The Incorporation of NMDA Receptors with a Distinct Subunit Composition at Nascent Hippocampal Synapses In Vitro

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Activity-dependent synaptic rearrangements during CNS development require NMDA receptor activation. The control of NMDA receptor function by developmentally regulated subunit expression has been proposed as one mechanism for this receptor dependence. We examined the phenotype of synaptic and extrasynaptic NMDA receptors during the development of synaptic load using the NMDA receptor 2B (NR2B)-selective antagonist ifenprodil. In cultured rat hippocampal neurons when relatively few synapses had formed, the ifenprodil block of EPSCs was less than whole-cell currents, the latter of which included both synaptic and extrasynaptic receptors. At the same developmental stage, we found that extrasynaptic receptors outnumbered synaptic receptors by 3:1; thus whole-cell currents were dominated by the extrasynaptic population. We used the macroscopic kinetics of ifenprodil block to distinguish between the receptor populations. The ifenprodil kinetics of whole-cell currents from neurons before and during the development of synaptic load was comparable with that of whole-cell currents in HEK293 cells transfected with NR1 and NR2B cDNA, indicating that extrasynaptic receptors are largely NR1/NR2B heteromers. In contrast, synaptic receptors included both a highly ifenprodil-sensitive (NR1/NR2B) component as well as a second population with lower ifenprodil sensitivity; the reduced ifenprodil block of EPSCs was attributable to synaptic receptors with lower ifenprodil sensitivity rather than to the appearance of ifenprodil-insensitive (NR1/NR2A) receptors. Our data indicate that the synaptic NMDA receptor complement changes quickly after synapse formation. We suggest that synapses containing predominately NR1/NR2B heteromers represent “immature” sites, whereas mature sites express NMDA receptors with a distinct, presumably triheteromeric, subunit composition.

Key words: hippocampus; ifenprodil; NMDA receptors; patch clamp; synapse formation; synaptic load

The NMDA receptor is implicated in developmentally associated synaptic rearrangements in the vertebrate CNS. Native NMDA receptors are assembled from NMDA receptor 1 (NR1) and NR2 subunits (ξ and ε in mouse; Kutsuwada et al., 1992). The expression pattern of these subunits changes during development (Monyer et al., 1994; Sheng et al., 1994), suggesting that NMDA receptors composed of particular subunit combinations may govern the time course of the critical period during cortical development (Sheetz and Constantine-Paton, 1994). In the hippocampus, mRNA for NR1 and NR2B is predominant at times when synapses are forming, whereas mRNA for NR2A is low and then increases to plateau levels later in development (McDonald and Johnston, 1990; Monyer et al., 1994). NMDA receptor-mediated synaptic responses are not present in hippocampal slices from neonatal NR2B−/− mice (Kutsuwada et al., 1996), implying that NR2B is required for channel formation or synaptic localization of functional NMDA receptors. Consistent with this expression pattern, mice lacking NR1 or NR2B die soon after birth (Forrest et al., 1994; Kutsuwada et al., 1996).

The expression pattern of NMDA receptor subunits could influence the development, maintenance, and stabilization of synapses by several potential mechanisms. In heterologous expression systems (Monyer et al., 1994; Krupp et al., 1998), NMDA receptor properties are dependent on subunit composition (for review, see McBain and Mayer, 1994). Likewise, changes in subunit composition may account for the acceleration of the decay of NMDA receptor-mediated EPSCs seen during development (Carmignoto and Vicini, 1992; Hestrin 1992). Receptor regulation by intracellular signaling cascades may also be subunit-specific. For example, the nonreceptor tyrosine kinases src and fyn differentially regulate receptors containing NR2A or NR2B subunits (Kohr and Seeburg, 1996). Alternatively, NMDA receptors may play a structural role by specific interactions of their intracellular C-terminal domains with postsynaptic density (PSD) proteins and subsynaptic signaling machinery (Sheng and Wyszynski, 1997; Wyszynski et al., 1997; Ziff, 1997). Such a structural role might explain the observation that mice lacking the long intracellular C-terminal domain of NR2A or NR2B show the same phenotype as the respective targeted deletions (Spengel et al., 1998).

Studies of recombinant NMDA receptors have provided pharmacological reagents that can distinguish between receptors containing different NR2 subunits. One of the most extensively studied of these is the noncompetitive antagonist ifenprodil. *Xenopus* oocytes expressing NR1/NR2B diheteromers are 400-fold more sensitive to ifenprodil than NR1/NR2A diheteromers (Williams, 1993). We took advantage of this selectivity and the kinetics of ifenprodil block to examine the role of NR2B-containing receptors during the period of synapse formation. NMDA recep-
tor EPSC's and whole-cell currents were recorded in rat hippocampal neurons that formed autapses in single-neuron microucultures. Our results indicated that highly ifenprodil-sensitive NR1/NR2B diheteromers constitute the initial extrasynaptic population, whereas a second population of less ifenprodil-sensitive receptors are incorporated quickly after synapse formation.

