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Use of Subunit-Specific Antisense Oligodeoxynucleotides to Define Developmental Changes in the Properties of *N*-Methyl-D-aspartate Receptors

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SUMMARY

Antisense oligodeoxynucleotides were used to determine whether alterations in the expression of *N*-methyl-D-aspartate (NMDA) receptor subunit mRNA are responsible for developmental changes in the sensitivity of receptors to agonists and antagonists. *Xenopus laevis* oocytes were injected with mRNA prepared from neonatal and adult rat cerebral cortex, and the effects of agonists and antagonists were determined under voltage-clamp conditions. Glycine-site antagonists like 7-chlorokynurenate and glutamate-site antagonists like CGP-39653 were more potent at NMDA receptors expressed from mRNA from adult rat cerebral cortex than those expressed from mRNA from 1-day-old rat. NMDA receptors from 1-day-old rat cerebral cortex were more sensitive to activation by glycine than were receptors from adult rat cerebral cortex. 7-Chlorokynurenate and CGP-39653 were more potent inhibitors of responses seen with heteromeric NR1/NR2A receptors than with NR1/NR2B receptors. Conversely, heteromeric NR1/NR2B receptors were more sensitive to activation by glycine than were NR1/NR2A receptors. We previously described a delay in the expression of the NR2A subunit in developing rat brain. Antisense oligodeoxynucleotides were used to determine whether the delayed expression of the NR2A subunit underlies changes in pharmacological properties observed during development. The properties of receptors seen when adult brain mRNA was coinjected with antisense oligodeoxynucleotides against the NR2A subunit were similar to those found in receptors from 1-day-old rat brain. These data suggest that changes in the sensitivity of NMDA receptors to antagonists and to glycine seen during development are a result of alterations in the expression of different species of NR2 subunit mRNA.

NMDA receptors are ligand-gated ion channels that contribute to synaptic transmission and are particularly important for certain forms of activity-dependent synaptic plasticity, such as long term potentiation, that underlie some forms of learning and memory (1). Excessive activation of NMDA receptors has been implicated in a number of diseases, including ischemic neuronal cell death, neurodegenerative diseases, and epilepsy (2, 3). Like many other ligand-gated channels, the functional and pharmacological properties of NMDA receptors change during postnatal development. Developmental changes in the kinetics of NMDA receptor-mediated excitatory postsynaptic currents (4, 5) and in the sensitivity of NMDA receptors to glycine, polyamines, ifenprodil, and voltage-dependent block by Mg^{2+} have been observed (6–9). The functional properties of a number of ligand-gated ion channels, including nicotinic acetylcholine receptors and $GABA_A$ receptors, have been found to change during development, and alterations in subunit composition seem to underlie these changes (10, 11).

Multiple genes encoding two families of NMDA receptor subunits have been cloned; these include the NMDAR1 (NR1), NR2A, NR2B, NR2C, and NR2D genes from rat brain (12-14) and the ζ1 (NR1) and ε1-4 (NR2A-D) genes from mouse brain (15–17). In *Xenopus laevis* oocytes, homomeric NR1 but not homomeric NR2 receptors mediate small responses to NMDA or glutamate and display many features of native NMDA receptors (12, 15). Much larger currents in response to exposure to NMDA or glutamate are seen after coexpression of NR1 with NR2 subunits, suggesting that native NMDA receptors are likely to be composed of NR1 and NR2 subunits (13, 16). Many properties of heteromeric NR1/NR2 receptors, such as their sensitivity to agonists or antagonists, Mg^{2+}, polyamines, or histamine, depend on the type of NR2 subunit included in a heteromeric complex (13, 14, 16–20). Developmental differences in the time course of expression of mRNA encoding NR2 subunits in rodent brain have been reported (21–23). NR1, NR2A, and NR2B subunits are the major subunits in rat cerebral cortex. NR1 and NR2B subunit mRNAs are expressed in fetal through adult cortex, whereas the NR2A subunit appears after birth. Increases in

ABBREVIATIONS: BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; 7-CK, 7-chlorokynurenate; GABA, γ-aminobutyric acid; NMDA, *N*-methyl-D-aspartate; ODN, oligodeoxynucleotide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

expression of NR2A subunits are seen between postnatal days 7 and 21 (21–23). Because NR2 subunits confer functional variability, differences in NR2 subunit composition may account for changes in the properties of native NMDA receptors seen during development. For examples, delayed expression of the NR2A subunit during development is thought to account for the late appearance of NMDA receptors with a low affinity for ifenprodil (9, 24).

To establish molecular correlates between changes in the properties of NMDA receptors seen during development and the expression of NMDA receptor subunits, the pharmacological properties of NMDA receptors were examined in oocytes injected with mRNA prepared from rat brains at different stages of development. In oocytes injected with mRNA from cerebral cortex of rats of different ages, differences in sensitivity to antagonists, including 7-CK and CGP-39653 [DL-(*E*)-2-amino-4-propyl-5-phosphono-3-pentanoic acid], and for glycine and NMDA were identified by current recordings using two-electrode voltage-clamp conditions. Antisense ODNs corresponding to NMDA receptor subunits were coinjected with cerebral cortex mRNA to determine which subunits are responsible for the changes in the properties of receptors that are observed during development. Antisense ODNs specific for the sequence of NR2A mRNA selectively blocked developmental changes in the sensitivity of NMDA receptors to 7-CK, CGP-39653, and glycine.

