-CPP, 500 μM AIDA, 500 μM EGLU, and 500 μM CPPG to block AMPA receptors, NMDA receptors, and group I–III mGluRs, respectively (Jane et al. 1996; Pellicciari et al. 1995). Following this treatment, functional AMPA receptor clusters were detected colocalized with FM1-43-stained terminals at 8–10 DIV (Fig. 6E; 8 synapses, 5 cells, 5 cultures), suggesting that glutamate receptor activation is unnecessary for the insertion and clustering of functional AMPA receptors during synapse formation.

**DISCUSSION**

**Distribution and density of glutamate receptors before and after synapse formation**

Since the glutamate receptor map sampled only functional receptors, there may have been nonfunctional receptors present in the dendritic membrane that avoided detection and contaminated our density estimates. According to our data, it is possible that there is an equal density of receptors in the membrane following synapse formation but that a much larger proportion of receptors at the synapse are functional. However, this is an unlikely scenario as antibody labeling of both AMPA and NMDA receptors has found that there is strong punctate staining at synaptic sites, suggesting that these proteins have a markedly higher density at synaptic sites (Craig et al. 1993; Rao and Craig 1997; Richmond et al. 1996). Thus the functional glutamate receptor clustering we observe is due to the physical clustering of receptors at the synapse. Furthermore in contrast with antibody-labeling studies that reported a number of extrasynaptic NMDA receptor clusters (Rao and Craig 1997; Rao et al. 1998), we found that all clusters of functional NMDA receptors colocalized with presynaptic terminals. Although there may be nonfunctional clusters of receptors outside of synaptic locations, this would require an improbable mechanism to maintain receptor clusters in a nonfunctional state within a pool of nonclustered functional receptors. Therefore we propose that all inserted AMPA and NMDA receptors are normally in a functional state and that the formation of AMPA and NMDA receptor clusters is regulated to occur only at developing synapses.

Interestingly, the glutamate receptor densities we report here are similar to the estimated AChR densities in the NMJ (Hall and Sanes 1993; Sanes and Lichtman 1999). We found that there is a pool of functional AMPA and NMDA receptors with a density of ~3/μm² in the extrasynaptic membrane after synaptogenesis, while the estimated density and number of synaptic AMPA and NMDA receptors were on the order of 10³/μm² and 400/synapse, respectively (see Table 1). These similarities suggest that ionotropic neurotransmitter receptors may be regulated to maintain a synaptic density on the order of 10⁴/μm² that is optimum for signal transmission. It will be interesting to determine if other ionotropic receptors are clustered at a similar density.

The only major difference in receptor density estimates is prior to synapse formation, which likely reflects differences in the mechanism of receptor clustering. At the NMJ, a relatively high density of AChRs (~1,000/μm²) are inserted into the muscle membrane prior to synapse formation, and these receptors aggregate to form a receptor cluster. Like AChRs in the NMJ, it is possible that the evenly distributed glutamate receptors prior to synaptogenesis aggregate to form synaptic clusters. However, unlike the NMJ, there is a relatively low density of glutamate receptors in the dendritic membrane prior to synapse formation (~3/μm²), and there is no decrease in extrasynaptic glutamate receptor density following synapse formation, including following the time points sampled in this study (>10 DIV; data not shown). These data suggest that the lateral aggregation of existing receptors is not the primary mechanism of glutamate receptor clustering and that glutamate receptor cluster formation must rely almost entirely on the insertion of new receptors. Thus the mechanism of glutamate receptor clustering in hippocampal synapses is different from the mechanism of AChR clustering at the developing NMJ. The newly inserted glutamate receptors in the developing clusters can be obtained in two ways: receptors continue to be inserted into the extrasynaptic membrane and diffuse into the postsynaptic site (Craig et al. 1994; Nusser et al. 1998) or receptors are inserted directly into the postsynaptic site (Craig et al. 1994; Richmond et al. 1996; Steward 1995; Tovar and Westbrook 1999). Monitoring the dendritic membrane with the glutamate receptor map may allow us to determine where these receptors are inserted.