MATERIALS AND METHODS

Neuronal cell culture. Microisland cultures were prepared as previously described (Bekkers and Stevens, 1991). Glass coverslips (31 mm; Biophysics Instruments, Millbrae, CA) were placed in 35-mm culture dishes (Roskilde, Denmark), coated with 0.15% agarose, and allowed to dry. Using an atomizer, a solution of poly-d-lysine (0.1875 mg/ml in 17 mM acetic acid; Sigma, St. Louis, MO) and collagen (0.05 mg/ml; Collagen Corp., Redwood City, CA) was sprayed on the agarose background to yield microdots of 100–1000 μm. After growth of glial feeder layers on the microdots, the CA1 region of hippocampi from postnatal day 0–1 rats were removed, enzymatically (papain; Collaborative Research, Bedford, MA) and mechanically dissected, and plated. Cultures were treated on day 1 with 0.2 mg/ml 5′-fluoro-2-deoxyuridine and 0.5 mg/ml uridine (FUDR; Sigma) to reduce glial proliferation, and then media were exchanged weekly.

Expression of recombinant NMDA receptors. HEK293 cells were transfected on cover slips on 31-mm glass coverslips (Krupp et al., 1998). and lymphocyte CD4 receptor cDNAs were transfected in a 4:4:1 ratio using the calcium phosphate method (Chen and Okayama, 1987). In cases in which two different NR2 subunits were transfected, the total amount of NR2 subunit (1 μg) was kept constant (i.e., for 1:100 NR2A:NR2B, 0.01 μg of NR2A and 0.09 μg of NR2B were transfected). The transfection was ended after 8–10 hr by replacing the solution with fresh media (DMEM plus 10% fetal calf serum, 1% glutamine, 1% penicillin-streptomycin, and FUDR). Kynurenic acid (3 mM; Sigma) and Dl-AP5 (1 mM; Tocris, Ballwin, MO) were added to prevent glutamate-induced excitotoxicity (Cik et al., 1993). Transfected cells were identified using CD4 receptor antibody-coated beads (Dynabeads, M-450 CD4; Dynal, Oslo, Norway). Before recording, 1 μl of Dynabead suspension was added to HEK293 cells in 1 ml of media and gently rocked for 15–30 min. NR1-1a and NR2B CDNA's were gifts from Jim Boulter and Stephen Heinemann (Salk Institute, La Jolla, CA). NR2A cDNA was a gift from Shigetada Nakanishi (Kyoto University, Kyoto, Japan). Bluescript cDNA encoding NR1-1a, NR2A, and NR2B was inserted into pcDNA1/AMP (Invitrogen, San Diego, CA; Krupp et al., 1998). Lyphocyte CD4 receptor cDNA was inserted into the JPA vector provided by John Adelman (Vollum Institute). NR1-1a, the predominantly expressed splice variant in the CNS (Laure et al., 1995), was used throughout these experiments.

Whole-cell recording and solutions. Whole-cell voltage-clamp recordings were performed on transfected HEK293 cells 12–72 hr after the end of the transfection reaction. Recordings from neurons were performed after 1–3 DIV. Cells were placed in a recording chamber at room temperature and continually perfused with an extracellular solution containing (in mM): NaCl (168), KCl (2.4), HEPES (10), D-glucose (10), glycine (0.001–0.01), and CaCl2 (1.3). The solution pH and osmolality were adjusted to final values of 7.4 and 325 mmol/kg, respectively. The solution containing (in mM): NaCl (168), KCl (2.4), HEPES (10), Cs4-BAPTA (5), Na2-ATP (2), and MgCl2 (3). Intracellular filling solution containing (in mM): Cs-methanesulfonate (125), CsCl (15), HEPES (10), Cs2-BAPTA (5), Na2-ATP (2), and MgCl2 (3). Intraacellular solution for synaptic recordings containing (in mM): K-glucurate (150), CsCl (6.23), MgCl2 (2), EGTA (10), HEPES (10), Na2-ATP (2), and NaGTP (0.2). The pH of intracellular solutions was adjusted to 7.4 with CsOH. The final osmolality was adjusted to 315 mOsm/kg by the pCa of this solution was calculated to be 7.0. Recording electrodes were made by an attached piezoelectric bimorph driven by a stimulus isolation unit (Winston Instruments, Palo Alto, CA). Flow pipe translations were made by an attached piezoelectric bimorph driven by a stimulus isolation unit (Winston Instruments). Neurons were equilibrated in 3 μM ifenprodil before and during agonist applications (1 mM glutamate, 100 msec duration). For synaptic recordings, neurons were equilibrated in 3 μM ifenprodil. For whole-cell experiments 300 nM 7-nitroquinoxaline-2,3-dione (CNQX; Sigma) and the AMPA receptor antagonist 6-cyano-7-nitroquinazoline-2,3-dione (CNQX, 5 μM; Tocris) were added to the extracellular solution. TTX was omitted from synaptic experiments.

