Glycine-Insensitive NMDA-Sensitive Receptor Expressed in *Xenopus* Oocytes by Guinea Pig Cerebellar mRNA

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The electrophysiological and pharmacological properties of *N*-methyl-D-aspartate (NMDA)-sensitive receptors expressed in *Xenopus* oocytes by injection of total poly(A)⁺ RNAs (mRNAs) from the cerebellum and cerebrum of guinea pigs were compared. The inward current induced by NMDA under voltage-clamp in cerebellar mRNA-injected oocytes was depressed in a voltage-dependent fashion by Mg²⁺ to show a negative slope conductance and selectively antagonized by D-2-amino-5-phosphonovalerate (D-APV) and phencyclidine (PCP). Glycine (0.01–10 μ M) did not potentiate NMDA-induced currents in cerebellar mRNA-injected oocytes, while it potentiated NMDA-induced currents in cerebral mRNA-injected oocytes in a dose-dependent fashion. 6-Cyano-7-nitroquinoxaline-2,3-dione and 7-chlorokynure-nate suppressed the NMDA response but significantly less potently in cerebellar mRNA-injected oocytes than in cerebral mRNA-injected oocytes. These results suggest that the NMDA-sensitive receptor expressed in *Xenopus* oocytes by guinea pig cerebellar mRNA resembles the cerebral NMDA receptor in its high sensitivities to Mg²⁺, PCP, and D-APV, but it is distinct from the cerebral NMDA receptor in responsiveness to glycine.

Excitatory amino acid receptors in the mammalian cerebrum are classified into either 3 subtypes, *N*-methyl-D-aspartate (NMDA), quisqualate (QA), and kainate (KA) receptors, or 2 subclasses, NMDA and non-NMDA types (Foster and Fagg, 1984). Among them, the NMDA receptor has attracted the most attention because of its possible role in high-order brain functions such as the long-term potentiation (LTP) in the hippocampus (Bliss and Gardner-Medwin, 1973; Bliss and Lomo, 1973; Collingridge and Bliss, 1987; Mayer and Westbrook, 1987).

The cerebral NMDA receptor may be characterized by the following 4 electrophysiological and pharmacological properties: (1) its response to NMDA is depressed in a voltage-dependent fashion by Mg²⁺ to show a negative slope conductance (Mayer et al., 1984; Nowak et al., 1984); (2) glycine potently enhances the NMDA response (Johnson and Ascher, 1987; Kleckner and Dingledine, 1988); (3) D-2-amino-5-phosphonovalerate (D-APV) is the specific antagonist of the NMDA receptor (Watkins and Evans, 1981); and (4) phencyclidine (PCP)

is the relatively specific open channel-blocker of the calcium channel linked to the NMDA receptor (Anis et al., 1983; Barker et al., 1983; Berry et al., 1984; Coan and Collingridge, 1985; Duchen et al., 1985; Harrison and Simmonds, 1985; Honey et al., 1985; Mayer and Westbrook, 1985; Thomson and Lodge, 1985).

However, there appears to be a regional heterogeneity in NMDA-sensitive receptors in the brain, as suggested by the following findings: (1) Rat cerebellar and spinal cord neurons are almost insensitive to quinolinate (QNL), an endogenous NMDA receptor agonist, while the cerebral cortical, hippocampal, and neostriatal neurons are sensitive to QNL (Perkins and Stone, 1983). (2) The potentiation by glycine of ³H-glutamate binding to NMDA receptors was significantly greater in the thalamus and cerebral cortex than in the cerebellum, septum, and striatum of the rat (Monaghan et al., 1988). (3) ³H-1-(1-(2-thienyl)cyclohexyl)-piperidine (³H-TCP), a potent radioligand for a PCP-binding site on the NMDA receptor, shows a low affinity for NMDA receptors in the rat cerebellum (Lawrence et al., 1988). Although these findings may be to suggest an NMDA receptor heterogeneity and might reflect multifunctional roles of NMDA receptors in the brain, direct electrophysiological or pharmacological evidence for the heterogeneity has not yet been presented.

For the purpose of electrophysiologically exploring a regional difference in the NMDA receptor between the cerebrum and cerebellum, the properties of the NMDA-sensitive receptor expressed in *Xenopus* oocytes by guinea pig cerebellar mRNA were compared with those of the cerebral NMDA receptor similarly expressed.