Materials and Methods

Isolation of mRNA from rat brain cerebral cortex. The cerebral cortex was dissected from brains of Sprague-Dawley rats at postnatal day 1 or from young adult rats (~14 weeks of age). When necessary, tissue from five or six animals was pooled. The tissue was frozen in liquid nitrogen and stored at −80°. Total RNA was prepared using a modification of the guanidinium thiocyanate/phenol extraction method (25). Tissue was homogenized in TRIzol solution (GIBCO BRL, Gaithersburg, MD) using a Polytron homogenizer (Omni International, Gainesville, VA). After chloroform extraction, samples were precipitated at room temperature with 1 volume of isopropanol, and RNA was recovered by centrifugation (12,000 × *g*, 10 min) at 4°. Pellets were washed with 70% ethanol, dried briefly, and dissolved in diethylpyrocarbonate-treated water. Poly(A)$^+$ RNA was selected by one round of oligo(dT) cellulose chromatography using mini-oligo(dT) cellulose spin column kits (5 Prime → 3 Prime, Inc., Boulder, CO). RNA was dissolved in RNase-free water and stored at a concentration of 1 μg/μl (3-μl aliquots) at −80°. The final concentration of RNA in each sample was calculated on the basis of its absorbance at 260 nm.

Preparation of cRNA. The NR1A clone (RN60) was a gift from Dr. S. Nakanishi (Kyoto University, Kyoto, Japan). The NR2A and NR2B clones were gifts from Dr. P. H. Seeburg (University of Heidelburg, Heidelburg, Germany). Capped cRNAs were synthesized from linearized plasmids containing the NR1A (12), NR2A, and NR2B (13) constructs using an *in vitro* transcription mMessage mMachine kit (Ambion, Austin, TX). Briefly, template cDNAs were linearized with *Not*1 (NR1A) or *Eco*R1 (NR2A and NR2B). The corresponding cRNA was synthesized from each cDNA template using T7 or T3 RNA polymerase in the presence of $m^2G(5')ppp(5')G$. Template cDNAs were removed with DNase 1, and cRNAs were extracted with phenol/chloroform and chloroform and then precipitated with ammonium acetate and isopropanol. cRNAs were dissolved in RNase-free water and stored in aliquots (2 μl each) at −80° until use.

Synthesis of ODNs. The antisense ODNs used in these experiments were phosphorothioate 20 mers, which are more stable than unmodified ODNs (26). Phosphorothioate ODNs were synthesized on a model 394 DNA synthesizer (Applied Biosystems, Norwalk, CT). The purity of the ODNs was evaluated by thin layer chromatography (SurePure Kit; United States Biochemical, Cleveland, OH). On the UV illuminated plate, a major band corresponding to full-length 20-mer ODN was detected. For some ODNs, one or two additional minor bands were seen. These observations suggested that the majority of the product was full-length 20 mer. The sequences for the ODNs used were anti-2A, 5′-ATC TGC CCA TGG TCG CCA CT-3′; anti-2B, 5′-TGG GCT TCA TCT TCA GCT AG-3′; controls, 5′-GCT AGT CTG ACC GGA ATC AC-3′; 5′-AGT GGC GAC CAT GGG CAG AT-3′; and 5′-CTA GCT GAA GAT GAA GCC CA-3′. The concentrations of ODNs were determined on the basis of their absorbance at 260 nm.

Preparation of *X. laevis* oocytes. Female adult *X. laevis* frogs (Nasco, Fort Atkinson, WI, and Xenopus 1, Ann Arbor, MI) were anesthetized in 0.15% tricaine (Sigma Chemical, St. Louis, MO). Ovarian lobes were removed through a small incision in the abdomen. Oocytes were then defolliculated by gentle agitation for 1.5–2 hr in Ca^{2+}-free buffer (82 mM NaCl, 2.5 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.5) containing 2 mg/ml collagenase (Type 1A, Sigma). The oocytes were washed and placed in a saline buffer (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5 mM HEPES, 2.5 mM sodium pyruvate, 50 μg/ml gentamycin, pH 7.5). Only stage V and VI oocytes (27) were used. Oocytes were injected with ~50 nl of rat cerebral cortex mRNA (50 ng) or cRNA from cDNA clones (5 ng of NR1A, 1 ng of NR2A or NR2B) and were maintained at 17° for 2–5 days before recordings were made. Oocytes injected with mRNA from cortex were maintained in ND96 medium (96 mM NaCl, 1 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, 50 μg/ml gentamycin, pH 7.5) supplemented with 5% horse serum (28). In some experiments, antisense or control ODNs were coinjected with rat brain mRNA or NMDA receptor cRNAs. Typically, 50 ng of rat cerebral cortex mRNA (~50 pmol of NR2A or NR2B mRNA) (23) or 1–3 fmol of *in vitro* synthesized NMDA receptor subunit mRNA was coinjected with 75 or 250 fmol of ODNs. The final intracellular concentrations of the ODNs were estimated to be 75 nM or 250 nM on the assumption that the total volume of an oocyte was 1 μl (29).

***X. laevis* oocytes recordings.** For electrophysiological recordings, oocytes were positioned in a recording chamber and perfused continuously with saline solution (96 mM NaCl, 2 mM KCl, 1.8 mM $BaCl_2$, 5 mM HEPES, pH 7.5). The recording solution contained $BaCl_2$ to reduce Ca^{2+}-activated Cl^- currents (30). In most recordings, oocytes were injected with BAPTA (80 nl of 40 mM, pH 7.0) to eliminate a slowly activating Cl^- current (18). Macroscopic currents were recorded using two-electrode voltage-clamp techniques with a GeneClamp 500 amplifier (Axon Instruments, Burlingame, CA). Electrodes were filled with 3 M KCl, and their resistance ranged from 0.6 to 2.5 MΩ. Data were recorded and analyzed using a TL-1 DMA interface with AxoTape software (Axon Instruments). Dose-response curves for antagonists were constructed in the presence of 100 μM NMDA and 10 μM glycine. Glycine dose-response curves were obtained in the presence of 100 μM NMDA, and NMDA concentration-response curves were obtained in the presence of 10 μM glycine. Data analysis was performed with Kaleidagraph graphics software (Abelbeck/Synergy, Reading, PA).

Materials. NMDA and 7-CK were purchased from Research Biochemicals (Natick, MA). Glycine was obtained from Sigma. CGP-39653 was a gift from Dr. R. Heckendorn (Ciba-Geigy, Basel, Switzerland). *In vitro* transcription mMessage mMachine kits were from Ambion. TRIzol reagent was purchased from GIBCO BRL. The mini-oligo(dT) cellulose spin column kits were from 5 Prime → 3 Prime, Inc.