Data analysis. All data were analyzed using Axograph software (Axon Instruments). Unless otherwise specified, currents from equilibrium drug application experiments were measured using a 500–1000 msec window after currents had reached steady-state amplitude. For NMDA receptor-mediated EPSC's, currents were measured using a 5–10 msec window centered at the peak of the EPSC. Measurements were performed on 5–10 consecutive EPSC's in the absence and presence of ifenprodil. Statistics were done using unpaired two-tailed t tests or ANOVA, and significance was set at p < 0.05. Data are reported as means ± SE.

RESULTS

NMDA receptor-mediated EPSC's are less sensitive to ifenprodil than whole-cell NMDA currents

We used whole-cell voltage-clamp recording to compare the sensitivity of synaptic and whole-cell NMDA currents with the subunit-specific antagonist ifenprodil. Using microisland cultures, functional synaptic contacts were detected 4–5 d after plating, as judged by the presence of spontaneous and evoked EPSC's. Neurons continued to form synapses for 2–3 weeks in vitro, as judged by the increase in EPSC amplitude and by the increase in the number of Glur1-like immunoreactive puncta (data not shown). At 5–7 DIV, ifenprodil (3 μM) reversibly reduced the slow NMDA receptor-mediated component of the EPSC (Fig. 1A). This concentration of ifenprodil provides a maximal and selective block of NR1/NR2B diheteromeric receptors in heterologous expression systems (Williams, 1993; see below). However, the inhibition of EPSC's was quite variable, ranging from 15 to 50% of control, as shown for two different neurons in Figure 1A. The average inhibition (Iifen) was 30.2 ± 2.2% of control (n = 22). Ifenprodil had no effect on the fast AMPA receptor-mediated component (94.1 ± 2.3% of control; n = 3; Fig. 1B). Additionally, ifenprodil did not alter the paired pulse ratio (P2/P1; 1.38 ± 0.16 for control and 1.25 ± 0.13 for ifenprodil; n = 4; 50 msec interstimulus interval), indicating that ifenprodil reduced NMDA receptor-mediated EPSC's by direct block of NMDA receptors rather than by a reduction of transmitter release.

For comparison, we measured the extent of ifenprodil block (3 μM) in a pure population of NR1/NR2B receptors by transfecting HEK293 cells with NR1 and NR2B cDNA. As expected, ifenprodil reduced steady-state NMDA-evoked (1 mM, 20 sec) currents to 17.5 ± 2.2% of control (n = 14; Fig. 1C,D). Before synapse formation (1–3 DIV) and after synapse formation has begun (5–7 DIV), Iifen from whole-cell currents in neurons was not significantly different from Iifen in recombinant receptors (11.6 ± 0.9%; n = 10; and 21.2 ± 2.1%; n = 17, respectively; Fig. 1C,D). Nonequilibrium agonist applications (1 mM glutamate, 100 msec) in the continuous presence of 3 μM ifenprodil resulted in a similar extent of block: Iifen was 15.1 ± 1.0% (n = 9) of control (data not shown). The extent of block of whole-cell currents in

acquired at a rate of 5 kHz and filtered at 0.05–2.5 kHz (eight-pole Bessel; Frequency Devices, Haverhill, MA).

NMDA (1 mM; Tocris), glutamate (1 mM; Sigma), and ifenprodil (1–10 μM; Dr. B. Stanton, Synthelabo) were dissolved in extracellular solution. Some aliquots of ifenprodil were solubilized in ethanol; final ethanol concentration was 0.025% (v/v). This concentration had no effect of whole-cell NMDA currents (data not shown). Drug applications were done using quartz flow pipes positioned 50–150 μm from the cell. Each flow pipe was controlled by a solenoid valve that, in turn, was controlled by an external timer (Winston Instruments, Palo Alto, CA). Flow pipe translations were made by an attached piezoelectric bimorph driven by a stimulus isolation unit (Winston Instruments). Neurons were equilibrated in 3 μM ifenprodil before and during agonist applications (1 mM glutamate, 100 msec duration). For synaptic recordings, neurons were equilibrated in 3 μM ifenprodil. For whole-cell experiments 300 nM 7-nitroquinoxaline-2,3-dione (CNQX; Sigma) and the AMPA receptor antagonist 6-cyano-7-nitroquinazoline-2,3-dione (CNQX, 5 μM; Tocris) were added to the extracellular solution. TTX was omitted from synaptic experiments.

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hippocampal neurons at 1–7 DIV is consistent with these currents resulting primarily from NR1/NR2B receptors. In neurons from 5–7 DIV, EPSCs were significantly less sensitive to block by ifenprodil than whole-cell currents. The difference in the ifenprodil sensitivity between synaptic and whole-cell NMDA receptors implies that the synaptic receptor complement includes NMDA receptors that are less sensitive or insensitive to ifenprodil. Synaptic and extrasynaptic NMDA receptors are differentially sensitive to ifenprodil