Materials and Methods

Messenger RNA was extracted from the cerebellum or cerebrum of adult male guinea pigs (350–500 gm, Hartley strain) according to the method of Chirgwin et al. (1979), but the cerebellar or cerebral microsomal fraction was used as a starting material as described previously (Sekiguchi et al., 1989). Briefly, the microsomal fraction was homogenized in 6 M guanidinium thiocyanate solution containing 5 mM Na-citrate and 0.5% Na-lauroylsarcosine, and ultracentrifuged at 160,000 $\times$ g for 17 hr at 20°C on a 5.7 M CsCl cushion containing 0.1 M Na₂-EDTA (pH 7.4). Precipitated total RNA was dissolved in 10 mM Tris-HCl buffer (pH 7.4) containing 5 mM EDTA and 0.1% Na₃-dodecylsulfate, and washed thoroughly with a phenol/chloroform/iso-amylalcohol mixture (25:24:1 by vol), then with a chloroform/iso-amylalcohol mixture (24:1 by vol). After the total RNA was precipitated by ethanol, mRNA was separated by oligo-dT cellulose column chromatography and precipitated by ethanol. The mRNA precipitate was dissolved in sterile water to be 1–2 mg mRNA/ml, and about 50 ng of mRNA (about 30 nl) was injected into each *Xenopus laevis* oocyte (at stage V or VI, a diameter of >1.0 mm, and with clear animal and vegetal hemispheres) basically by the method of Gundersen et al. (1984a).

The oocytes injected with mRNA were incubated at 19°C for 2–4 d

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in modified Barth's medium consisting of (in mM) NaCl, 88; KCl, 1; NaHCO₃, 2.4; HEPES (pH 7.6), 15; Ca(NO₃)₂, 0.3; CaCl₂, 0.41; MgSO₄, 0.82, supplemented with streptomycin-SO₄, 100 mg/liter, and penicillin-K, 10,000 U/liter, and then treated with collagenase (1 mg/ml) for 2–3 hr at room temperature (22–24°C) to defolliculate. (In some experiments, the oocytes were first treated with collagenase, then mRNA was injected to examine whether the collagenase treatment affects receptors' expression, but no difference was noted.) The defolliculated oocyte was placed (one oocyte at a time) in a recording well (0.5 ml capacity) and superfused at 5 ml/min with frog Ringer's solution consisting of 125 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, and 5 mM HEPES (pH 7.4).

In all experiments in which the effect of antagonists or glycine was to be compared between cerebral mRNA-injected oocytes and cerebellar mRNA-injected oocytes, mRNAs were extracted respectively from 10 cerebra and 10 cerebella of 10 guinea pigs and injected into oocytes from a single mother frog.

Each test substance was dissolved in frog Ringer's solution at various concentrations and applied to oocytes by constant flow superfusion (5 ml/min). Since the NMDA responses of oocytes injected with cerebral mRNA, but not with cerebellar mRNA, were too small, the frog Ringer's solution containing 10 μ M glycine was superfused for cerebral mRNA-injected oocytes, except for experiments to determine the effect of the amino acid itself. All solutions used were prepared with double-distilled autoclaved water (12 M Ω /cm or more) to minimize contaminations with Mg²⁺, etc.

Responses of oocytes to test substances were recorded at room temperature by a voltage-clamp technique (clamped at –60 mV unless otherwise stated) using 2 glass microelectrodes both filled with 3 M KCl at 5–10 M Ω , one for current recording and the other for current injection through the voltage-clamp unit (CEZ-1100, Nihon Kohden, Tokyo). Current signals from oocytes were recorded by the recorder responding up to 1 kHz (WS-641G, Nihon Kohden) and also stored on magnetic tape (FE-35S, Sony, Tokyo). Intracellular recordings from oocytes were also performed using a single glass microelectrode (filled with 3 M KCl, 5–10 M Ω), but the results were employed only for a supplementary purpose.

NMDA, QA, DL-2-amino-5-phosphonovaleric acid (DL-APV), D-APV, and γ -D-glutamylglycine (DGG) were purchased from Funakoshi Chemical Co. (Tokyo, Japan). L-Glutamic acid diethylester (GDEE) was obtained from Sigma Chemical Co. (St. Louis, MO), and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and 7-chlorokynurenic acid (7-Cl-KYN) were from Tocris Neuramin (Essex, England). Phencyclidine was a gift from Dr. Toshitaka Nabeshima of the Department of Pharmacology, Faculty of Pharmaceutical Sciences, Meijo University (Nagoya, Japan). The other chemicals used, including L-glutamic acid, L-aspartic acid, and collagenase, were obtained from Wako Pure Chemical Industries (Tokyo).

Results

Membrane potentials and responsiveness of mRNA-injected and noninjected oocytes

The mean resting membrane potential (RMP) was -50.2 ± 1.7 ($\pm$ SEM) mV for 50 randomly selected oocytes injected with cerebellar mRNA and defolliculated, -51.8 ± 1.8 mV for 20 oocytes injected with cerebral mRNA and defolliculated, and -51.0 ± 2.9 mV for 11 randomly selected defolliculated oocytes to which no mRNA was injected.