Results

Developmental changes in sensitivity of NMDA receptors to 7-CK and CGP-39653. 7-CK is an antagonist of the glycine site on the NMDA receptor (31), whereas CGP-39653 is a

competitive antagonist at the glutamate binding site (32). Experiments were carried out to determine whether there are developmental changes in sensitivity of the receptors to these antagonists. Oocytes were injected with cerebral cortex mRNA from 1-day-old or adult rats and voltage-clamped at a potential of −70 mV. Application of 100 μM NMDA and 10 μM glycine produced inward currents (25–200 nA) (Fig. 1A). The effects of 7-CK and CGP were studied alone and in the presence of 100 μM NMDA and 10 μM glycine. Neither antagonist elicited a current at the highest concentration tested (100 μM for 7-CK and 3 μM for CGP-39653). The inhibitory effects of 7-CK and CGP-39653 on NMDA-induced currents were concentration dependent (Fig. 1). NMDA-induced currents in oocytes injected with adult mRNA were more sensitive to inhibition by 7-CK than were currents in oocytes injected with day 1 mRNA (Fig. 1B and Table 1). CGP-39653 also was a more potent antagonist at receptors expressed from adult mRNA than at receptors from day-1 mRNA (Fig. 1C and Table 1).

Sensitivity of NMDA receptors to glycine and NMDA during development. The potencies of glycine and NMDA at NMDA receptors were determined after injection of oocytes with mRNA from 1-day and adult rat brain. Concentration-response curves for glycine were obtained in the presence of 100 μM NMDA. Receptors from 1-day-old rat brain were more sensitive to glycine than were receptors from adult brain (Fig. 2 and Table 1). Concentration-response curves for NMDA were determined in the presence of 10 μM glycine (Fig. 2). NMDA had a similar potency on receptors expressed from 1-day-old and adult cerebral cortex (Fig. 2 and Table 1).

Properties of heteromeric NMDA receptors expressed in *X. laevis* oocytes. NR1, NR2A, and NR2B are the major subunits in developing and adult rat cerebral cortex (21–23). To test the hypothesis that differences in subunit composition account for differences in sensitivity to glycine, 7-CK, and CGP-39653, the potencies of these compounds at heteromeric NR1/NR2A and NR1/NR2B receptors were determined in *X. laevis* oocytes. The NR1 isoform used in these experiments was NR1A, the major splice variant found in rat brain (33). 7-CK was more potent in inhibiting NMDA-induced currents from heteromeric NR1A/NR2A channels than from NR1A/NR2B channels (Fig. 3A). The IC_{50} value for NR1A/NR2B was similar to that seen with oocytes injected with mRNA from 1-day-old rat cerebral cortex (Table 1). Similarly, CGP-39653 was a more potent antagonist of heteromeric NR1A/NR2A receptors than NR1A/NR2B receptors (Fig. 3B). CGP-39653 displayed similar potency at heteromeric NR1A/NR2B receptors and receptors from 1-day-old rat brain (Fig. 3 and Table 1). Glycine was a more potent coagonist on NR1A/NR2B than on NR1A/NR2A receptors (Fig. 3C). NMDA was equally potent at NR1A/NR2A and NR1A/NR2B receptors in *X. laevis* oocytes (Fig. 3D).

Use of antisense ODNs to define developmental changes. Experiments with antisense ODNs were carried out to test the hypothesis that differences in subunit compo-