Once synapses have formed, whole-cell currents reflect openings of synaptic as well as extrasynaptic NMDA receptors. Differences in subunit composition between these two receptor populations could explain the discrepancy in ifenprodil sensitivity if whole-cell currents predominantly reflect the gating of extrasynaptic receptors. We took advantage of the single-axon input of microisland cultures to directly compare the ifenprodil sensitivity of the EPSC (synaptic) and whole-cell (synaptic plus extrasynaptic) current on the same neuron. As shown in Figure 2A, there was not an obvious correlation between ifenprodil block of EPSCs and whole-cell currents on a given neuron, but the peak whole-cell current was 55.6 ± 17.7 times larger than the EPSC in the same cell (Fig. 2B; n = 7). If all the receptors were synaptic, the EPSC would be expected to be fivefold smaller than the whole-cell current, assuming that the weighted average probability of transmitter release ($P_r$) at these terminals is ~0.2 (Rosenmund et al., 1993). However, the corrected amplitude of EPSCs is still an order of magnitude less than the whole-cell current, indicating that most of the functional NMDA receptors are extrasynaptic at this early stage of development.

To determine the ratio of extrasynaptic to synaptic receptors, we measured the peak current in response to 1 mM NMDA before and after irreversibly blocking the EPSC (in excess of 90%) with the use-dependent antagonist MK-801 (Huebner and Bean, 1988). Using this protocol (Fig. 2C), the contribution of synaptic receptors was eliminated from the whole-cell response. In four cells, the whole-cell current remaining after block of the EPSC was 74.5 ± 2.1% of control, indicating that extrasynaptic receptors outnumbered synaptic receptors by 3:1 when synaptic load is relatively low. These data indicate that the small fraction of synaptic receptors at this developmental stage has little impact on the ifenprodil sensitivity of the whole-cell current.

Macroscopic kinetics of ifenprodil block

The similar degree of block of neuronal whole-cell currents and currents from NR1/NR2B recombinant receptors suggests that extrasynaptic receptors are NR1/NR2B diheteromers. However, the pharmacological similarity does not exclude other possibilities, such as multiple NMDA receptor types within the extrasynaptic population. A comparison of the macroscopic kinetic characteristics of ifenprodil block provides a more sensitive test of this hypothesis. Figure 3A shows the onset of ifenprodil block during a steady-state application of NMDA. The onset of block accelerated with the ifenprodil concentration and reached a similar
maximum block in both native receptors and in NR1/NR2B diheteromers. In neurons, the onset of block in 1, 3, or 10 μM ifenprodil was fitted with a single exponential with time constants of 1.55 ± 0.09 (n = 7), 1.41 ± 0.19 (n = 18), and 0.32 ± 0.03 (n = 7) sec, respectively. The time constants for NR1/NR2B receptors were 2.12 ± 0.58 (n = 5), 0.95 ± 0.08 (n = 8), and 0.51 ± 0.27 (n = 5) sec, respectively (Fig. 3A). The onset of block was slightly slower in NR1/NR2B diheteromers at 1 μM ifenprodil but not statistically different from neurons at higher concentrations. In some cases, a high concentration of ifenprodil (10 μM) resulted in a fast relaxation in native and recombinant receptors, possibly reflecting a different mode of block, as previously observed at higher ifenprodil concentrations (Legendre and Westbrook, 1991).

To examine recovery from ifenprodil block, NMDA receptors were activated with 1 mM NMDA and then blocked with 3 μM ifenprodil in the continuous presence of NMDA (Fig. 3B). After washout of ifenprodil, test pulses of NMDA (1 sec duration) were applied every 10 sec to monitor recovery. The time required for recovery to 50% of the control amplitude was 47.2 ± 5.8 sec (n = 6) for neurons at ≤7 DIV and 52.4 ± 4.2 sec for NR1/NR2B diheteromers (n = 5; Fig. 3C). Thus the macroscopic kinetics of ifenprodil binding and unbinding are comparable in receptors from 5–7 DIV neurons and recombinant NR1/NR2B receptors, suggesting that the extrasynaptic receptor complement is composed largely, if not exclusively, of NR1/NR2B diheteromers.

**Differential ifenprodil sensitivity at “mature” synapses**

The expression of the NR2A subunit is low in rat brain before the seventh postnatal day and then increases to plateau levels 12–21 d after birth (Monyer et al., 1994; Sheng et al., 1994). A similar pattern also occurs in vitro (Zhong et al., 1994; Li et al., 1998). Because recombinant NR1/NR2A receptors are insensitive to 3 μM ifenprodil (Williams, 1993), we examined whether the ifenprodil sensitivity of NMDA receptor-mediated EPSCs decreased during this period. NMDA receptor-mediated EPSCs at ≥13 DIV were significantly less sensitive to ifenprodil (I_{fen} = 43.1 ± 3.6% of control; n = 14) compared with EPSCs at ≤7 DIV (Fig. 4A,B). Because recombinant NR1/NR2A receptors are insensitive to 3 μM ifenprodil (Williams, 1993), the expression of NR2A subunit is low in rat brain before the seventh postnatal day and then increases to plateau levels 12–21 d after birth (Monyer et al., 1994; Sheng et al., 1994). A similar pattern also occurs in vitro (Zhong et al., 1994; Li et al., 1998). Because recombinant NR1/NR2A receptors are insensitive to 3 μM ifenprodil (Williams, 1993), we examined whether the ifenprodil sensitivity of NMDA receptor-mediated EPSCs decreased during this period. NMDA receptor-mediated EPSCs at ≥13 DIV were significantly less sensitive to ifenprodil (I_{fen} = 43.1 ± 3.6% of control; n = 14) compared with EPSCs at ≤7 DIV (Fig. 4A,B). AMPA receptor-mediated EPSCs at mature synapses were unaffected by 3 μM ifenprodil (95.6 ± 4.5% of control currents; n = 4), nor was the paired pulse ratio (data not shown). We blocked synaptic receptors from ≥13 DIV neurons with MK-801 (10 μM) and found that the ifenprodil sensitivity of extrasynaptic receptors (I_{fen} = 20.6 ± 4.9%; n = 4; data not shown) was not different from the whole-cell ifenprodil sensitivity in ≤7 DIV neurons. Thus the differential NMDA receptor distribution is even more pronounced at ≥13 DIV than at ≤7 DIV.