In oocytes that did not receive mRNA, 0.1–2 mM NMDA or L-aspartate (Asp) applied by superfusion sometimes induced small hyperpolarizations (1–3 mV), but no depolarizing response was induced by NMDA, Asp, QA, or glutamate (Glu), even when these agonists were applied at concentrations as high as 1 mM (data not shown).

Inward currents induced by excitatory amino acids

The oocytes injected with cerebellar mRNA became responsive to all excitatory amino acids tested. Either intracellularly recorded depolarizing responses or smooth/oscillating inward current responses under voltage-clamp to QA and Glu were ob-

served in all 105 cerebellar mRNA-injected oocytes (mRNAs from 8 separate batches), and those to NMDA and Asp were observed in 102 of 105 oocytes; the remaining 3 oocytes responded neither to NMDA nor to Asp, but only to QA and Glu.

QA and Glu exhibited both smooth and oscillating inward currents or depolarizations; oscillating responses were observed in 32 out of 128 QA applications and 18 out of 74 Glu applications. The smooth and oscillating inward currents induced by QA (50 μ M) are illustrated in Figure 1C (left) and D, respectively. The record in Figure 1D was obtained from the same cell as in Figure 1C. Though not shown here, the shape of Glu-induced oscillating inward currents was similar to the oscillating QA response shown in Figure 1D. On the other hand, NMDA and Asp primarily showed smooth responses (e.g., see Fig. 1A, a); oscillating responses were observed in only 2 of 156 NMDA applications and in 3 of 68 Asp applications. As seen in Figure 1, A–C, smooth inward current responses to excitatory amino acids were elicited after latencies as short as 5–20 sec. Although no large desensitization to NMDA, Asp, Glu, or QA was observed in all oocytes tested, a slight (<15%) initial decline in current amplitude was usually noted in repetitive applications of NMDA similar to those described by Verdoorn et al. (1989). Therefore, only the NMDA responses stabilized after this initial decline were employed.

Figure 1A, a and b illustrates the dose dependence of the smooth inward current responses to 1–1000 μ M NMDA of a cerebellar mRNA-injected oocyte in normal frog Ringer's solution containing neither Mg²⁺ nor glycine (a) and a cerebral mRNA-injected oocyte in the frog Ringer's solution containing no Mg²⁺ and 10 μ M glycine (b). As shown in Figure 1A, a and b, the oocytes injected with cerebral and cerebellar mRNAs showed approximately equivalent responses in the NMDA concentration range of 1–1000 μ M, when 10 μ M glycine was added to the medium superfusing cerebral mRNA-injected oocytes.

The mean response to 100 μ M NMDA of cerebral mRNA-injected oocytes in the frog Ringer's solution with no added glycine was 2.3 ± 0.7 nA ($n = 5$); see Figure 5A, b, for example. On the basis of this finding, we decided to make routine additions of 10 μ M glycine to the frog Ringer's solution superfusing cerebral, but not cerebellar, mRNA-injected oocytes.

The ED₅₀ value for NMDA in either cerebral mRNA-injected oocytes or cerebellar mRNA-injected oocytes was found to change from about 30 μ M to about 200 μ M depending primarily upon a seasonal biological variation in the mother frog (see results for Figs. 6 and 7). However, despite this variation in ED₅₀, the oocytes which were collected from one mother frog and injected with cerebral and cerebellar mRNAs showed approximately similar responses to NMDA, provided that glycine at ~ 10 μ M was added to the superfusion medium for cerebral mRNA-injected oocytes.

Effects of D-APV on NMDA-induced inward currents

The effects of D-APV on NMDA-induced inward currents were comparatively studied in the oocytes injected with cerebellar and cerebral mRNAs. As shown in Figure 1E, 0.1–10 μ M of D-APV suppressed the response to 100 μ M NMDA of cerebellar mRNA-injected oocytes (closed circles) slightly more effectively than that of cerebral mRNA-injected oocytes (open circles). There was a significant difference ($p < 0.05$, Student's *t*-test) between the 2 values (open and closed circles in Fig. 1E) at 10 μ M D-APV. The concentration of D-APV to achieve 50% suppression (IC₅₀) of responses to 100 μ M NMDA was $14.3 \pm$

