0026-895X/94/010161-8$03.00/0

MOLECULAR PHARMACOLOGY, 46:161-168, 1994.

Mechanisms Influencing Stimulatory Effects of Spermine at Recombinant *N*-Methyl-D-Aspartate Receptors

KEITH WILLIAMS

Department of Pharmacology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104-6084

Received February 21, 1994; Accepted April 15, 1994

SUMMARY

Stimulatory effects of spermine at heteromeric *N*-methyl-D-aspartate (NMDA) receptors expressed from cloned subunits were studied by voltage-clamp recording in *Xenopus* oocytes. At NR1A/NR2B receptors, in the presence of a saturating concentration of glycine, the magnitude of spermine stimulation was dependent on the concentration of NMDA or glutamate. In oocytes voltage-clamped at −25 mV, spermine markedly enhanced the response to 100 μM NMDA but had little or no effect on the response to 10 μM NMDA. This effect was not related to the size of the macroscopic currents, the quantity or ratio of injected receptor subunit RNAs, or a voltage-dependent block by spermine. Spermine induced a small (1.2–1.5-fold) decrease in the affinity of NR1A/NR2B receptors for NMDA and glutamate. This decrease was sufficient to counteract the stimulatory effect of spermine at low concentrations of NMDA and glutamate, resulting in no net effect of spermine or a decrease in macroscopic currents in the presence of spermine with low concentrations of agonist. Spermine did not alter the affinity of NR1A/NR2A receptors for NMDA. Endogenous polyamines could act as a bidirectional gain control at some native NMDA receptors containing the NR1A and NR2B subunits, by dampening the response to low concentrations of glutamate and enhancing the response to high concentrations of glutamate. Alternatively, polyamines could enhance the decay of NMDA receptor-mediated responses by increasing the rate of dissociation of glutamate from the receptor.

The endogenous polyamine spermine has multiple effects on the NMDA subtypes of glutamate receptor. These include so-called "glycine-independent" and "glycine-dependent" stimulation and voltage-dependent inhibition (1–5). Glycine-independent stimulation, which is seen in the presence of saturating concentrations of glycine, involves an increase in the size of whole-cell currents, possibly due to an increase in the channel opening frequency of NMDA receptors (1, 4, 5). Glycine-dependent stimulation involves a small (2–3-fold) increase in the affinity of the NMDA receptor for glycine and is seen in the presence of subsaturating concentrations of glycine (4–6). The inhibitory effect of spermine is voltage dependent, being more pronounced at hyperpolarized membrane potentials, and may represent an open-channel block of the NMDA receptor and/or screening of surface charges (1, 2, 4, 5). The stimulatory and inhibitory effects of spermine measured in electrophysiological studies may correspond to the stimulation and inhibition, respectively, of binding of open-channel blockers and the increase in affinity for glycine seen in radioligand binding assays (7–11).

At native NMDA receptors expressed on cultured neurons, there is considerable variability in the degree of stimulation by spermine on individual neurons (1–4). This may be due to the expression of multiple forms of the NMDA receptor composed of different combinations of subunits. Molecular cloning has identified two families of NMDA receptor subunits. The NR1 (NMDAR1) family consists of eight splice variants of the NR1 gene (12–15), seven of which were originally termed NMDA R1A-R1G (14). The NR2 family consists of NR2A, NR2B, NR2C, and NR2D cDNAs isolated from rat brain (16, 17). Equivalent cDNAs, termed ζ1 (NR1) and ε1–ε4 (NR2A–D), have been cloned from mouse brain (18–21). NR1 but not NR2 subunits can form functional homomeric NMDA receptors when expressed in *Xenopus* oocytes, but macroscopic currents activated by these receptors are very small (12). Much larger responses are seen with receptors coexpressed from combinations of NR1 and NR2 subunits (16, 17, 19, 20). NR2A and NR2B are the major NR2 subunits expressed in adult rodent hippocampus and cerebral cortex (16, 17, 22, 23). The properties of recombinant NR1A/NR2A and NR1A/NR2B receptors, as defined by their affinity for the atypical antagonist ifenprodil, are similar to those of the two major subtypes of NMDA receptor identified in adult rat forebrain and in cultured neocortical neurons (24, 25). In addition, the single-channel conductances and open- and closed-time distributions of NR1/NR2A and NR1/NR2B receptor channels are very similar to

This work was supported by United States Public Health Service Grants NS30000 and GM34781.

ABBREVIATIONS: NMDA, *N*-methyl-D-aspartate; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

those of native NMDA receptors on hippocampal neurons (26). Thus, heteromers containing NR1/NR2A and NR1/NR2B subunits may be the predominant forms of the NMDA receptor in the cerebral cortex and hippocampus.

Homomeric NR1 receptors expressed from splice variants, such as NR1A, that do not contain a 5′ insert exhibit glycine-independent stimulation by spermine. In contrast, homomeric NR1 receptors expressed from variants containing a 5′ insert do not show glycine-independent stimulation by spermine (27). Recently, it was found that heteromeric NMDA receptors expressed from NR1A plus NR2B subunits, but not receptors expressed from NR1A plus NR2A subunits, show glycine-independent stimulation by spermine (5). Voltage-dependent inhibition is seen at both types of receptor (5). Thus, inclusion of an NR1 variant such as NR1A in a heteromeric receptor is necessary for glycine-independent stimulation by polyamines, but the manifestation of this stimulatory effect is controlled by the type of NR2 subunit present in the receptor complex.

In the present work, the effects of spermine on heteromeric NMDA receptors of defined subunit composition have been studied. It was found that the stimulatory effect of spermine is markedly dependent on the concentration of NMDA used to activate NR1A/NR2B receptors. At high concentrations of NMDA spermine can enhance the response of these receptors by 50–100%, but this effect is masked at low concentrations of NMDA, probably due to a decrease in the affinity of the receptor for NMDA. These observations have implications for understanding the effects of spermine on native NMDA receptors studied on cultured neurons and for determining a possible role of endogenous polyamines at NMDA receptors at intact synapses.