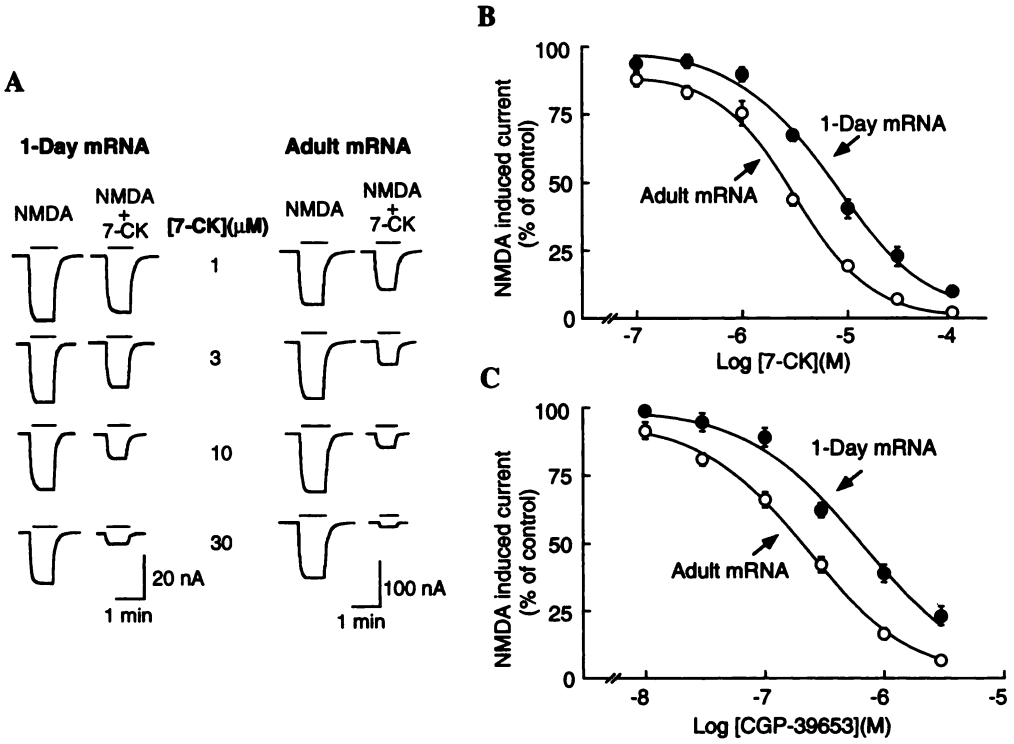

Fig. 1. Effects of 7-CK and CGP-39653 on NMDA receptors expressed in oocytes. A, Representative inward currents induced by NMDA (100 μM) and glycine (10 μM) in the presence of increasing concentrations of 7-CK. Oocytes were injected with mRNA prepared from 1-day-old or adult rat cerebral cortex and voltage-clamped at −70 mV. *Horizontal bars above each trace*, duration of NMDA and antagonist applications. B and C, Inhibition by 7-CK and CGP-39653 of currents expressed from adult and 1-day-old rat cerebral cortex mRNA. The indicated concentrations of 7-CK and CGP-39653 were applied with 100 μM NMDA and 10 μM glycine. Data are expressed as percentage of control currents determined in the absence of antagonist. Control currents were the average of currents determined before and after application of each concentration of antagonist. *Points*, mean ± standard error of results from four to seven oocytes. Curves were fit according to the equation $I = I_{max} - I_{max}/[1 + (IC_{50}/x)^n]$, where I is current response, x is concentration of antagonist, I_{max} is the maximal response of NMDA in the absence of antagonists, and n is the Hill coefficient. When error bars are not shown, they are smaller than the symbol.

TABLE 1

Potencies of 7-CK, CGP-39653, glycine, and NMDA at NMDA receptors expressed in *Xenopus* oocytes

The IC_{50} or EC_{50} is expressed with 95% confidence intervals (four to seven experiments).

	7-CK	CGP-39653	Glycine	NMDA
	IC_{50} (μM)	IC_{50} (nM)	EC_{50} (μM)	EC_{50} (μM)
Adult cortex	3.1 (2.8–3.4)	257 (143–372)	0.78 (0.49–1.1)	31 (24–37)
NR1A/NR2A	1.1 (0.7–1.4)[a]	97 (61–133)[c]	1.4 (1.2–1.7)[b]	26 (19–32)
Adult + anti-2B	0.91 (0.65–1.2)[a]	130 (97–163)[c]	1.7 (1.1–2.3)[c]	NA
NR1A/NR2B	8.9 (7.6–10.2)[a]	635 (453–818)[b]	0.25 (0.18–0.31)[c]	24 (17–29)
Adult + anti-2A	8.1 (7.3–8.8)[a]	745 (620–871)[b]	0.22 (0.15–0.28)[b]	NA
1-Day cortex	7.7 (5.6–9.8)[b]	665 (468–862)[b]	0.20 (0.14–0.24)[b]	27 (22–31)

[a] $p < 0.001$ compared with adult cortex, alternate Welch t test.
[b] $p < 0.01$ compared with adult cortex, alternate Welch t test.
[c] $p < 0.05$ compared with adult cortex, alternate Welch t test.

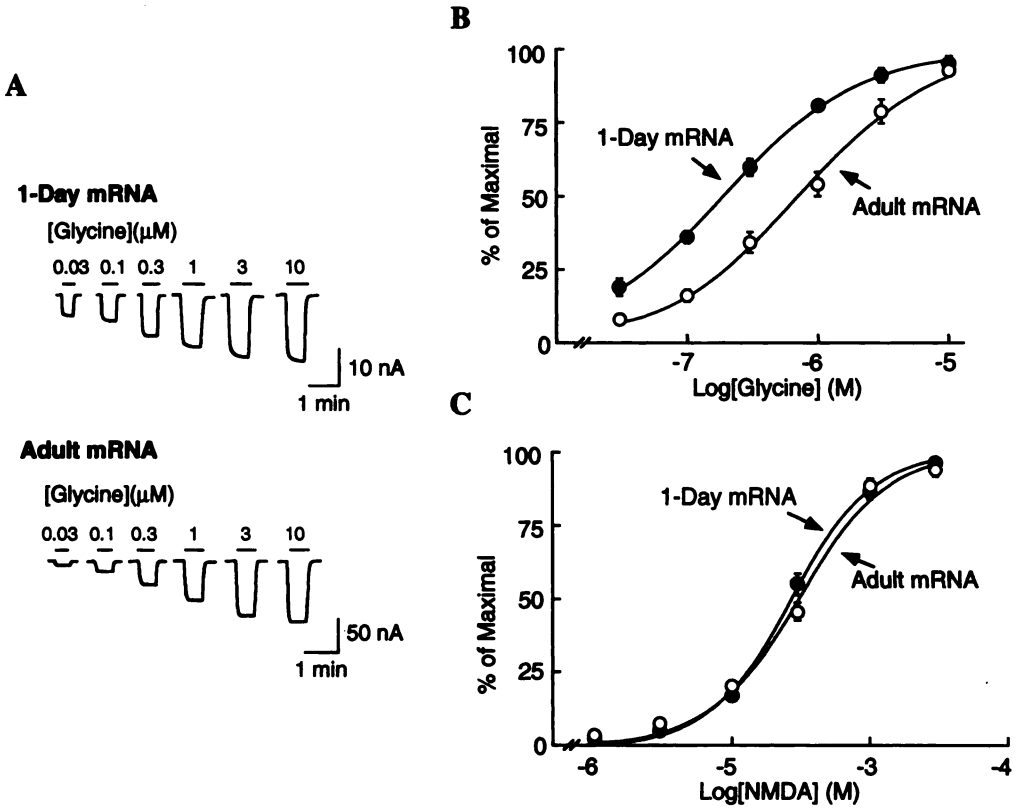

Fig. 2. Activation by glycine and NMDA of NMDA receptors expressed in oocytes injected with mRNA from adult and 1-day-old rat cerebral cortex. A, Inward currents generated by application of NMDA (100 μM) with increasing concentrations of glycine (0.03–10 μM) in oocytes injected with mRNA from 1-day-old and adult rat cortex. Oocytes were injected with cerebral cortex mRNA and recorded at a clamped potential of −70 mV. *Horizontal bars above each trace*, duration of NMDA (100 μM) and glycine (0.03–10 μM) applications. B and C, Concentration-response curves for glycine and NMDA potentiation in oocytes injected with mRNA from adult and 1-day-old rat cerebral cortex. The glycine curves were obtained by the application of the indicated concentrations of glycine together with 100 μM NMDA. The NMDA curves were determined by applying increasing concentrations of NMDA together with 10 μM glycine. Data are expressed as a percentage of maximal current for each oocyte. *Points*, mean ± standard error of results from four to seven oocytes. Curves were fit according to the equation $I = I_{max}/[1 + (EC_{50}/x)^n]$, where x is concentration of agonist, I_{max} is the maximal response for an individual oocyte, and n is the Hill coefficient.