Figure 1. Responses to NMDA and QA of the oocytes injected with guinea pig cerebellar and cerebral mRNAs under voltage clamp, and the effects of D-APV. *A*, The inward currents induced by 1–1000 μ M NMDA in the oocytes injected with cerebellar (*a*) and cerebral (*b*) mRNAs. NMDA was applied by superfusion for 30 sec (bars), and the oocytes were clamped at -60 mV (also for *B–E*). *B*, The inhibitory effect of 1–100 μ M D-APV on responses to 100 μ M NMDA in the oocytes injected with cerebellar (*a*) and cerebral (*b*) mRNAs. D-APV was applied together with NMDA for 30 sec (also for *C* and *E*). *C*, A smooth response to 50 μ M QA in a cerebellar mRNA-injected oocyte (*left*), and the lack of the inhibitory effect of 25 μ M D-APV thereon (*right*). *D*, An oscillating response to 50 μ M QA in the same cerebellar mRNA-injected oocyte as that of *C*. *E*, The dose dependence of the blocking effects of D-APV on NMDA responses in the oocytes injected with cerebellar (closed circles) and cerebral (open circles) mRNAs. Mean values ($\pm$ SEM) from 3 oocytes are plotted. Ordinate: the percentage of the mean control current in the absence of D-APV [35.9 ± 4.9 nA ($n = 3$) and 33.6 ± 2.4 nA ($n = 3$) for the oocytes injected with cerebellar and cerebral mRNAs, respectively]. Glycine (10 μ M) was added to the superfusion medium for cerebral mRNA-injected oocytes. Calibrations: vertical, 50 nA for *A*, *C*, and *D*, and 25 nA for *B*; horizontal, 1 min for all.

2.8 μ M (mean $\pm$ SEM, $n = 3$) and 3.16 ± 0.68 μ M ($n = 3$) for the oocytes injected with cerebral and cerebellar mRNAs, respectively (values of mean $IC_{50} \pm$ SEM in the text were calculated by averaging the IC_{50} values individually estimated from dose-inhibition curves such as that in Fig. 1*E*). However, NMDA responses of cerebral mRNA-injected oocytes ($n = 3$) were consistently abolished by 100 μ M D-APV in such a manner as in Figure 1*E* (open circle), whereas the currents induced by 100 μ M NMDA in cerebellar mRNA-injected oocytes ($n = 3$) were not abolished, but rather were increased or unchanged, resulting in a mean suppression of about 35% at 100 μ M D-APV (Fig. 1*E*, closed circles).

In contrast to NMDA responses, QA-induced smooth inward currents in cerebellar mRNA-injected oocytes were much less sensitive to 25 μ M D-APV (Fig. 1*C*), indicating that the inward currents induced by NMDA in cerebellar mRNA-injected oocytes were not mediated by QA receptors.

Mg²⁺- and voltage-dependence of NMDA-induced currents

We wanted to determine whether NMDA-induced inward currents in cerebellar mRNA-injected oocytes depend on both Mg ions and the membrane potential in a similar manner as those in cerebral mRNA-injected oocytes. As mentioned above, since NMDA responses of cerebral mRNA-injected oocytes had to

be recorded in the presence of glycine, the absolute ED_{50} value for NMDA could not be estimated. Thus, the concentration of NMDA was tentatively fixed at 100 μ M, and the mRNA-injected oocytes which showed comparable responses to 100 μ M NMDA (that is, the responses of cerebellar mRNA-injected oocytes in the absence of glycine were similar to those of cerebral mRNA-injected oocytes in the presence of 10 μ M glycine) were employed for this and subsequent comparative studies between the oocytes injected with cerebellar and cerebral mRNAs, unless otherwise stated.

As shown in Figure 2*A*, *a*, *b*, and 2*B*, NMDA responses of cerebellar mRNA-injected oocytes (closed circles) had a sensitivity to Mg^{2+} similar to that of cerebral mRNA-injected oocytes. The apparent IC_{50} value estimated at this concentration (100 μ M) of NMDA was 29.9 ± 6.1 μ M ($n = 3$) and 39.8 ± 12.2 μ M ($n = 3$) for cerebellar and cerebral mRNA-injected oocytes, respectively.

Responses to QA of cerebellar mRNA-injected oocytes were confirmed to be insensitive to Mg ions. As shown in Figure 2*B*, closed squares, the smooth inward current induced by 100 μ M QA in cerebellar mRNA-injected oocytes was little affected by 1–1000 μ M Mg^{2+} , suggesting again that responses to NMDA and QA were mediated by distinct classes of receptor.

Figure 3*A* shows the currents induced by 100 μ M NMDA in

Figure 2. Dose dependence of the Mg^{2+} effect. *A*, The inward currents induced by $100 \mu M$ NMDA applied for 15 sec together with 0, 10, 100, and $1000 \mu M$ $MgCl_2$ to cerebellar (*a*) and cerebral (*b*) mRNA-injected oocytes, clamped at -60 mV. Calibrations: vertical, 25 nA; horizontal, 1 min. *B*, Dose-response curves of the blocking action of Mg^{2+} on the inward current induced by $100 \mu M$ NMDA in the oocytes injected with cerebellar (closed circles) and cerebral (open circles) mRNAs clamped at -60 mV. Glycine ($10 \mu M$) was present in the external medium for cerebral mRNA-injected oocytes. Mean values ($\pm$ SEM) from 3 cerebellar mRNA-injected oocytes and 3 cerebral mRNA-injected oocytes are plotted. Ordinate: percentage of the mean control current in the absence of Mg^{2+} [10.0 ± 0.8 nA ($n = 3$) and 14.5 ± 2.0 nA ($n = 3$) for cerebellar and cerebral mRNA-injected oocytes, respectively]. The lack of the Mg^{2+} effect on the inward current induced by $100 \mu M$ QA in cerebellar mRNA-injected oocytes ($n = 3$) is also shown (closed squares).