Materials and Methods

Oocyte injection and recording. Defolliculated stage V-VI oocytes were prepared from *Xenopus laevis* as described previously (25). Capped cRNAs were synthesized from linearized cDNA templates containing the NR1A, NR2A, and NR2B clones, using a commercially available *in vitro* transcription kit (mMessage mMachine; Ambion Inc., Austin, TX) with appropriate RNA polymerases. Most of the 5′ untranslated region was removed from the NR2 clones, as described previously (see Ref. 24), before synthesis of cRNAs. This resulted in better expression of heteromeric channels, as assessed by the size of macroscopic NMDA-induced currents (24). In most experiments oocytes were injected with NR1A plus NR2 cRNAs in a ratio of 1:5 (2 or 4 ng of NR1A plus 10 or 20 ng of NR2A or NR2B). In some experiments the ratio or quantity of injected RNAs was varied as described in Results. After injection, oocytes were maintained in a saline solution (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5 mM Na-HEPES, 2.5 mM sodium pyruvate, 50 μg/ml gentamycin, pH 7.5) at 18° for 1–5 days before recording. The saline solution was replaced daily.

Macroscopic currents were recorded with a two-electrode voltage-clamp using an OC-725 amplifier (Warner Instruments, Hamden, CT), as described previously (24). Electrodes were filled with 3 M KCl and had resistances of 0.5–1 MΩ (current-injecting electrode) or 1–6 MΩ (voltage-sensing electrode). Oocytes were continuously superfused (≈10 ml/min) with a Mg^{2+}-free saline solution (96 mM NaCl, 2 mM KCl, 1.8 mM $BaCl_2$, 10 mM Na-HEPES, pH 7.5), and NMDA, glutamate, and spermine were applied in the same solution. NMDA and glutamate were applied in solutions containing 10 μM glycine. The extracellular solution contained $BaCl_2$ rather than $CaCl_2$, and oocytes were injected with BAPTA (50–100 nl of 40 mM K-BAPTA, pH 7.5) on the day of recording to eliminate Ca^{2+}-activated Cl^- currents (24, 28, 29). In some oocytes, NMDA induced very large inward currents (>1 μA), particularly when oocytes were used at 3–5 days after injection of RNAs. In these oocytes steady state responses to NMDA were not obtained and a slowly rising phase of the inward current was seen, presumably because the concentration of BAPTA was insufficient to prevent activation of secondary Cl^- currents by Ca^{2+} released from intracellular stores (see Refs. 24 and 29). Such oocytes were not used to study the effects of spermine.

Data acquisition and analysis were carried out using an MP-100 interface with AcqKnowledge software (Biopac Systems, Goletta, CA) on a Macintosh computer (24). I-V curves were constructed by ramping the command potential from −100 to +40 mV over a period of 12 sec during steady state responses induced by NMDA or NMDA plus spermine at −100 mV, as described previously (5). This slow ramp protocol allows the detection of both the stimulatory and inhibitory effects of spermine (5). Leak currents, measured in ramps before and after test ramps, were digitally subtracted.

Data from concentration-response curves were fit to the logistic function

$$I = I_{max}/1 + ([\text{agonist}]/EC_{50})^{n_H}$$

where I is the response to NMDA or glutamate, I_{max} is the maximum response, [agonist] is the concentration of NMDA or glutamate, and n_H is the Hill slope. Values for I_{max}, EC_{50}, and n_H were derived using this procedure.

Materials. NMDA was purchased from Research Biochemicals Inc. (Natick, MA). Glycine and L-glutamate were purchased from Sigma Chemical Co. (St. Louis, MO). Spermine tetrahydrochloride was purchased from Aldrich Chemical Co. (Milwaukee, WI). The plasmid pN60 containing the rat NR1A clone (originally termed NMDAR1 or NMDA R1A) (12, 14) was a gift from Dr. S. Nakanishi (Kyoto University, Kyoto, Japan). The NR2A and NR2B cDNA clones (16) were a gift from Dr. P. H. Seeburg (University of Heidelberg, Heidelberg, Germany).

Results

Dependence of stimulation by spermine on the concentration of NMDA. Spermine has both stimulatory and inhibitory effects at NR1A/NR2B receptors expressed in oocytes (5). To study the stimulatory effects of spermine at NR1A/NR2B receptors, oocytes were voltage-clamped at −25 mV to minimize the voltage-dependent inhibition, which, at a concentration of 100 μM spermine, is negligible or absent at this holding potential (Ref. 5 and see below). Responses were measured in the presence of a saturating concentration (10 μM) of glycine, which allows the detection of glycine-independent but not glycine-dependent spermine stimulation. Previously, it was found that spermine (100 μM) enhanced the response to 100 μM NMDA at NR1A/NR2B receptors (5). Subsequently, the surprising discovery was made that spermine had virtually no effect on macroscopic currents induced by 10 μM NMDA at these receptors (Fig. 1A).

In the experiments shown throughout this paper, responses to NMDA were measured in the presence of extracellular Ba^{2+} (substituted for Ca^{2+}) in oocytes injected with BAPTA. These recording conditions eliminate Ca^{2+}-activated Cl^- currents, which otherwise may interfere with quantitative measurements of NMDA responses and effects of modulators such as spermine (24, 28). A dependence of spermine stimulation on the concentration of NMDA was also seen with oocytes that were not injected with BAPTA and with an extracellular solution containing $CaCl_2$ (data not shown). Thus, the differential effect of spermine on responses to 10 and 100 μM NMDA is not related to the absence of extracellular Ca^{2+} or the chelation of intracellular Ca^{2+} by BAPTA.