sition of NMDA receptors are responsible for differences in the properties of receptors expressed from oocytes injected with mRNA from neonatal and adult rat cortex. Oocytes were injected with brain mRNA and an antisense ODN to either NR2A or NR2B to block the expression of the NR2A or NR2B subunit, respectively.

The sequences for antisense ODNs directed against NR2A (anti-2A) and NR2B (anti-2B) surrounded the initiation codon (−10 to +10) of the sequences of the NR2A and NR2B subunits (13). Control ODNs included random ODNs with the same GC content and sense ODNs. Coinjection of antisense ODNs to NR2A or NR2B with corresponding cloned full-length NMDA receptor subunit mRNA markedly reduced the NMDA-induced currents in *X. laevis* oocytes. The NMDA-induced currents in oocytes were not affected by coinjection of control ODNs (Table 2). Coinjection of either anti-2A or anti-2B ODNs with adult cortical mRNA reduced the NMDA-induced currents. However, coinjection of anti-2A and anti-2B ODNs together with mRNA extracted from adult rat cerebral cortex blocked >95% of NMDA-induced current in oocytes in comparison to oocytes injected with control ODNs (Table 2). Coinjection of anti-2B ODN had no effect on NMDA-induced currents in oocytes injected with NR1A/NR2A mRNA, and coinjection of anti-2A ODN had no effect

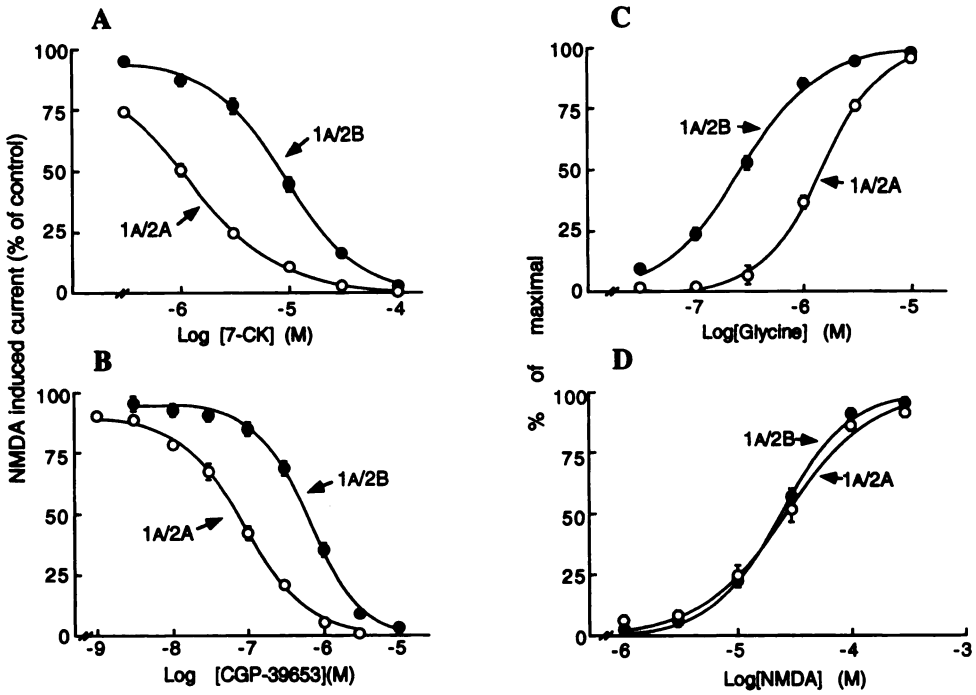

Fig. 3. Differences in potency of blockade of antagonists 7-CK and CGP-39653 and of activation by glycine and NMDA at heteromeric NMDA receptors expressed in oocytes. A and B, Effects of increasing concentrations of the NMDA receptor antagonists 7-CK and CGP-39653 on NMDA receptors expressed in oocytes injected with mRNA for NR1A/NR2A (○) and NR1A/NR2B (●). *Points*, mean ± standard error of results from four to seven oocytes. Data are expressed as percentage of control currents determined in the absence of antagonist. Curves were fit as described in the legend to Fig. 1. C and D, Concentration-response curves for activation of heteromeric NMDA receptors by glycine and NMDA. Dose-response curves for glycine in oocytes injected with mRNA for NR1A and NR2A or for NR1A and NR2B were determined by application of increasing concentrations of glycine together with 100 μM NMDA (C). The NMDA concentration-response curves were determined by application of increasing concentrations of NMDA in the presence of 10 μM glycine (D). *Points*, mean ± standard error of results from four to seven oocytes. Curves were fit as described in the legend to Fig. 2.

TABLE 2

Effects of antisense ODNs on NMDA-induced currents in *Xenopus* oocytes

The currents were measured by the application of 100 μM NMDA and 10 μM glycine at holding potential of −70 mV. Results are mean ± standard error (seven to nine experiments).