Figure 3. Voltage and Mg^{2+} dependence of the NMDA response. *A*, Inward currents induced by $100 \mu M$ NMDA in a cerebellar mRNA-injected oocyte at clamped membrane potentials of -60 , -30 , and $+10$ mV (RMP = -54 mV) in the presence (*a*) and absence (*b*) of $25 \mu M$ external Mg^{2+} . NMDA was applied by superfusion for 15 sec (bars) at $100 \mu M$. Calibrations: vertical, 12.5 nA for the left 4 records and 25 nA for the right 2 records; horizontal, 30 sec. *B*, The $I-V$ relations obtained by averaging 3–4 observed values in different cerebellar mRNA-injected oocytes in the presence (closed circles) and absence (open circles) of $25 \mu M$ Mg^{2+} . Mean values ($\pm$ SEM) from 3–4 oocytes are plotted. Abscissa, membrane potential; ordinate, the current normalized at a membrane potential of -40 mV; the mean currents at -40 mV were 30.7 ± 1.3 nA ($n = 4$) and 35.0 ± 1.2 nA ($n = 4$) in the presence and absence of Mg^{2+} , respectively.

a cerebellar mRNA-injected oocyte at voltage-clamped membrane potentials of -60 , -30 , and $+10$ mV in the presence (*a*) and absence (*b*) of external $25 \mu M$ Mg^{2+} . Figure 3*B* shows the mean $I-V$ relations obtained with $100 \mu M$ NMDA in the absence (open circles) and presence of $25 \mu M$ Mg^{2+} (closed circles) in 3–4 cerebellar mRNA-injected oocytes and normalized at a membrane potential of -40 mV. As shown in Figure 3*B*, closed circles, a negative slope conductance is seen in the presence of $25 \mu M$ Mg^{2+} in a hyperpolarized membrane potential range.

Effects of PCP on NMDA-induced inward currents

Figure 4 shows the dose dependence of inhibition by PCP of the inward currents induced by $100 \mu M$ NMDA in the oocytes injected with cerebellar mRNA and cerebral mRNA. PCP dose-dependently suppressed NMDA responses in both cerebellar and cerebral mRNA-injected oocytes, but the inhibitory potency of PCP on $100 \mu M$ NMDA was about 60 times stronger in cerebellar mRNA-injected oocytes; that is, apparent IC_{50} values were 0.53 ± 0.37 nM ($n = 3$) and 33.0 ± 4.0 nM ($n = 3$) for cerebellar and cerebral mRNA-injected oocytes, respectively.

Effect of glycine on NMDA-induced inward currents

Whether glycine can potentiate the responses of cerebellar mRNA-injected oocytes to NMDA was examined in comparison with cerebral mRNA-injected oocytes. This series of comparative studies first revealed a marked difference in the sensitivity of NMDA response to glycine between the oocytes injected with cerebellar and cerebral mRNAs. As shown in Figure 5*A*, *a* and *B*, 0.1 – $10 \mu M$ glycine did not potentiate NMDA-induced inward currents in cerebellar mRNA-injected oocytes. The lack of potentiation by 0.1 – $10 \mu M$ glycine was confirmed in a total of 9 cerebellar mRNA-injected oocytes. Moreover, glycine was also tested at higher concentrations such as 50 and $100 \mu M$, but NMDA responses of cerebellar mRNA-injected oocytes were not potentiated at all (data not shown).

In contrast, NMDA responses in cerebral mRNA-injected oocytes were clearly and dose-dependently potentiated by glycine (Fig. 5*A*, *a* and open circles in *B*), the concentration of glycine to achieve a 5-fold increase being $0.63 \pm 0.20 \mu M$ ($n = 3$). As mentioned earlier, NMDA also induced only a small (~ 1.5 nA) inward current in the absence of added glycine in this oocyte (Fig. 5*A*, *b*, leftmost trace), and such a small current

Figure 4. Effect of PCP on NMDA-induced inward currents. *A*, Inward current induced by 100 μ M NMDA applied for 30 sec (bars) together with 0–1000 nM PCP to the oocytes injected with cerebellar (*a*) and cerebral (*b*) mRNAs, clamped at -60 mV. Calibrations: vertical, 25 nA; horizontal, 1 min. *B*, Dose–response curves of the inhibitory action of PCP on the inward currents induced by 100 μ M NMDA in the oocytes injected with cerebellar (closed circles) and cerebral (open circles) mRNAs, clamped at -60 mV. All values plotted are the means ($\pm$ SEM) of 3 observations. Glycine (10 μ M) was present in the external medium for cerebral mRNA-injected oocytes. Ordinate: the percentage of the mean control current in the absence of PCP [26.7 ± 1.1 nA ($n = 3$) and 36.7 ± 0.8 nA ($n = 3$) for the oocytes injected with cerebellar and cerebral mRNAs, respectively].