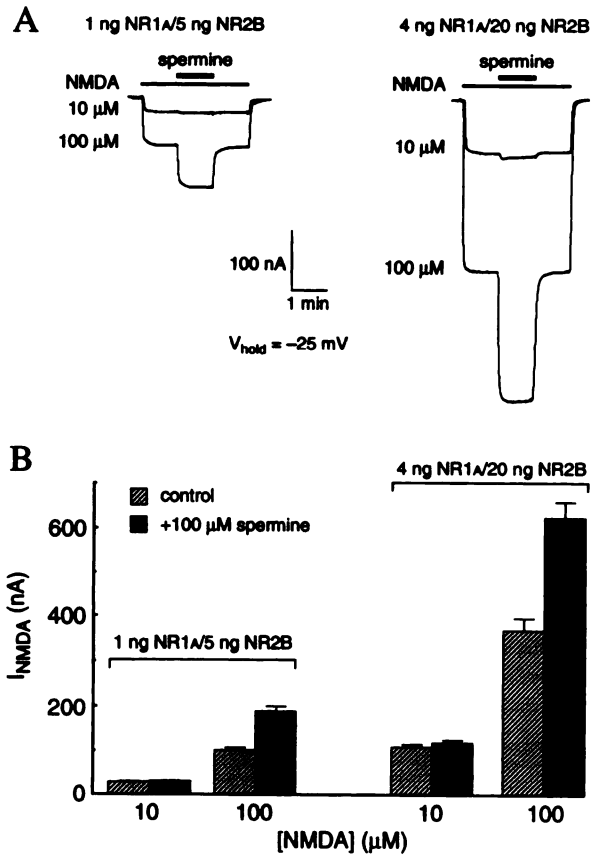

Fig. 1. Effects of spermine at NR1A/NR2B receptors activated by different concentrations of NMDA. A, Superimposed recordings of inward currents induced by 10 and 100 μM NMDA (with 10 μM glycine) in the absence and presence of 100 μM spermine. Oocytes were injected with 1 plus 5 ng (*left*) or 4 plus 20 ng (*right*) of NR1A plus NR2B cRNAs. B, Currents induced by 10 and 100 μM NMDA, measured using the protocol described for A. Values are mean ± standard error from seven oocytes for each combination of subunits. Oocytes were voltage-clamped at −25 mV.

A number of factors could be responsible for the differential effects of spermine seen with high and low concentrations of NMDA at NR1A/NR2B receptors. These include a dependence on the absolute size of NMDA-induced responses, possibly involving activation of secondary currents, the expression of mixed populations of NMDA receptors, differences in the inhibitory effects of spermine, or a change in the affinity of the receptor for NMDA in the presence of spermine. These potential mechanisms are considered below.

Response size, receptor number, and subunit stochiometry. One possibility that may account for the differential effects of spermine on responses to 10 and 100 μM NMDA is that the effect of spermine is dependent on the actual size of the macroscopic current, rather than the concentration of NMDA. To test this possibility, the effects of spermine on responses to 10 and 100 μM NMDA were studied in oocytes injected with different amounts of NR1A plus NR2B cRNAs (1 plus 5 ng or 4 plus 20 ng) in a constant ratio (NR1A:NR2B, 1:5). In oocytes injected with 1 plus 5 ng of RNA, control responses to 100 μM NMDA (100 ± 7 nA, $n = 7$) were very similar to those seen with 10 μM NMDA in oocytes injected with 4 plus 20 ng of RNA (109 ± 6 nA, $n = 7$) (Fig. 1). However, in oocytes injected with small (1 plus 5 ng) or large (4 plus 20 ng) amounts of RNA, spermine enhanced the response to 100 μM NMDA but had little or no effect on the response to 10 μM NMDA (Fig. 1). In oocytes injected with 1 plus 5 ng of RNA, spermine potentiated responses by 6 ± 2% (10 μM NMDA) and by 87 ± 2% (100 μM NMDA). In oocytes injected with 4 plus 20 ng of RNA, spermine potentiation was 10 ± 3% (10 μM NMDA) and 71 ± 4% (100 μM NMDA). Thus, the stimulatory effect of spermine is not dependent on the size of the macroscopic current or on the number of expressed receptors; rather, it is dependent on the concentration of NMDA.

Another possibility that could account for the observed effects of spermine and NMDA is that, after injection with NR1A plus NR2B cRNAs, oocytes express mixed populations of NMDA receptors, with a subpopulation being sensitive to spermine and high concentrations of NMDA. The NR2B subunit does not form functional homomeric receptors (16, 17, 20, 24). Under the recording conditions used in the present study, responses of homomeric NR1A receptors in oocytes injected with 4 ng of NR1A cRNA were very small (1–3 nA) or undetectable (data not shown). Thus, expression of homomeric NR1A or NR2B receptors does not contribute to the NMDA current measured in oocytes injected with NR1A plus NR2B subunits and cannot account for the NMDA-dependent effects of spermine. Another possibility is that oocytes injected with NR1A plus NR2B subunits express mixed populations of receptors having different NR1A:NR2B stochiometries. To test this hypothesis, oocytes were injected with NR1A plus NR2B subunits in different ratios (1:1, 1:5, and 1:10). The effects of spermine were not different in oocytes injected with NR1A:NR2B subunits at different ratios, suggesting that coexpression of NR1A/NR2B receptors having different subunit stochiometries does not underlie the differential effects of spermine on responses to 10 and 100 μM NMDA.

Voltage dependence and inhibitory effects of spermine. Spermine has both stimulatory and inhibitory effects at NR1A/NR2B receptors (5). Inhibition is strongly voltage dependent, being more pronounced at hyperpolarized membrane potentials. Experiments were carried out with voltage ramps to examine stimulatory and inhibitory effects of spermine at NR1A/NR2B receptors, using 10 and 100 μM NMDA to activate the receptors (Fig. 2). Control responses to 100 μM NMDA were 3–4-fold larger than responses to 10 μM NMDA (Fig. 2, A and B), but the I-V curves had the same shape and the same reversal potential at each concentration of NMDA (Fig. 2C). Spermine had dual effects on the response to 100 μM NMDA, i.e., a net inhibition of the NMDA response at membrane potentials more negative than −70 mV and a net stimulation at more positive potentials (Fig. 2B). This profile presumably reflects the sum of a voltage-independent stimulatory effect of spermine and a separate, but overlapping, voltage-dependent block (5). In contrast to effects seen with 100 μM NMDA, spermine inhibited the response to 10 μM NMDA, with inhibition occurring only at membrane potentials more negative than approximately −30 mV, but stimulation by spermine was not seen even at a membrane potential of +40 mV (Fig. 2A). Thus, a difference in the inhibitory effect of spermine cannot account for the lack of stimulation by spermine when 10 μM NMDA is used as the agonist. Activation of secondary currents or changes in the voltage dependence of spermine block are not responsible for the differential effects of spermine on responses to 10 and 100 μM NMDA.