In addition, our data suggest that the ratio of AMPA to NMDA receptor density is close to one in all dendritic locations, including the synapse. This raises the possibility that the level of AMPA and NMDA receptor expression and/or insertion may be coordinated to produce such a constant ratio. Previous reports estimated the number and density of synaptic NMDA receptors to be much lower than we report here (Jahr 1992; Silver et al. 1992; Spruston et al. 1995; Stern et al. 1992). However, these calculations all relied on a relatively high value of the NMDA receptor pₒ (~0.3) (Hessler et al. 1993; Jahr 1992). Critically, a number of other reports have suggested that this value is significantly lower (pₒ < 0.04) (Huettner and Bean 1988; Rosenmund et al. 1993, 1995). These discrepant reported pₒ values may have arisen because of different experimental preparations, each of which has its limitations. Some of these reports used outside-out patches, which removes the channel from its native environment and requires equilibrating doses of glutamate, while others used synaptic transmission, which involves multiple synapses and may include transmission failures. Since an accurate estimation of receptor density is dependent on this value, we attempted to overcome the limitations of previous studies by measuring the NMDA receptor pₒ using direct, saturating glutamate applications to NMDA receptors in their native synaptic environment. Under these conditions, we estimated the NMDA receptor pₒ to be ~0.03. When this low pₒ is taken into account, the estimated number and density of synaptic NMDA receptors is much higher than previously calculated.
Importantly, few glutamate receptor clusters sampled in this study were on dendritic spines. However, dendritic spines occur infrequently at the culture ages from which we collected our data as most synapses are made directly on the dendritic shaft at early stages of culture development (Boyer et al. 1998; Papa et al. 1995). A similar situation occurs in the hippocampus in vivo, where the vast majority of synapses are made on dendritic shafts until spine synapses form after postnatal day 15 (Boyer et al. 1998; Cotman et al. 1973; Fiala et al. 1998; Harris et al. 1992; Pokorny and Yamamoto 1981; Steward and Falk 1991). In fact, it has been proposed that during early synaptogenesis, synapses are initially formed on dendritic shafts from which dendritic spines eventually develop (Fiala et al. 1998). Thus the process of glutamate receptor clustering that we describe here may be similar to how it occurs during synaptogenesis in vivo.

**Signal for clustering and activation of AMPA receptors at developing synapses**

A number of electrophysiological studies have found that there are more NMDA than AMPA receptor-mediated synaptic events during early development and that plasticity inducing paradigms causes synapses that show NMDA receptor-only mediated events to then display both receptor components (Durand et al. 1996; Isaac et al. 1997; Liao and Malinow 1996; Wu et al. 1996). These data have been interpreted to mean that the activation of synaptic NMDA receptors is the signal that triggers AMPA receptor insertion and clustering at that synapse during its formation. Such a mechanism would require NMDA receptors to cluster prior to AMPA receptors. As such, immunolabeling has been used to determine the order of AMPA and NMDA receptor clustering during synapse formation. A number of reports have found that a proportion of synapses contain only NMDA receptors, particularly during early development (Gomperts et al. 1998; He et al. 1998; Liao et al. 1999; Petralia et al. 1999; Takumi et al. 1999). This evidence has been used to support the idea that synaptic NMDA receptor activation is necessary for AMPA receptor insertion into the synapse.

Surprisingly, with the glutamate receptor map, we did not locate any NMDA receptor-only synapses during an early stage of synaptogenesis in cultured hippocampal neurons. Although we did locate several synapses that contained only AMPA receptor clusters, we found that the majority of glutamatergic synapses contains both AMPA and NMDA receptor clusters. Thus functional AMPA and NMDA receptors appear to cluster at a similar time course in developing glutamatergic synapses. The reason for the discrepancy between our data and the immunocytochemical data are unclear, although possibilities include that the glutamate receptor map technique may detect more AMPA receptor clusters due to its greater sensitivity than immunolabeling, that there are synapses that contain only nonfunctional NMDA receptors that avoid detection with our glutamate receptor map, and that those synapses that contain only NMDA receptors do not stain with FM1-43 and are therefore both pre- and postsynaptically “silent” (Malgaroli 1999). Furthermore functional NMDA receptor-only synapses may exist at this time, but the proportion of these synapses must be low to have avoided detection. Regardless, the most direct approach to understanding the colocalization of functional receptors is to apply glutamate directly to the synapse and record the resulting response.