Figure 5. Effects of glycine on NMDA responses in the oocytes injected with cerebellar and cerebral mRNAs. *A*, Inward currents induced by 100 μ M NMDA applied for 30 sec (bars) together with 0, 0.1, 1, and 10 μ M glycine to the oocytes injected with cerebellar (*a*) and cerebral (*b*) mRNAs, clamped at -60 mV. Calibrations: vertical, 25 nA; horizontal, 1 min. *B*, Dose–response curves of the potentiating action of glycine on responses to 100 μ M NMDA in the oocytes injected with cerebellar (closed circles) and cerebral (open circles) mRNAs, clamped at -60 mV. Mean values ($\pm$ SEM) from 3 oocytes are plotted. Ordinate, the mean inward current expressed as the ratio to the mean control value in the absence of glycine [48.3 ± 10.9 nA ($n = 3$) and 2.2 ± 0.8 nA ($n = 3$) for the oocytes injected with cerebellar and cerebral mRNAs, respectively].

was increased by nearly 10-fold on average by 10 μ M glycine (Fig. 5*B*, open circle).

Effects of 7-Cl-KYN and CNQX on NMDA-induced inward currents

The glycine binding site on cerebral NMDA receptors is known to be blocked specifically by 7-Cl-KYN (Kemp et al., 1988; Donald et al., 1988). The effect of this antagonist was tested in order to confirm that the lack of the glycine effect reflects the characteristic property of the NMDA-sensitive receptors expressed in cerebellar mRNA-injected oocytes.

In this series of experiments (Figs. 6, 7), however, the test concentration of NMDA had to be raised to 300 μ M for cerebellar mRNA-injected oocytes and to 150 μ M (in the presence of 10 μ M glycine) for cerebral mRNA-injected oocytes in order to obtain equivalent responses. This was because the oocytes used for these experiments had a relatively low ability to translate mRNAs, a seasonal biological variation which is particularly prominent in the summer.

As illustrated in Figure 6*A*, 7-Cl-KYN suppressed the inward current induced by NMDA in oocytes injected with cerebellar mRNA (*a*) or cerebral mRNA (*b*). Figure 6*B* shows the dose–response relations obtained from 3–4 mRNA-injected oocytes. Apparent IC_{50} values estimated from Figure 6*B* were 225.7 ± 30.9 μ M ($n = 4$) and 11.0 ± 2.8 μ M ($n = 3$) for the oocytes injected with cerebellar (closed circles) and cerebral (open circles) mRNAs, respectively.

CNQX is also known to be an antagonist of the glycine binding site on cerebral NMDA receptors (Birch et al., 1988; Verdoorn et al., 1989). Figure 7*A* exemplifies the dose-dependent suppressive effect of CNQX (1–150 or 300 μ M) on the inward currents induced by NMDA in the oocytes injected with cerebellar (*a*) and cerebral mRNA (*b*). The dose–response curves obtained from 3 mRNA-injected oocytes are illustrated in Figure 7*B*. As shown in Figure 7*B*, CNQX only partially suppressed NMDA responses in cerebellar mRNA-injected oocytes (closed circles, $IC_{50} > 300$ μ M, $n = 3$), although it completely inhibited those in cerebral mRNA-injected oocytes (open circles; $IC_{50} = 7.6 \pm 1.5$ μ M, $n = 3$).

Figure 6. Antagonistic action of 7-Cl-KYN on NMDA responses in the oocytes injected with cerebellar and cerebral mRNAs. *A*, Effect of 1–300 μ M 7-Cl-KYN on the inward currents induced by 300 μ M NMDA in a cerebellar mRNA-injected oocyte (*a*) and the effect of 1–150 μ M 7-Cl-KYN on the inward currents induced by 150 μ M NMDA in a cerebral mRNA-injected oocyte (*b*), both clamped at -60 mV. Calibrations: vertical, 25 nA; horizontal, 1 min. *B*, Dose-response of the blocking action of 7-Cl-KYN (1–300 μ M for cerebellar mRNA-injected oocytes and 1–150 μ M for cerebral mRNA-injected oocytes) on the responses to 300 μ M NMDA of cerebellar mRNA-injected oocytes (closed circles) and to 150 μ M NMDA of cerebral mRNA-injected oocytes (open circles). Mean values ($\pm$ SEM) from 3–4 oocytes are plotted. Ordinate: the mean percentage of the control response in the absence of 7-Cl-KYN [35.4 ± 7.9 nA ($n = 4$) and 48.7 ± 8.3 nA ($n = 3$) for the oocytes injected with cerebellar and cerebral mRNAs, respectively].