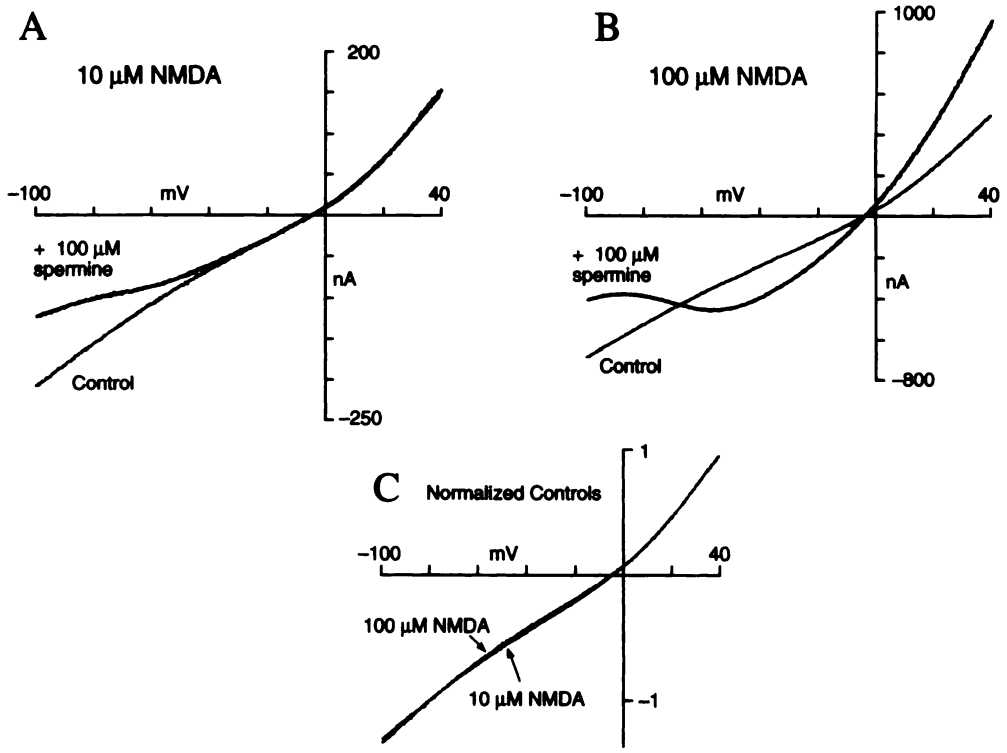

Fig. 2. I-V relationships for effects of spermine at NR1A/NR2B receptors. A and B, I-V curves for 10 μM NMDA (A) and 100 μM NMDA (B) were measured by voltage ramps in the absence (control) and presence of 100 μM spermine, in the same oocyte. Note the different scales on the *abscissae* in A and B. Leak currents have been subtracted. C, Control I-V curves for 10 and 100 μM NMDA have been normalized to the current measured at −80 mV.

Concentration-response relationships for NMDA. The difference in the magnitude of spermine stimulation seen with 10 and 100 μM NMDA could be due to a decrease in the affinity of the NMDA receptor for NMDA. If this decrease in affinity balances the stimulatory effect of spermine at a concentration of 10 μM NMDA, the net result would be no change in the size of the macroscopic current in the presence of spermine. To test this hypothesis, concentration-response curves for NMDA were measured in the absence and presence of spermine. The effects of 100 μM spermine on responses to 10 concentrations of NMDA (3–300 μM) were determined using the protocol shown in Fig. 3A. At concentrations of NMDA below 10 μM, spermine had little or no effect on the response to NMDA (Fig. 3, A, B, and D). A progressive increase in the magnitude of spermine stimulation was seen at concentrations of 15–300 μM NMDA (Fig. 3, B and D). Spermine increased the EC_{50} for NMDA from 20 μM to 30 μM, resulting in a small rightward shift of the concentration-response curve (Fig. 3, B and C).

These results are consistent with the hypothesis that spermine decreases the affinity of NR1A/NR2B receptors for NMDA, masking the stimulatory effect of spermine at low concentrations of NMDA. However, if spermine does not alter the affinity for NMDA but the magnitude of spermine stimulation is dependent on the concentration of NMDA, then an *apparent* decrease in affinity for NMDA would result. A computer simulation of the NMDA concentration-response curve was generated to determine whether the small (1.5-fold) change in affinity for NMDA is sufficient to account for the observed effects of spermine at different concentrations of NMDA. In this model it was assumed that the stimulatory effect of spermine is not dependent on the concentration of NMDA and that the apparent concentration dependence results from a concomitant decrease in the affinity of the receptor for NMDA (Fig. 4). Thus, a theoretical concentration-response curve was generated in which the response to NMDA was increased by 86% (the mean increase seen at 300 μM NMDA) at all concentrations of NMDA, accompanied by a 1.5-fold decrease in the affinity for NMDA (Fig. 4). The theoretical curve generated with this model (Fig. 4A) is virtually identical to that measured experimentally in the presence of spermine, and changes in the magnitude of NMDA currents calculated from this curve (Fig. 4B) match those seen in experiments with spermine. Thus, a decrease in the affinity for NMDA is sufficient to account for the NMDA-dependent effects of spermine.

To determine the specificity of the interactions between spermine and NMDA, effects of spermine on responses to L-glutamate, which is likely to be the endogenous agonist at NMDA receptors, were also examined. Similar to effects seen with NMDA, the degree of stimulation by spermine was dependent on the concentration of glutamate (Fig. 5). At concentrations of glutamate above 1 μM, spermine enhanced the response to glutamate, with maximal effects occurring at 10–30 μM glutamate (Fig. 5). At concentrations of glutamate below 1 μM, a small decrease in the response to glutamate was seen in the presence of spermine (Fig. 5, *inset*). Spermine increased the EC_{50} for glutamate from 2.1 to 2.5 μM (Fig. 5). These results are consistent with a decrease in the affinity of NR1A/NR2B receptors for glutamate that attenuates the response and masks the stimulatory effect of spermine at low concentrations of glutamate.