Importantly, data regarding the time course of AMPA and NMDA receptor cluster formation are purely correlational, and it is difficult to draw causative information from such results. For example, in our culture system, NMDA receptor-only synapses may be rapidly converted to synapses that contain both AMPA and NMDA receptors. Therefore a direct test to determine whether NMDA receptor activation is necessary for AMPA receptor insertion, clustering, and activation is to study functional AMPA receptor expression in synapses after NMDA receptor blockade during neuronal development.

Following both pharmacological blockade and genetic removal of NMDA receptor activation, we found that functional AMPA receptors were clustered at newly formed synapses. In contrast with the electrophysiological studies that suggest that NMDA receptor activation is essential for functional AMPA receptor clustering (Durand et al. 1996; Isaac et al. 1997; Liao and Malinow 1996; Wu et al. 1996), work with antibody labeling in cultured neurons indicates that AMPA receptor clustering can occur independently of activity or glutamate receptor activation (Craig et al. 1994; Mammen et al. 1997). This discrepancy may be due to the fact that following NMDA receptor blockade, AMPA receptor clusters are inactive and “silent” on presynaptic stimulation, as antibody labeling gives no information regarding the functional status of the receptors. Since the glutamate receptor map technique sampled functional AMPA receptors, we ruled out the possibility that AMPA receptor clusters following NMDA receptor blockade are in an inactive state. Furthermore our results are not likely attributable to differences between slice and culture preparations, considering that normal synaptic AMPA receptor current has been observed in hippocampal slices (Kutsuwada et al. 1996) and in brain stem trigeminal complex slices (Li et al. 1994) following the genetic removal of functional NMDA receptors. Thus NMDA receptor activation is not necessary for AMPA receptor insertion, clustering, and activation during synapse formation.

These results appear to contradict studies that found synapses that show NMDA receptor-only synaptic events during early development and that can be activated to demonstrate both AMPA and NMDA receptor responses (Durand et al. 1996; Isaac et al. 1997; Liao and Malinow 1996; Wu et al. 1996). However, it is theoretically possible that individual synapses containing both AMPA and NMDA receptors could show NMDA receptor-only responses due to the presynaptic release profile of glutamate (Choi et al. 2000) and the different activation kinetics of AMPA and NMDA receptors (Dubé and Liu 1999) and not to the absence of functional AMPA receptors. Such proposed synapses have been observed in hippocampal slices (Choi et al. 2000). Therefore we speculate that during synaptogenesis, functional AMPA receptors do indeed cluster independently of NMDA receptor activation and that NMDA receptor-only events may result from presynaptic effects and the differential responses of AMPA and NMDA receptors to relatively prolonged release profiles of glutamate (Dubé and Liu 1999).

Our data further suggest that neither neuronal activity nor activation of any known subtype of glutamate receptor is necessary for AMPA receptor insertion, clustering, and activation during synapse formation. Although we have not ruled out
the possibility of an activity-independent, vesicularly released molecule that may trigger this process, we hypothesize that an activity-independent, contact-mediated surface signal may be sufficient for AMPA receptor clustering and activation during synapse formation.

APPENDIX

To estimate accurately the number and density of glutamate receptors, it is necessary to determine the dendritic surface area affected by each iontophoretic glutamate application. This calculation requires estimations of the spread of glutamate and of the glutamate concentration dependency of channel opening. The spread of glutamate can be estimated by solving the diffusion equation with the appropriate boundary conditions. Since the distance between the tip of the electrode and the dendritic membrane is significantly smaller than the distance between the tip of the electrode and the surface boundaries of the recording chamber, the diffusion process is equivalent to the release of glutamate from a point source into an infinite space. The solution of the diffusion equation with the boundary conditions described in the preceding text gives the concentration of glutamate \( C \) at any time \( t \) and position relative to the electrode tip \( r \).