	Current
	nA
1A/2A with control ODN (random)	308 ± 73
1A/2A with anti-2A ODN	6 ± 1[a]
1A/2A with anti-2B ODN	292 ± 60
1A/2B with control ODN (random)	296 ± 62
1A/2B with anti-2A ODN	279 ± 58
1A/2B with anti-2B ODN	7 ± 1[a]
Adult mRNA with control ODN (random)	102 ± 14
Adult mRNA with anti-2A ODN	40 ± 7[b]
Adult mRNA with anti-2B ODN	50 ± 5[b]
Adult mRNA with anti-2A and anti-2B ODN	4 ± 1[c]

[a] $p < 0.005$ compared with control ODN, alternate Welch *t* test.
[b] $p < 0.01$ compared with adult mRNA with control ODN, alternate Welch *t* test.
[c] $p < 0.001$ compared with adult mRNA with control ODN, alternate Welch *t* test.

on the NMDA-induced response in oocytes injected with NR1A/NR2B mRNA (Table 2). Thus, antisense ODNs to either NR2A or NR2B selectively blocked the expression of the NR2A or NR2B subunit, respectively.

Injection of anti-2B ODN with mRNA from adult rat cerebral cortex resulted in 7-CK concentration-response curves that were shifted to the left. The IC_{50} value for 7-CK in the presence of anti-2B ODN was similar to that for heteromeric NR1A/NR2A receptors (Table 1). In contrast, injection of anti-2A ODNs with mRNA from adult cortex shifted the 7-CK inhibition curve to the right, with an IC_{50} value that was similar to the IC_{50} values for receptors from 1-day-old rat cortex and for NR1A/NR2B (Fig. 4B and Table 1). Similar results were obtained for inhibition of NMDA currents by CGP-39653 (Fig. 4C and Table 1). The effects of anti-2A and anti-2B ODNs on the potency of glycine at NMDA receptors from adult cortex were also determined. In the presence of anti-2A ODN, the potency of glycine was increased to that seen with receptors from 1-day-old animals or with NR1A/NR2B heteromeric receptors (Fig. 5 and Table 1). Anti-2B ODN reduced the potency of glycine to a value comparable to that seen with NR1A/NR2A receptors (Table 1).

Discussion

The functional and structural properties of a number of ligand-gated ion channels, including nicotinic acetylcholine receptors and $GABA_A$ receptors, have been found to change during development (10, 11). The switch in subunit composition in nicotinic acetylcholine receptors (from γ subunits in neonates to ε subunits in adults) accounts for changes in the gating properties of channels observed during development (10). The delayed expression of α1 subunits in $GABA_A$ receptors may underlie the delayed appearance of benzodiazepine sites in developing hippocampus (11). Developmental changes in the kinetics of NMDA-mediated responses and in the sensitivity of receptors to Mg^{2+}, glycine, and polyamines have also been observed (4–8). The subunit composition of NMDA receptors *in vivo* is not known. Coimmunoprecipita-