The deactivation of NMDA receptor-mediated EPSCs was faster at mature synapses, as described in other preparations (Carmignoto and Vicini, 1992; Hestrin, 1992). This acceleration was attributable to a larger fast component of deactivation rather than a change in time constants (≤7DIV: τ₁ = 252.2 ± 21.4 msec; ≥13 DIV: τ₁ = 81.2 ± 11.4 msec).
The age-dependent reduction in ifenprodil sensitivity of NMDA receptors such as ifenprodil-insensitive NR1/NR2A receptors. However, native receptors can contain both NR2A and NR2B subunits (Sheng et al., 1994). Whether triheteromeric receptors composed of NR1, NR2A, and NR2B are as sensitive to ifenprodil as NR1/NR2B receptors is unknown. To address this question, we transfected HEK293 cells with 1:1, 1:10, and 1:100 ratios of cDNA for NR2A, and NR2B are as sensitive to ifenprodil as NR1/NR2B receptors. As expected, recombinant NR1/NR2A receptors in our experiments were not blocked by ifenprodil (100.7 ± 5.7% of control; n = 10; Fig. 5A,B), whereas NR1/NR2B receptors were highly sensitive (Figs. 2C, 5B). Cells transfected with both NR2A and NR2B showed intermediate sensitivities. $\Delta_{\text{max}}$ was 75 ± 4.1% of control (n = 33) for a 1:1 ratio of NR2A:NR2B and 76.4 ± 4.2% of control (n = 19) for a 1:10 ratio. The inhibition by ifenprodil for the 1:100 ratio was greater (35 ± 2.7% of control; n = 15; Fig. 5B). However, the responses from these cells appeared to fall into two groups (Fig. 5C,D). In eight cells, $I_{\text{ifen}}$ was 10 ± 3.1% of control, similar to results from NR1/NR2B diheteromers, whereas the remaining seven cells had an intermediate ifenprodil sensitivity of triheteromeric receptors containing NR1, NR2A, and NR2B.

Ifenprodil sensitivity of triheteromeric receptors containing NR1, NR2A, and NR2B

A comparison of the ifenprodil sensitivity of native receptors with recombinant NR1/NR2 diheteromers is straightforward if native receptors consist of either highly sensitive NR1/NR2B receptors or ifenprodil-insensitive NR1/NR2A receptors. However, native receptors can contain both NR2A and NR2B subunits (Sheng et al., 1994). Whether triheteromeric receptors composed of NR1, NR2A, and NR2B are as sensitive to ifenprodil as NR1/NR2B receptors is unknown. To address this question, we transfected HEK293 cells with 1:1, 1:10, and 1:100 ratios of cDNA for NR2A:NR2B along with NR1 and compared the ifenprodil sensitivity with cells transfected with NR1/NR2A and NR1/NR2B. As expected, recombinant NR1/NR2A receptors in our experiments were not blocked by ifenprodil (100.7 ± 5.7% of control; n = 10; Fig. 5A,B), whereas NR1/NR2B receptors were highly sensitive (Figs. 2C, 5B). Cells transfected with both NR2A and NR2B showed intermediate sensitivities. $\Delta_{\text{max}}$ was 75 ± 4.1% of control (n = 33) for a 1:1 ratio of NR2A:NR2B and 76.4 ± 4.2% of control (n = 19) for a 1:10 ratio. The inhibition by ifenprodil for the 1:100 ratio was greater (35 ± 2.7% of control; n = 15; Fig. 5B). However, the responses from these cells appeared to fall into two groups (Fig. 5C,D). In eight cells, $I_{\text{ifen}}$ was 10 ± 3.1% of control, similar to results from NR1/NR2B diheteromers, whereas the remaining seven cells had an intermediate ifenprodil sensitivity was slow and monophasic (Fig. 3B); thus recovery of the ifenprodil-sensitive component of the EPSCs would be expected to be similarly slow. Likewise, if receptors with distinct ifenprodil sensitivities contribute to the EPSC, the recovery time course might be expected to be multiphasic.