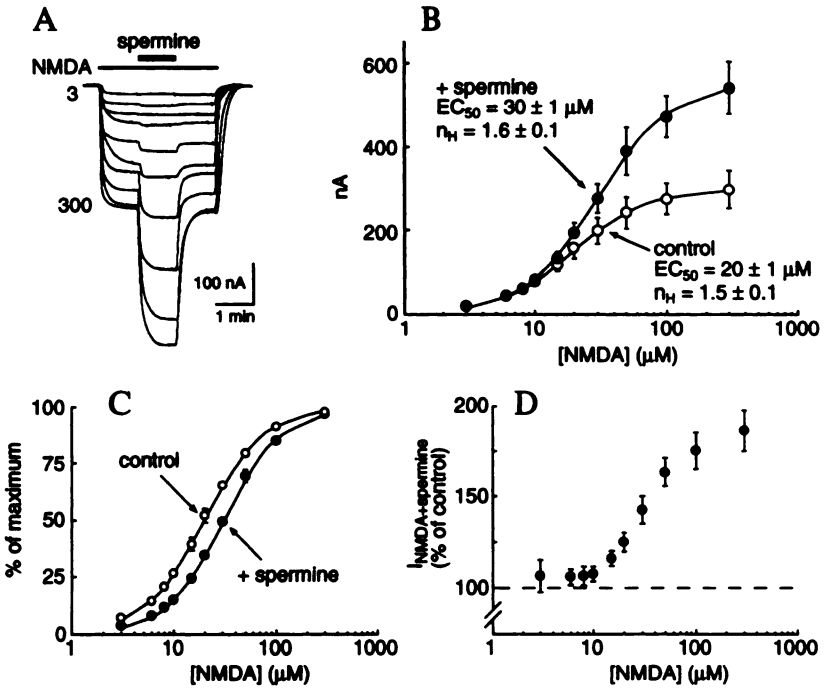

Fig. 3. Concentration-response analysis for NMDA at NR1A/NR2B receptors. A, Traces of inward currents induced by various concentrations of NMDA (3–300 μM; all with 10 μM glycine) in the absence and presence of 100 μM spermine, in an oocyte expressing NR1A/NR2B receptors and voltage-clamped at −25 mV, are superimposed. B, Concentration-response curves for NMDA were measured using the protocol described for A; values are mean ± standard error from five oocytes. C, Data are expressed as a percentage of the maximum response in the absence and presence of spermine. D, Responses measured in the presence of spermine are expressed as a percentage of the control response at each concentration of NMDA. The *smooth curves* in B and C were derived by fitting the data to a logistic function, as described in Materials and Methods.

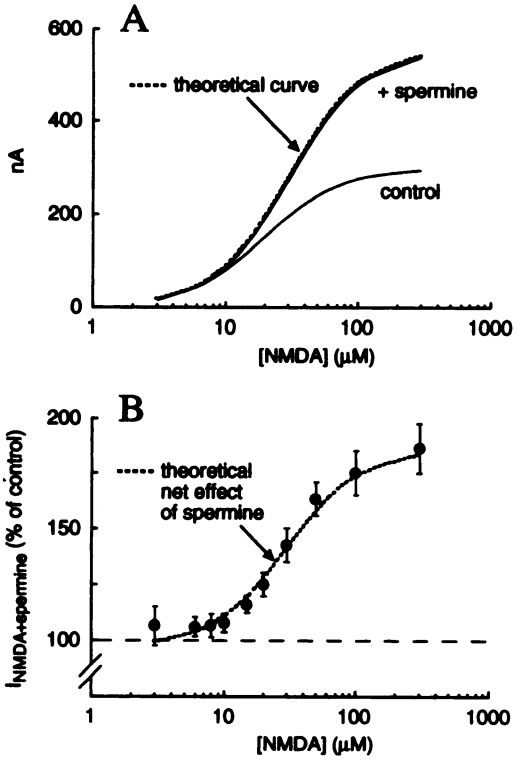

Fig. 4. Theoretical effect of spermine on responses to NMDA. A, *Solid lines*, fitted curves for NMDA-induced currents measured in the absence (control) and presence of spermine, replotted from Fig. 3; *dashed line*, theoretical curve in which the affinity of the control curve is decreased by 1.5-fold together with an 86% increase in the magnitude of the NMDA current at all concentrations of NMDA. The *curves* were derived from a logistic function, as described in Materials and Methods and Results. B, Theoretical curve from A replotted as a percentage of the control response, together with the experimental data for spermine stimulation, which are replotted from Fig. 3.

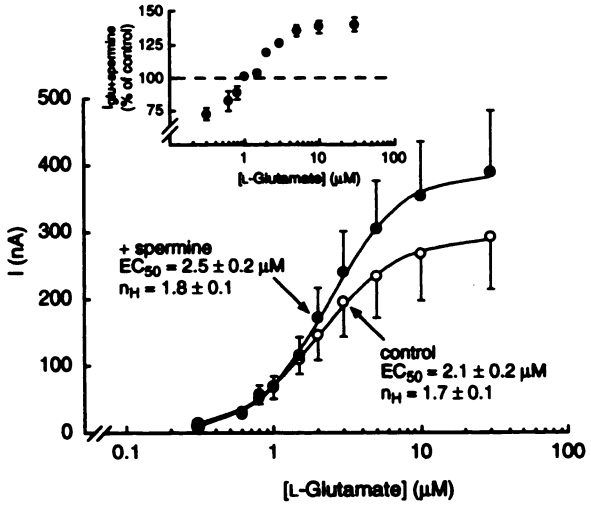

Fig. 5. Concentration-response analysis for glutamate at NR1A/NR2B receptors. Responses to various concentrations of L-glutamate (all with 10 μM glycine) were measured in the absence (control) and presence of 100 μM spermine, in oocytes expressing NR1A/NR2B receptors and voltage-clamped at −25 mV. The *smooth curves* were derived by fitting the data to a logistic function as described in Materials and Methods. *Inset*, responses measured in the presence of spermine are expressed as a percentage of the control response at each concentration of glutamate. Values are mean ± standard error from six oocytes.

Concentration-response relationships at NR1A/NR2A receptors. Experiments were carried out to determine whether spermine also decreases the affinity of NR1A/NR2A receptors for NMDA. In oocytes voltage-clamped at −25 mV, spermine produced a small (10–20%) decrease in the response to all concentrations of NMDA (Fig. 6). This is presumably due to a voltage-dependent block that, with 100 μM spermine, is not fully relieved at NR1A/NR2A receptors at a membrane potential of −25 mV (see Ref. 5). However, in contrast to effects seen at NR1A/NR2B receptors, spermine did not alter the affinity of NR1A/NR2A receptors for NMDA (Fig. 6).