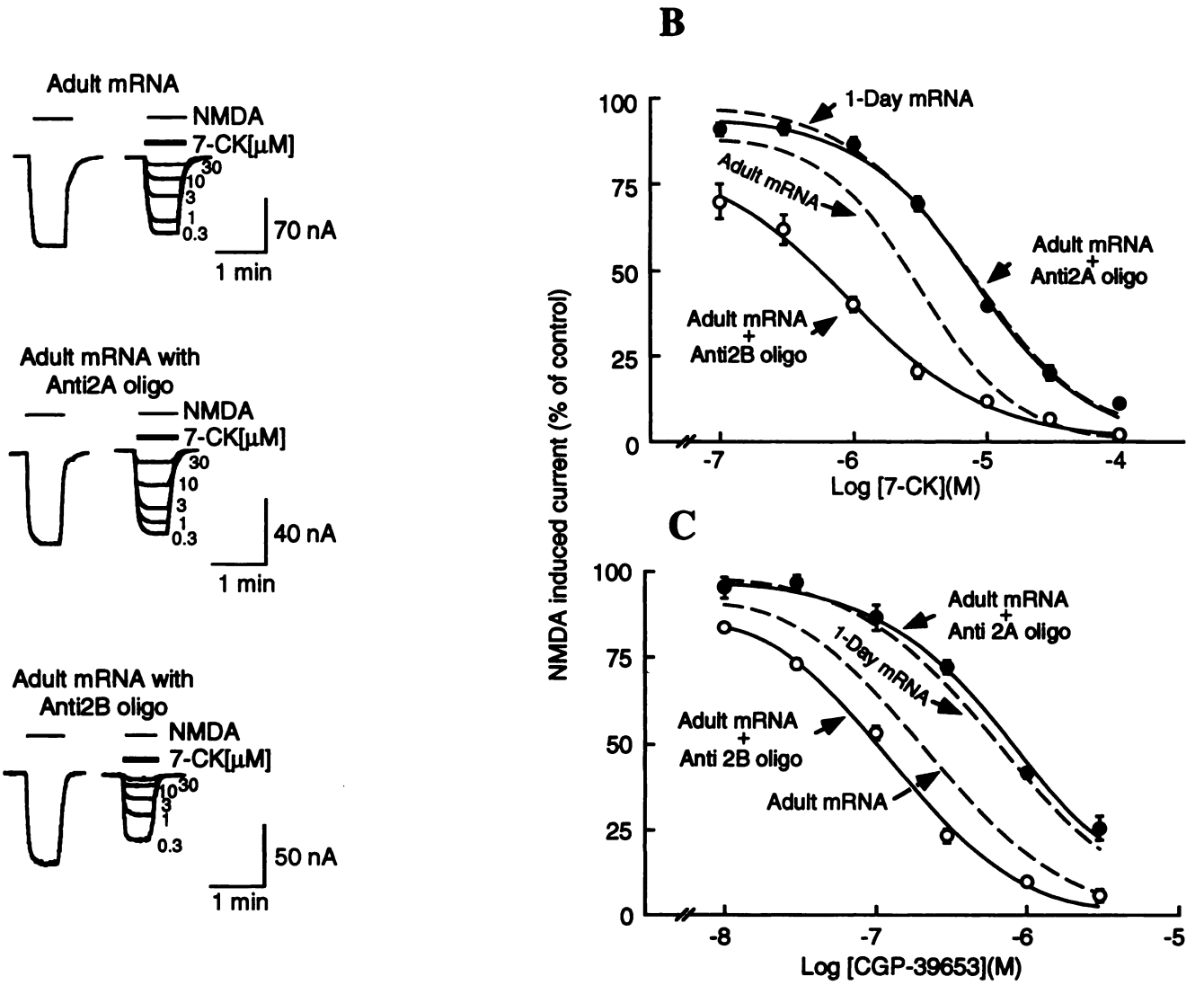

Fig. 4. Effects of antisense ODNs on the inhibition of currents by 7-CK and CGP-39653 in oocytes injected with mRNA from 1-day-old and adult rat cerebral cortex. A, Effect of 7-CK on the inward currents induced by NMDA from oocytes injected with adult cerebral cortex mRNA with control ODNs, adult cerebral cortex mRNA with anti-2A ODNs, and adult cerebral cortex mRNA with anti-2B ODNs. Oocytes were injected with a mixture of adult cerebral cortex mRNA together with indicated ODNs 2–5 days before recording and were voltage-clamped at −70 mV. *Horizontal bars above each trace*, duration of NMDA (100 μM) and 7-CK (0.3–30 μM) applications. B and C, Concentration-response curves for inhibition of NMDA-induced currents by 7-CK (B) and CGP-39653 (C) in oocytes injected with mRNA from adult rat cerebral cortex with anti-2A ODNs or anti-2B ODNs. *Points*, mean ± standard error of current responses from four to seven oocytes. Curves were fit as described in the legend to Fig. 1. *Dashed lines*, fitted dose-response curves of inhibition by 7-CK and CGP-39653 on NMDA receptors expressed from adult and 1-day-old rat cerebral cortex mRNA replotted from Fig. 1 for comparison.

tion with subunit-specific antibodies has demonstrated coassociation of NR1, NR2A, and NR2B subunits in rat cortex (34). A preferential coassembly of NR1, NR2A, and NR2C subunits expressed in *X. laevis* oocytes has also been reported (35). *X. laevis* oocytes have been widely used as a model system with which to study receptors and ion channels. The use of oocytes allows a detailed and quantitative pharmacological analysis of neuronal excitatory amino acid receptors. The properties of NMDA receptors expressed in oocytes injected with rat brain mRNA are similar to those of native neuronal receptors (36).

In the current study, NMDA receptors generated from cortical mRNA from 1-day-old and adult rat brain were expressed in *X. laevis* oocytes, and the pharmacological properties of the receptors, including glycine modulation and inhibition by competitive and noncompetitive antagonists, were examined. Developmental changes were observed in the potencies of glycine and the antagonists 7-CK and CGP-39653 at NMDA receptors expressed in oocytes. NMDA receptors expressed from 1-day-old rat cortex mRNA were more sensitive to glycine activation and less sensitive to block by antagonists than were receptors expressed from adult cortical mRNA. Heteromeric NR1/NR2A and NR1/NR2B receptors expressed in oocytes also differed in sensitivity to glycine and the antagonists. Similar results were obtained with mouse (17) and rat (20) NR1/NR2A and NR1/NR2B receptors. Heteromeric NR1/NR2B receptors had pharmacological properties similar to those of receptors expressed using mRNA from cortex of 1-day-old rats. Receptors expressed using mRNA from cortex of adult rats had pharmacological profiles intermediate between those of NR1/NR2B and NR1/NR2A receptors (Table 1). In neonatal rat cortex, levels of