Figure 7. Antagonistic action of CNQX on NMDA responses in the oocytes injected with cerebellar and cerebral mRNAs. *A*, Effect of 1–150 or 300 μ M CNQX on the inward currents induced by 300 μ M NMDA in a cerebellar mRNA-injected oocyte (*a*) and the effect of 1–150 μ M CNQX on the inward currents induced by 150 μ M NMDA in a cerebral mRNA-injected oocyte (*b*), both clamped at -60 mV. Calibrations: vertical, 25 nA; horizontal, 1 min. *B*, Dose-response of the blocking action of CNQX (1–300 μ M for cerebellar mRNA-injected oocytes and 1–150 μ M for cerebral mRNA-injected oocytes) on the responses to 300 μ M NMDA of cerebellar mRNA-injected oocytes (closed circles) and to 150 μ M NMDA of cerebral mRNA-injected oocytes (open circles). Mean values ($\pm$ SEM) from 3–4 oocytes are plotted. Ordinate: the mean percentage of the control response in the absence of CNQX [38.0 ± 3.0 nA ($n = 3$) and 26.2 ± 2.5 nA ($n = 3$) for the oocytes injected with cerebellar and cerebral mRNAs, respectively].

Discussion

The *Xenopus laevis* oocyte is now established as an effective expression system for excitatory amino acid receptors (Gundersen et al., 1984a, b; Houamed et al., 1984; Parker et al., 1985; Saito et al., 1987; Sugiyama et al., 1987; Verdoorn et al., 1987, 1989; Kleckner and Dingledine, 1988; Kushner et al., 1988; Sekiguchi et al., 1989).

NMDA receptors expressed in *Xenopus* oocytes by injection of rat cerebral mRNA have been proved to have essentially the same pharmacological and electrophysiological properties as neuronal NMDA receptors in the rat cerebrum (Verdoorn et al., 1987; Kushner et al., 1988). On the basis of this methodological support, we carried out the present study for the purpose of examining the regional difference of NMDA receptors by pharmacologically comparing NMDA responses in oocytes injected with mRNAs from the cerebellum and cerebrum of the guinea pig.

Growing evidence suggests possible differences between NMDA-sensitive receptors in the cerebellum and NMDA receptors in other brain regions. In addition to those mentioned in our introductory remarks, the following findings may also

suggest the difference between the cerebellar NMDA-sensitive receptor and the cerebral NMDA receptor. (1) Rat cerebellar membranes show a low binding capacity to the specific ligand of cerebral NMDA receptors, ^3H -3-(($\pm$)-2-carboxypiperazine-4-yl)propyl-1-phosphonic acid (^3H -CCP) (Cotman et al., 1987). (2) The number of PCP-binding sites is much smaller than that of NMDA receptors in the rat cerebellum (Jarvis et al., 1987; Kushner et al., 1988). (3) NMDA does not act as an agonist but is an antagonist of Asp-induced depolarizations and climbing fiber responses in the guinea pig cerebellar slices (Kimura et al., 1985). (4) DL-APV is not potent enough to abolish the NMDA or Asp response of Purkinje cells *in vitro* (Kimura et al., 1985; Sekiguchi et al., 1987).

On the other hand, as for the similarity to cerebral NMDA receptors, the Mg^{2+} and voltage dependence of the NMDA response was reported for Purkinje cell dendrites in guinea pig cerebellar slices (Sekiguchi et al., 1987) and for intracerebellar nuclei neurons in cerebellar slices from immature rats (Gardette and Crepel, 1986), and the potentiating action of glycine was demonstrated in cultured granule cells from the rat cerebellum (Wroblewski et al., 1989).

In the present study, NMDA responses in the oocytes injected with cerebellar and cerebral mRNAs were found to have the following properties: (1) NMDA-induced inward current in cerebellar mRNA-injected oocytes was not potentiated by glycine, whereas, under exactly the same experimental condition, the NMDA response of cerebral mRNA-injected oocytes was dose-dependently potentiated by glycine (see Fig. 5). (2) NMDA-induced inward current in cerebellar mRNA-injected oocytes was voltage and Mg^{2+} dependent to show a negative slope conductance (see Fig. 3). (3) NMDA-induced inward current in cerebellar mRNA-injected oocytes was strongly blocked by PCP (Fig. 4).