As expected, for EPSCs at ≥7 DIV, in which ifenprodil produced a large degree of block (22% of control; Fig. 4A), recovery from ifenprodil block was monophasic, with a time constant (τslow) of 88.6 ± 21.1 sec (n = 11; Fig. 4D). In contrast, for EPSCs showing a lower ifenprodil sensitivity at ≥13 DIV (59% of control; Fig. 4B), a rapid initial component of recovery was apparent, followed by a slow component (see Fig. 4C for normalized comparison of recovery). The slow component was fitted with a single exponential with a time constant of 78.8 ± 16.1 sec (n = 8), similar to τslow at ≤7 DIV (Fig. 4D). We could not determine the time constant of the fast component, because higher stimulation rates caused changes in the amplitude of the EPSC, consistent with altered transmitter release and/or receptor desensitization. However, the fast component was fully developed at 0.3 Hz (data not shown), suggesting a time constant of ≤1 sec. The biphasic recovery was not limited to neurons at ≥13 DIV. A rapid component of recovery was observed for EPSCs at ≤7 DIV that had a lower ifenprodil sensitivity, in the range for EPSCs ≥13 DIV.

The slow component of recovery at both ≤7 and ≥13 DIV is consistent with the presence of NR1/NR2B receptors at these synapses, whereas the reduced ifenprodil sensitivity and fast component of recovery indicate that receptors with a different subunit composition, possibly NR1/NR2A/NR2B triheteromers, are also present. Consistent with this hypothesis, the fast component was larger for EPSCs that were less sensitive to ifenprodil. For EPSCs in which the block by ifenprodil suggested a homogeneous population of NR1/NR2B receptors (Iifen < 25% of control), the fast component contributed 12.6 ± 1.9% (n = 7). For EPSCs that were less ifenprodil-sensitive (Iifen > 25% of control), the fast component was 33.3 ± 3.7% (n = 16; Fig. 4E).

Figure 3. Macroscopic kinetic properties of NMDA receptors in ≤7 DIV neurons and recombinant NR1/NR2B receptors are similar. A, Detail of relaxations in 1, 3, and 10 μM ifenprodil, as indicated. The arrowhead indicates that the steady-state amplitude reduction is approximately the same for these three concentrations. The relaxations were fit with single exponentials, and the results are plotted below the raw data in A. B, Protocol for measuring the recovery from ifenprodil block of whole-cell currents. This example is from a neuron at 6 DIV. Bars above the current are as in Figure 1C. C, Bar graphs of half-recovery times using the protocol shown in B, showing the similarity in the recovery from block between native NMDA receptors at ≤7 DIV and recombinant NR1/NR2B receptors.

$\tau_2 = 43.7 ± 4.5$ msec; $\tau_1/\tau_2$ amplitude = 1.1 ± 0.14; ≥13 DIV; $\tau_1$ = 303.3 ± 31.4 msec; $\tau_2$ = 52.4 ± 2.1 msec; $\tau_1/\tau_2$ amplitude = 1.7 ± 0.26.

The age-dependent reduction in ifenprodil sensitivity of EPSCs could result from a homogenous NMDA receptor population characterized by decreased ifenprodil sensitivity or from the presence of a pharmacologically distinct class of synaptic NMDA receptors such as ifenprodil-insensitive NR1/NR2A receptors. We used the recovery from ifenprodil block to distinguish between these possibilities. Recovery for whole-cell cur-
ceptors as synaptic load is increasing may indicate that NMDA
eromeric) receptors. The addition of less ifenprodil-sensitive re-
the rapid appearance of less ifenprodil-sensitive (possibly trihet-
and highly ifenprodil-sensitive. However, the synaptic NMDA
complement being composed largely of NR1/NR2B dihetero-
synaptic load. Our data are consistent with the NMDA receptor
whole-cell currents from neurons during the development of

ifenprodil recovered less in the first interval after ifenprodil removal than that EPSCs that were less sensitive to ifenprodil block.

sensitivity (48.4 ± 5.4% of control). The receptor complement in
the highly ifenprodil-sensitive cells most likely represented NR1/
NR2B cDNA, these cells probably did not receive sufficient NR2A-
containing plasmid to affect the whole-cell current. However, the
cells with intermediate ifenprodil sensitivity suggest an additional
population of triheteromeric receptors.

For ratios of 1:1 and 1:10, the intermediate ifenprodil sensitivity
could result from a mixed population of ifenprodil-insensitive
NR1/NR2A and ifenprodil-sensitive NR1/NR2B receptors. In
this case, the recovery from block should match NR1/NR2B
receptors. We tested this possibility by examining the recovery
from block from cells transfected with a 1:1 ratio of NR2A:NR2B
(Fig. 6A). The half-recovery times for cells transfected with a 1:1
ratio of NR2A:NR2B was much faster (0.49 ± 0.28 sec; n = 7)
than NR1/NR2B diheteromers (Fig. 6B). Thus these experi-
ments indicate the presence of triheteromeric NR1/NR2A/
NR2B receptors with an intermediate ifenprodil sensitivity.