Receptors expressed from combinations of NR1A,

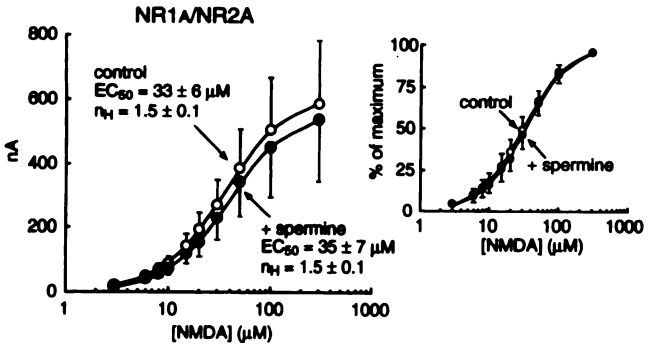

Fig. 6. Concentration-response analysis for NMDA at NR1A/NR2A receptors. Responses to various concentrations of NMDA (3–300 μM; all with 10 μM glycine) were measured in the absence (control) and presence of 100 μM spermine, in oocytes expressing NR1A/NR2A receptors and voltage-clamped at −25 mV. *Inset*, data are expressed as a percentage of the maximum response in the absence and presence of spermine. Values are mean ± standard error from four oocytes.

NR2A, and NR2B subunits. Stimulatory effects of spermine at heteromeric NMDA receptors are controlled by the inclusion of different NR2 subunits. Inclusion of NR2B in NR1A/NR2B receptors allows the expression of glycine-independent stimulation, which is also seen with homomeric NR1A receptors, whereas this effect is abolished by the inclusion of NR2A in NR1A/NR2A receptors (5). Coexpression of NR1A, NR2A, and NR2B subunits in oocytes may be expected to lead to the expression of mixed populations of NR1A/NR2A and NR1A/NR2B heteromers. Alternatively, coexpression of these three subunits may result in the generation of a unique heteromeric complex containing a combination of NR1A, NR2A, and NR2B subunits, with either the NR2A or NR2B subunit controlling the effects of spermine. To examine these possibilities, the effects of spermine were studied in oocytes injected with NR1A plus NR2A (4 plus 10 ng), NR1A plus NR2B (4 plus 10 ng), or NR1A plus NR2A plus NR2B (4 plus 10 plus 10 ng) subunit RNAs and voltage-clamped at −25 mV (Fig. 7). Control responses in all three groups of oocytes were similar. NMDA at 10 μM produced currents of 68 ± 14 nA, 63 ± 8 nA, and 76 ± 15 nA and 100 μM NMDA produced currents of 316 ± 60 nA, 240 ± 34, nA, and 297 ± 50 nA (mean ± standard error, $n = 7$) in oocytes injected with NR1A plus NR2A, NR1A plus NR2B, and NR1A plus NR2A plus NR2B subunits, respectively. At NR1A/NR2A receptors, spermine produced a small inhibition of the response to 10 and 100 μM NMDA (Fig. 7). At NR1A/NR2B receptors, spermine had no effect on the response to 10 μM NMDA but markedly enhanced the response to 100 μM NMDA. In oocytes injected with NR1A plus NR2A plus NR2B subunits, the effects of spermine were intermediate between those seen at NR1A/NR2A and NR1A/NR2B receptors (Fig. 7). With 100 μM NMDA, currents measured in the presence of spermine were 89 ± 1% of control (NR1A plus NR2A), 169 ± 11% of control (NR1A plus NR2B), and 137 ± 2% of control (NR1A plus NR2A plus NR2B). The results shown in Fig. 7 are consistent with the coexpression of NR1A/NR2A and NR1A/NR2B receptors in oocytes injected with NR1A plus NR2A plus NR2B subunits, and they provide no evidence for the expression of a unique heteromeric receptor complex.

Discussion

A main finding from the present work was that the stimulatory effect of spermine at NR1A/NR2B receptors is dependent

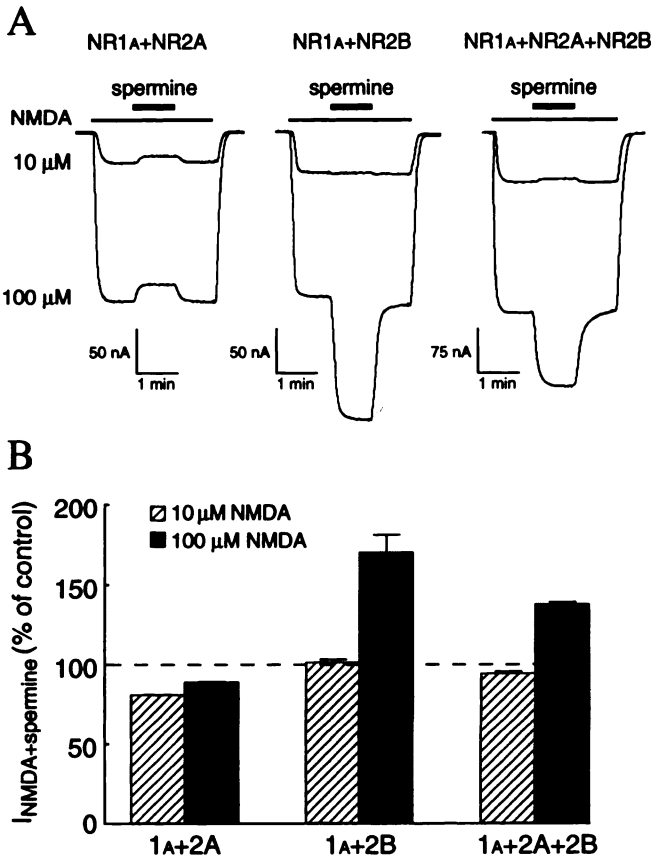

Fig. 7. Receptors expressed from combinations of NR1 and NR2 subunits. A, Inward currents induced by 10 and 100 μM NMDA (with 10 μM glycine) in the absence and presence of 100 μM spermine, in oocytes injected with NR1A plus NR2A (*left*), NR1A plus NR2B (*center*), or NR1A plus NR2A plus NR2B (*right*) subunit RNAs. Oocytes were injected with 4 ng of NR1A cRNA and 10 ng of NR2 cRNAs and were voltage-clamped at −25 mV. B, Responses to 10 and 100 μM NMDA measured in the presence of spermine, expressed as a percentage of the control response to each concentration of NMDA. Values are mean ± standard error from seven oocytes for each combination of subunits.

on the concentration of NMDA or glutamate used to activate the receptors. This effect is not related to the size of the macroscopic currents, the number of expressed receptors, the subunit stochiometry, or the voltage-dependent block by spermine. The dependence of spermine stimulation on the concentration of NMDA probably arises through a small decrease in the affinity of the receptor for NMDA in the presence of spermine. At low concentrations of NMDA or glutamate, the decrease in agonist affinity can counteract the stimulatory effect of spermine on macroscopic currents. Spermine is a tetra-amine with no anionic groups and lacks the structural features associated with competitive antagonists acting at the glutamate/NMDA binding site (30). The effect of spermine on the affinity for NMDA is, therefore, unlikely to be due to a competitive interaction at this site and probably involves allosteric modulation of the NMDA binding site. An unequivocal determination of whether spermine decreases the affinity for NMDA and glutamate, and of the mechanisms underlying this effect, will require measurements of the binding and unbinding rates of agonists in the presence of spermine.