The lack of the glycine effect on the NMDA response of oocytes injected with guinea pig cerebellar mRNA (Fig. 5) contrasts with the absolute requirement of glycine for appreciable NMDA responses of oocytes injected with cerebral mRNAs from the guinea pig (Fig. 5) and the rat (Kleckner and Dingledine, 1988). Before concluding that this lack of the glycine effect reflects the distinct nature of NMDA-sensitive receptors in the cerebellum, at least 2 possibilities must be considered: (1) NMDA activated not only NMDA-sensitive receptors but also glycine-insensitive non-NMDA receptors, and (2) glycine which leaked out of oocytes already potentiated the NMDA response in cerebellar mRNA-injected oocytes.

The first possibility, however, is unlikely because (1) the NMDA response of cerebellar mRNA-injected oocytes was highly sensitive to D-APV (Fig. 1), Mg^{2+} (Fig. 2), and PCP (Fig. 4), and was dependent upon membrane potential (Fig. 3), while smooth QA responses were not sensitive to D-APV (Fig. 1) or Mg^{2+} (Fig. 2); (2) 1 μM PCP suppressed the depolarizations induced by 50 μM NMDA to 13% of control and that by 50 μM QA only to 71%; (3) 10 μM DGG depressed the NMDA response to 45% and the QA response to 74%; and (4) 30 μM GDEE suppressed the NMDA response to 98% and the QA response to 51%. (Findings 2–4 were from our separate experiments using intracellular recordings of cerebellar mRNA-injected oocytes.)

The second possibility also seems to be unlikely, although no direct evidence can be adduced at present. Our findings against this possibility may be that 7-Cl-KYN, which is said to be a specific antagonist of the glycine binding site on cerebral NMDA receptors (Donald et al., 1988; Kemp et al., 1988), was significantly less effective on the NMDA response of cerebellar mRNA-injected oocytes than that of cerebral mRNA-injected oocytes (Fig. 6), and CNQX, which is reported to be the mixed competitive–noncompetitive antagonist of the glycine binding site (Birch et al., 1988; Verdoorn et al., 1989), was significantly less potent in cerebellar mRNA-injected oocytes than in cerebral mRNA-injected oocytes (Fig. 7). In addition, there is neither a compelling reason nor experimental support to postulate that an effective amount of glycine leaks out only from cerebellar mRNA-injected oocytes but not from cerebral mRNA-injected oocytes. The existence of a diffusion barrier against added glycine only in cerebellar mRNA-injected oocytes is also unlikely because both cerebellar mRNA-injected and cerebral mRNA-injected oocytes were similarly defolliculated by collagenase treatment.

On this basis, it may be at least suggested that the NMDA-sensitive receptor expressed in oocytes by injection of guinea pig cerebellar mRNA is much less sensitive to glycine than the cerebral NMDA receptor similarly expressed in oocytes.

If the present findings were to suggest the absence of the glycine binding site on the cerebellar NMDA-sensitive receptor,

one may expect a heterogeneity in NMDA receptors similar to that of the GABA_A receptor (Levitan et al., 1988) and the glycine receptor (Akagi and Miledi, 1988). However, as demonstrated by Monaghan et al. (1988), glycine potentiates the agonist binding and reduces the antagonist binding to NMDA receptors in the rat brain. These authors further proposed the hypothesis that glycine causes a conversion of NMDA receptors from an antagonist-preferring conformation (relatively unresponsive to Glu) to an agonist-preferring conformation (highly responsive to Glu). If this were the case, glycine-insensitive, NMDA-sensitive receptors in the cerebellum would be in a state similar to the agonist-preferring conformation regardless of the presence or absence of glycine.

According to Bristow et al. (1986) and Wroblewski et al. (1989), strychnine-insensitive glycine binding sites are present in the granule cell layer of the rat cerebellum. Whether these glycine binding sites are expressed in *Xenopus* oocytes, however, is unclear because a potentiating action of glycine was not observed in the present study.

As demonstrated in this study, the *Xenopus* oocyte system is suitable for exploration of receptor heterogeneity due to regional differences and will be useful for ontogenic and developmental investigations of receptors.

In conclusion, the NMDA-sensitive receptor expressed in *Xenopus* oocytes by injection of mRNA extracted from guinea pig cerebellum resembles the cerebral NMDA receptor in voltage-dependent sensitivity to Mg^{2+} and high susceptibilities to D-APV and PCP, but it is distinct from the cerebral NMDA receptor in responsiveness to glycine. The physiological role of this NMDA-sensitive receptor in synaptic transmission, including the long-term depression (Ito, 1984), in the cerebellum and its anatomical and functional relations to cerebellar non-NMDA receptors are matters of interest for further investigations.

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