For an iontophoretic source continuously applied from 0 to \( n \) ms

\[
C(r, t) = \frac{q}{4\pi Dr} \text{erfc} \left( \frac{r}{2\sqrt{Dt}} \right) \]

For time following iontophoretic application \( (t > n \text{ ms}) \)

\[
C(r, t) = \frac{q}{4\pi Dr} \left[ \text{erf} \left( \frac{r}{2\sqrt{D(t-n)}} \right) - \text{erf} \left( \frac{r}{2\sqrt{Dt}} \right) \right]
\]

where \( D \) is the diffusion constant, \( q \) is the rate of glutamate release during iontophoresis, and \( n \) is the length of time of the glutamate application (1 ms in this study). Since the glutamate-evoked AMPA receptor current reaches its peak before 3 ms following the release of glutamate, we checked the solution at \( t = 3 \text{ ms} \) (Fig. A1B, —). Within 3 ms after initial glutamate release, the predicted glutamate concentration is highly localized and falls to 10% of its peak value 1.8 \mu m away from the electrode tip following a \(-100 \text{ nA} \) iontophoretic pulse.

The concentration dependency of AMPA receptor opening can be estimated by performing a dose-response paradigm at a single synapse (Fig. A1A). We converted iontophoretic current to glutamate concentration by assuming that the concentration of glutamate required to activate 50% of AMPA receptors (\( EC_{50} \)) in our system is similar to that in previous studies (480 \mu M) (Jonas et al. 1993; Patneau and Mayer 1990). Therefore we assumed that the iontophoretic current that resulted in the half-maximal response (about \(-60 \text{ nA} \)) produced a glutamate concentration of 480 \mu M in the synaptic cleft at the time the peak AMPA receptor-mediated current was recorded (3 ms). We further calculated the concentration and time course of glutamate diffusion 0.5 \mu m away from the tip of the iontophoresis electrode following increasing iontophoretic pulses from \(-12 \text{ to } -200 \text{ nA} \) (Fig. A1A, left inset). Following these applications, the peak glutamate concentration under the tip of the iontophoresis electrode was estimated to range from 100 to 1,600 \mu M, respectively, and to return to baseline within 2 ms. These simulations indicate that the glutamate application is rapid and approaches the time course of synaptic transmission (Clements et al. 1992). Since the maximum AMPA receptor \( p_o \) has been estimated to be 0.58 (Diamond and Jahr 1997) and the response evoked by \(-200 \text{ nA} \) current was the maximum, we further converted the original dose-response curve into probability of channel opening as a function of glutamate concentration (Fig. A1A, main).

We used the estimated concentration-dependency of AMPA receptor opening (Fig. A1A) and the glutamate concentration profile (Fig. A1B, —) to estimate the AMPA receptor \( p_o \) along the dendrite 3 ms following initiation of glutamate application (Fig. A1B, - - -). The \( p_o \) decreased to baseline at \(-1.5 \mu m \) from the electrode tip, indicating that AMPA receptors along a 3 \mu m dendritic segment are activated within 3 ms after the release of glutamate. Thus the peak glutamate-evoked response is derived from AMPA receptors within a 3 \mu m segment of the dendrite. This theoretical calculation was supported by experimental observation (see Fig. 1B).

In principle, similar calculations can be carried out to determine the length of dendrite in which NMDA receptors are activated by glutamate applications. However, since the NMDA receptor has a much longer first latency (the time from receptor binding to its first opening) than the AMPA receptor (Dzubay and Jahr 1996), glutamate diffuses away by the time the NMDA receptor response reach its maximum \((\sim 10 \text{ ms}) \). Therefore it is not practical to predict the dendritic length whose NMDA receptors will be affected by the spread of glutamate by these methods. Since the results of the AMPA receptor map matched the results of the theoretical calculations, we used the width of the distribution of NMDA receptor-mediated peak in the NMDA receptor map (Fig. 1D) to approximate this value to be 8 \mu m.

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