DISCUSSION

The microdot culture system allowed us to compare EPSCs and
whole-cell currents from neurons during the development of
synaptic load. Our data are consistent with the NMDA receptor
complement being composed largely of NR1/NR2B dihetero-
mers before synapse formation. Soon after synapse formation
begins, the majority of NMDA receptors are still extrasynaptic
and highly ifenprodil-sensitive. However, the synaptic NMDA
receptor complement differs in its ifenprodil sensitivity because of
the rapid appearance of less ifenprodil-sensitive (possibly trihet-
eromeric) receptors. The addition of less ifenprodil-sensitive re-
ceptors as synaptic load is increasing may indicate that NMDA
receptors incorporating NR2A subunits are localized at synaptic
sites soon after synapses become functional.

Comparison with previous data

Our results rely on the subunit specificity of ifenprodil. Previous
results in heterologous expression systems indicate that ifenprodil
at the concentrations used in our experiments maximally blocks
NR1/NR2B receptors without affecting NR1/NR2A receptors
(Williams, 1993). This was confirmed in our experiments with
recombinant receptors expressed in HEK293 cells. Whole-cell
currents in cultured hippocampal neurons were blocked by ifen-
prodil as previously demonstrated (Legendre and Westbrook,
1991). Based on their similar degree of block and macroscopic
kinetics, we conclude that extrasynaptic receptors at 5–7 DIV are
largely composed on NR1/NR2B diheteromers, consistent with the
early expression of the NR2B subunit (Monyer et al., 1994;
Kew et al., 1998).

Ifenprodil has recently been used to study native NMDA
receptor subunit composition as well as development changes in
NMDA receptor phenotype. In whole-cell recordings of acutely
isolated cortical neurons, a developmental shift in ifenprodil
sensitivity and a reduction in glycine affinity were coincident with
an increase in NR2A expression (Kew et al., 1998). These authors
also reported that ifenprodil had a biphasic dose–response curve in
neurons from older animals. Decreased glycine affinity is
consistent with increased expression of NR2A (Kutsuwada et al.,
1992). A two-component mechanism was also responsible for
ifenprodil block of EPSCs in CA1 pyramidal neurons in slices
from 7- to 28-d-old mice (Kirson and Yaari, 1996). The ratio of
high to low ifenprodil-sensitive components decreased in neurons
from animals older than 35 d. Plant et al. (1997) found that

Figure 4. Synaptic NMDA receptor complement can be composed of two dis-
tinct types of NMDA receptors. A, Plot of peak NMDA EPSC amplitude as a func-
tion of recording time from a neuron at 7 DIV. The plot represents the amplitude of
the last 10 EPSCs before the 3 μM ifen-
prodil (ifen) application followed by the
amplitude of 10 EPSCs in ifenprodil. The
solid bar represents the ifenprodil applica-
tion. The arrowhead points to the first
EPSC after ifenprodil removal. B, As in A,
except from a neuron at 15 DIV. A, B, in-
sets, EPSCs in control and in 3 μM ifen-
prodil. Calibration: 400 pA, 1 nA, respec-
\[\text{tively}, 100 \text{msec for both. Note that in this}
example the first EPSC after ifenprodil
removal had recovered more than half of
the total extent of block in the 10 sec
interval between EPSCs (arrowhead).}
Stimulus frequency is 0.1 Hz, and current
amplitudes were measured with a 5 msec
window around the peak. Ifenprodil was
applied with a flow pipe positioned 50–
100 μm from the soma. In both cases the
recovery from block was fitted with a sin-
gle exponential starting at the first data
point after ifenprodil removal. C, Normal-
ized plots from A and B to show the extent
of recovery after ifenprodil removal. The
arrow indicates the last EPSC in
ifenprodil. D, Comparison of the slow re-
covery time constants of EPSCs from
\(\geq 7\) DIV and \(\geq 13\) DIV neurons. E, EPSCs
that were more sensitive to block by

ifenprodil.
EPSCs in GABAergic forebrain neurons in slice recordings from 14- to 17-d-old rats were highly variable in their sensitivity to ifenprodil (3 μM; range, 48–93% block). These results are consistent with our results indicating that synapse formation triggers heterogeneity in the synaptic NMDA receptor population.

Using whole-cell currents to inform us about synaptic NMDA receptors initially seemed like a reasonable strategy, because synaptic NMDA receptors outnumber extrasynaptic receptors by 4:1 on neurons at 9–14 DIV (Rosenmund et al., 1995). However, direct measurement of the extrasynaptic/synaptic ratio at 5–7 DIV indicated that the majority of receptors were extrasynaptic. Whole-cell currents at this stage predominantly reflect the properties of extrasynaptic receptors and thus do not provide an accurate sampling of synaptic receptors. The lower ifenprodil sensitivity of synaptic receptors compared with extrasynaptic receptors implies that there is rapid incorporation of pharmacologically distinct NMDA receptors into synaptic sites. Using a different approach, Stocca and Vicini (1998) have come to similar conclusions regarding NMDA receptor-mediated EPSCs in cortical slices. Whether the incorporation of new, presumably triheteromeric receptors occurs at synapses already containing NR1/NR2B receptors remains to be determined.