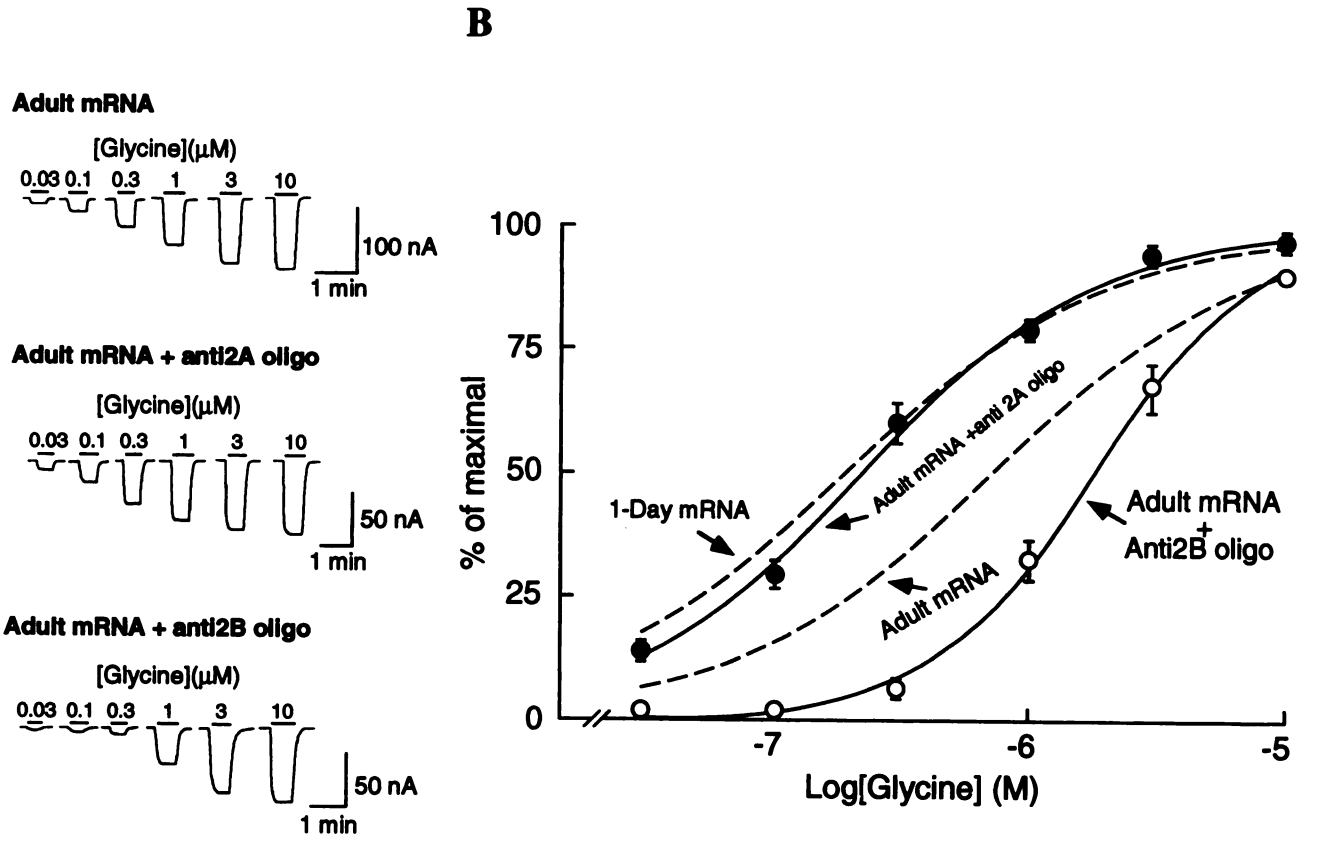

Fig. 5. Potencies of glycine at NMDA receptors in oocytes injected with mRNA from adult cerebral cortex with antisense ODNs. A, Inward currents generated by application of NMDA (100 μM) and increasing concentrations of glycine (0.03–10 μM) in oocytes injected with mRNA from adult cortex, mRNA from adult cortex with anti-2A ODNs, and mRNA from adult cortex with anti-2B ODNs. Oocytes were injected with cerebral cortex mRNA with or without antisense ODNs 2–5 days before recording and were recorded at a clamped potential of −70 mV. *Horizontal bars above each trace*, duration of NMDA (100 μM) and glycine (0.03–10 μM) applications. B, Concentration-response curves for glycine potentiation of NMDA-induced currents in oocytes injected with mRNA from adult cerebral cortex with anti-2A ODNs or with anti-2B ODNs. Increasing concentrations of glycine were applied with 100 μM NMDA. *Points*, mean ± standard error of results from four to seven oocytes. Data are expressed as a percentage of maximal response currents for each oocyte. Curves were fit as described in the legend to Fig. 2. *Dashed lines*, fitted curves for potentiation of NMDA-induced currents by glycine in oocytes injected with adult and 1-day-old rat cerebral cortex mRNA replotted from Fig. 2 for comparison.

NR2B mRNA are much higher than levels of NR2A mRNA, whereas the expression levels of these subunits are similar in adult cortex (21–23).

Antisense ODNs specific for NR2A and NR2B subunit mRNA were used to investigate the roles of NR2 subunits in modulating the pharmacological properties of NMDA receptors during development. Injection of adult cortex mRNA with anti-2B ODNs resulted in the expression of NMDA receptors with a pharmacological profile similar to that seen for NR1A/NR2A receptors. NMDA receptors expressed after injection of adult cortex mRNA with anti-2A ODNs resulted in receptors with a pharmacological profile that was similar to that of NMDA receptors from 1-day-old rat mRNA or from heteromeric NR1/NR2B receptors. These findings suggest that an increasing incorporation of NR2A subunits in native NMDA receptors underlies changes in the pharmacological properties of NMDA receptors in developing cortex. Results from studies with antisense ODNs indicate that changes in the expression of NR2 subunits are entirely sufficient to account for changes in the pharmacological properties of NMDA receptors seen in oocytes expressed from neonatal and adult cortical mRNAs.

Developmental changes in the sensitivity of hippocampal NMDA receptors to Mg^{2+} and glycine have been reported (6, 7). During development, NMDA receptors become more sensitive to block by Mg^{2+} and less sensitive to activation by glycine. A developmental decrease in the activation of cortical NMDA receptors by glycine was seen in the current study. Interestingly, a developmental increase in the potency of 7-CK for inhibiting NMDA-induced currents was also seen. Because 7-CK is a glycine-site antagonist, the increase in the potency of 7-CK inhibition may be due to a developmental decrease in the sensitivity of NMDA receptors to glycine. NMDA receptors play an important role in synapse elimination and sensory map formation during development (37). A reduced level of Mg^{2+} block and greater sensitivity to glycine in neonatal rat brain would facilitate the participation of native NMDA receptors in these developmental events. NR2 subunits may be regarded as modulatory because their expression leads to changes in the properties of NMDA receptors (e.g., sensitivity to antagonists, voltage-dependent block by Mg^{2+}, deactivation kinetics, and single-channel characteristics). NR2 subunits do not produce a viable receptor/channel complex in the absence of NR1 (38).

In the current study, receptors from neonatal rat cortex had similar properties to heteromeric NR1/NR2B receptor channels, whereas receptors from adult cerebral cortex had pharmacological properties that seemed to result from ex-

pression of both NR1/NR2B and NR1/NR2A receptors (Table 1). The 2–3-fold differences in sensitivities to agonists and antagonists between neonatal receptors and receptors in adults do not distinguish between receptors formed from all three subunits and mixtures of receptors formed from NR1 and either NR2A or NR2B. The predominance of the expression of NR2B subunits early in development indicates that in neonates, NR2B may be a major factor in controlling the properties of NMDA receptors, and that this subunit may play an important role in early brain development. Transient expression and disappearance of NR2B subunit mRNA in cerebellum during postnatal development may also indicate that NR2B subunits have a developmental role (21–23). The late expression of NR2A subunits may play an important role in modulating NMDA receptor function at later developmental stages. Results from the current study on NMDA receptors expressed in *X. laevis* oocytes, in conjunction with the alteration in expression of subunit mRNA observed during development, suggest that NMDA receptors in neonates are assembled from NR1 and NR2B subunits, whereas adult NMDA receptors are composed of NR1, NR2A, and NR2B subunits.

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