A decrease in the affinity of the NMDA receptor for glutamate in the presence of spermine is consistent with results of radioligand binding studies in which spermine, spermidine, and

related polyamines were found to decrease the affinity for glutamate and to increase the affinity for glutamate site antagonists (31–34). The decrease in affinity for glutamate seen in binding assays was, like that observed at NR1A/NR2B receptors, very small (1.3-fold) (32), and until now its significance was unclear. The present work has shown that a small change in affinity for glutamate is sufficient to negate the stimulatory effect of 100 μM spermine at low concentrations of glutamate.

In contrast to its effect at NR1A/NR2B receptors, spermine did not alter the affinity of NR1A/NR2A receptors for NMDA. Because spermine stimulation is seen at NR1A/NR2B but not NR1A/NR2A receptors, it is possible that the effect of spermine on the affinity for NMDA is mediated via the same polyamine binding site responsible for spermine stimulation. It is hypothesized that this site is present on heteromeric NR1A/NR2B receptors and on homomeric NR1A receptors (5, 27) but is not present or is not functional on heteromeric NR1A/NR2A receptors (Fig. 8). Furthermore, this polyamine binding site is probably distinct from the site that mediates voltage-dependent inhibition and from the site that produces an increase in affinity for glycine, because these effects are seen at both NR1A/NR2A and NR1A/NR2B receptors (Fig. 8).

Spermine is present in high concentrations in the brain (35) and can be released from neuronal tissues by depolarization (36–38). Polyamines could play a role as neuromodulators in the brain, but there is as yet no direct evidence of a role for polyamines in synaptic transmission. The NMDA receptor may represent the first identifiable target for polyamines at excitatory synapses. The results of the present work and those of previous studies (1–6) indicate that spermine has four mechanisms of action at the NMDA receptor, i.e., glycine-independent stimulation, voltage-dependent block, an increase in the affinity for glycine (glycine-dependent stimulation), and a decrease in the affinity for NMDA and glutamate (Fig. 8). The net effect of spermine on NMDA receptors thus depends on the concentrations of glutamate, glycine, and spermine and the membrane potential, as well as the types of subunits present in the receptor complex (Fig. 8). Given these variables, it is difficult to predict the effects of polyamines on NMDA receptors at intact synapses. However, we have previously shown that the voltage-dependent blocking effect of 100 μM spermine is masked in the presence of physiological concentrations of Mg^{2+} (5). Assuming that glycine is present at saturating concentrations in the synapse (39), the predominant effects of spermine may involve glycine-independent stimulation and a decrease in the affinity for glutamate. Thus, at receptors containing the NR2B subunit, high micromolar concentrations of spermine would attenuate responses to low concentrations of glutamate and potentiate responses to high concentrations of glutamate. Synaptically released polyamines could act as a bidirectional gain control, dampening low levels of synaptic activity and boosting high levels of activity if there is wide variability in the concentration of glutamate in the synaptic cleft.

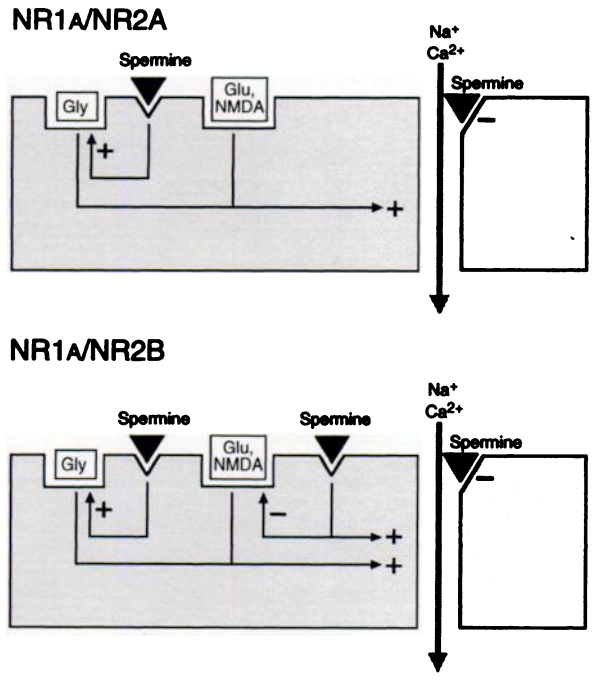

Fig. 8. Schematic models of recombinant NMDA receptors, illustrating the effects of spermine. At both NR1A/NR2A and NR1A/NR2B receptors, spermine acts as a voltage-dependent blocker and increases the affinity for glycine (*Gly*), probably acting at two distinct polyamine binding sites. At NR1A/NR2B receptors, spermine also acts at a third binding site to produce glycine-independent stimulation and to decrease the affinity of the receptor for NMDA and glutamate (*Glu*).

It is possible that during synaptic transmission the concentration of glutamate in the synaptic cleft is relatively high (≈1 mM) (40). In that case, an effect of spermine on the affinity of the receptor for glutamate would not alter receptor activation, because glutamate would be present at saturating concentrations. However, if the concentration of glutamate declines very rapidly, then the duration of postsynaptic currents is controlled by the kinetic and activation properties of postsynaptic glutamate receptors (40–42). Furthermore, the decay of the NMDA receptor-mediated excitatory postsynaptic current is controlled by the slow unbinding of glutamate from NMDA receptors (43). Thus, under conditions where glutamate is present at high concentrations for a very brief time, an effect of spermine on the affinity of NMDA receptors for glutamate could be manifested as an increase in the rate of dissociation of glutamate from the receptor and subsequent shortening of the NMDA receptor-mediated component of the postsynaptic potential. Such an effect, together with the stimulatory effect of spermine, could serve to alter the onset, magnitude, and duration of NMDA receptor-mediated responses. In this scenario, polyamines could be thought of as NMDA receptor/channel lubricants, smoothing the onset and decay of NMDA receptor responses.