**Role of NR2B and implications for synapse formation and maturation**

Mice lacking NR2B die soon after birth and have no detectable NMDA receptor component of the EPSP (Kutsuwada et al., 1996). This probably results from an inability to form functional channels rather than a general defect in localization of synaptic receptors, because cultured neurons from these mice have NMDA receptor-mediated EPSCs, with properties that are consistent with expression of NR1/NR2A diheteromers (Tovar et al., 1998).

Proteins that bind to intracellular domains of NMDA receptor subunits have recently been identified (for review, see Gomperts, 1996; Ziff 1997). Many of these proteins have homology to signal transduction molecules, whereas others may anchor these transduction molecules to the PSD. Mice lacking the NR2A subunit have attenuated long-term potentiation (LTP) and are deficient in some learning paradigms but are otherwise viable (Sakimura et al., 1995). The deficiency in LTP in mice lacking NR2A could result from the lack of an anchoring or association site for molecules important in LTP. The NR2A and NR2B subunits both contain a PDZ-binding sequence required for attachment to that class of “scaffolding” proteins (Kornau et al., 1995). However, in hippocampal neurons, the commonly expressed PDZ-containing protein, PSD-95, must not be initially responsible for clustering of NMDA receptors at synapses, because immunocytochemical colocalization of PSD-95 with NMDA receptor subunits does not occur until 21 DIV (Rao et al., 1998). Our results indicate that synapses containing AMPA and NMDA receptor components have formed long before that time.

In contrast to our work, Rao and Craig (1998) used immunocytochemistry to demonstrate that NR2A- and NR2B-containing clusters become localized at sites opposite clusters of the presynaptic marker synaptophysin after 14 DIV. They inferred from this that synaptic localization of NMDA receptors does not occur until that time and after localization of AMPA receptors (as inferred from clusters of the AMPA receptor subunit GluR1). However, our results indicate that functional synapses form after 5 d in culture, that NMDA receptors are present at these nascent synapses, and that NR2B subunits initially predominate at these sites. The differences between the physiological and immunocytochemical results may arise from an inability to detect with...
For NR1/NR2B cells are reiterated from Figure 3C. In synaptic junction (NMJ), where acetylcholine receptor channel kinetics are directly analogous to the developing neuromuscular junctions. This is attributed to changes in the synaptic receptor complement at active synapses. A corollary of this hypothesis, not tested here, is that the synaptic receptor subunit expression that occurs in cerebellar granule cells (Ozaki et al., 1997). In support of the idea that NMDA receptor activity results in changes in the receptor complement at active synapses, hippocampal neurons cultured in AP5 show a heightened sensitivity to ifenprodil, as if NMDA receptors at all synaptic sites were pure NR1/NR2B diheteromers (Chavis and Westbrook, 1998).

Our evidence indicates that nascent synapses are homogenous with respect to their NMDA receptor complement. A model of cortical development holds that changes in NMDA receptor subunit composition are important in governing the period during which synaptic rearrangements can occur (Sheetz and Constantine-Paton, 1994). An elaboration of this model is that sites with more than one type of NMDA receptor are the very sites that are capable of being modified, and they are marked by the presence of NR2A-containing receptors. This is supported by the apparent speed with which NR2A-containing receptors appeared at synapses in this study (also see Stocca and Vicini, 1998). Blockade of NMDA receptors in vivo prevents the formation of topographic maps (Cline and Constantine-Paton, 1987; Kleinschmidt et al., 1987) and also prevents the change from high to low NMDA EPSC ifenprodil sensitivity (Chavis and Westbrook, 1998). If NMDA channel gating acts on the same mechanisms in these systems, neural activity at synapses containing diheteromeric and triheteromeric NMDA receptors may be required for the formation of correlation-based topographic maps. Thus NR1/NR2B-only synapses may mark new synapses available for further elaboration, and synapses with diheteromeric and triheteromeric receptors may mark synapses that are subject to correlation-based refinement. The limit of correlation-based synaptic refinement (i.e., the critical period duration) may occur when synapses contain only triheteromeric receptors.

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**Figure 6.** HEK293 cells transfected with NR1, NR2A, and NR2B recover quickly from ifenprodil block. A, Protocol for measuring the recovery from block from triply transfected cells (with a 1:1 ratio of NR2A:NR2B) and the recovery from block of an NR1/NR2B-transfected cell for comparison. Data for block recovery are shown in B. Recovery data for NR1/NR2B cells are reiterated from Figure 3C and collected using the method illustrated in Figure 3B. This method could not be used for triply transfected cells, because often the recovery was completed in the interval between the removal of ifenprodil and the first application of NMDA alone. Instead, recovery was measured by returning to the NMDA solution lacking ifenprodil. An example using this method of measuring recovery from block from NR1/NR2B receptors is shown for comparison. Bars above the current are as in Figure 1C.
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