The subunit composition of native NMDA receptors is not known, but they may be tetramers or pentamers containing combinations of NR1 and NR2 subunits. In the present work, coinjection of oocytes with three subunits, i.e., NR1A, NR2A, and NR2B, led to the expression of receptors having the characteristics of mixed populations of NR1A/NR2A and NR1A/NR2B receptors, rather than a single population in which the NR2A or NR2B subunit controls the effects of spermine. These data do not exclude the possibility that two populations of receptors are expressed, each containing combinations of NR1A, NR2A, and NR2B in different ratios, with the effects of NR2A predominating in one population and those of NR2B predominating in the other. Nonetheless, it seems likely that NR2 subunits are important determinants of the polyamine sensitivity of native NMDA receptors (this work and Ref. 5),

in addition to influencing the kinetics of receptor activation (16) and the affinities for glycine (5, 17, 20), glutamate site antagonists (17, 20), Mg^{2+} and MK-801 (16, 17, 20), and ifenprodil (24).

Acknowledgments

The author is grateful to Lena Zappia for technical assistance and to Dr. Dolan Pritchett for advice on the preparation of cDNA plasmids and cRNAs.

References

1. Rock, D. M., and R. L. MacDonald. The polyamine spermine has multiple actions on *N*-methyl-D-aspartate receptor single-channel currents in cultured cortical neurons. *Mol. Pharmacol.* **41**:83–88 (1992).
2. Rock, D. M., and R. L. MacDonald. Spermine and related polyamines produce a voltage-dependent reduction of *N*-methyl-D-aspartate receptor single-channel conductance. *Mol. Pharmacol.* **42**:157–164 (1992).
3. Lerma, J. Spermine regulates *N*-methyl-D-aspartate receptor desensitization. *Neuron* **8**:343–352 (1992).
4. Benveniste, M., and M. L. Mayer. Multiple effects of spermine on *N*-methyl-D-aspartic acid receptor responses of rat cultured hippocampal neurones. *J. Physiol. (Lond.)* **464**:131–163 (1993).
5. Williams, K., A. M. Zappia, D. B. Pritchett, Y. M. Shen, and P. B. Molinoff. Sensitivity of the *N*-methyl-D-aspartate receptor to polyamines is controlled by NR2 subunits. *Mol. Pharmacol.* **45**:803–809 (1994).
6. McGurk, J. F., M. V. L. Bennett, and R. S. Zukin. Polyamines potentiate responses of *N*-methyl-D-aspartate receptors expressed in *Xenopus* oocytes. *Proc. Natl. Acad. Sci. USA* **87**:9971–9974 (1990).
7. Ransom, R. W., and N. L. Stec. Cooperative modulation of [^{3}H]MK-801 binding to the *N*-methyl-D-aspartate receptor-ion channel complex by L-glutamate, glycine, and polyamines. *J. Neurochem.* **51**:830–836 (1988).
8. Reynolds, I. J., and R. J. Miller. Ifenprodil is a novel type of *N*-methyl-D-aspartate receptor antagonist: interaction with polyamines. *Mol. Pharmacol.* **36**:758–765 (1989).
9. Sacaan, A. I., and K. M. Johnson. Spermine enhances binding to the glycine site associated with the *N*-methyl-D-aspartate receptor complex. *Mol. Pharmacol.* **36**:836–839 (1989).
10. Williams, K., C. Romano, and P. B. Molinoff. Effects of polyamines on the binding of [^{3}H]MK-801 to the *N*-methyl-D-aspartate receptor: pharmacological evidence for the existence of a polyamine recognition site. *Mol. Pharmacol.* **36**:575–581 (1989).
11. Romano, C., and K. Williams. Modulation of NMDA receptors by polyamines, in *Neuropharmacology of Polyamines* (C. J. Carter, ed.). Academic Press, London, 81–106 (1994).
12. Moriyoshi, K., M. Masu, T. Ishii, R. Shigemoto, N. Mizuno, and S. Nakanishi. Molecular cloning and characterization of the rat NMDA receptor. *Nature (Lond.)* **354**:31–37 (1991).
13. Anantharam, V., R. G. Panchal, A. Wilson, V. V. Kolchine, S. N. Triestman, and H. Bayley. Combinatorial RNA splicing alters the surface charge on the NMDA receptor. *FEBS Lett.* **305**:27–30 (1992).
14. Sugihara, S., K. Moriyoshi, T. Ishii, M. Masu, and S. Nakanishi. Structures and properties of seven isoforms of the NMDA receptor generated by alternative splicing. *Biochem. Biophys. Res. Commun.* **185**:826–832 (1992).
15. Hollmann, M., J. Boulter, C. Maron, L. Beasley, J. Sullivan, G. Pecht, and S. Heinemann. Zinc potentiates agonist-induced currents at certain splice variants of the NMDA receptor. *Neuron* **10**:943–954 (1993).
16. Monyer, H., R. Sprengel, R. Schoepfer, A. Herb, M. Higuchi, H. Lomeli, N. Burnashev, B. Sakmann, and P. H. Seeburg. Heteromeric NMDA receptors: molecular and functional distinction of subtypes. *Science (Washington D. C.)* **256**:1217–1221 (1992).
17. Ishii, T., K. Moriyoshi, H. Sugihara, K. Sakurada, H. Kadotani, M. Yokoi, C. Akazawa, R. Shigemoto, N. Mizuno, M. Masu, and S. Nakanishi. Molecular characterization of the family of the *N*-methyl-D-aspartate receptor subunits. *J. Biol. Chem.* **268**:2836–2843 (1993).
18. Yamazaki, M., H. Mori, K. Araki, K. J. Mori, and M. Mishina. Cloning, expression and modulation of a mouse NMDA receptor subunit. *FEBS Lett.* **300**:39–45 (1992).
19. Meguro, H., H. Mori, K. Araki, E. Kushiya, T. Kutsuwada, M. Yamazaki, T. Kumanishi, M. Arakawa, K. Sakimura, and M. Mishina. Functional characterization of a heteromeric NMDA receptor channel expressed from cloned cDNAs. *Nature (Lond.)* **357**:70–74 (1992).
20. Kutsuwada, T., N. Kashiwabuchi, H. Mori, K. Sakimura, E. Kushiya, K. Araki, H. Meguro, H. Masaki, T. Kumanishi, M. Arakawa, and M. Mishina. Molecular diversity of the NMDA receptor channel. *Nature (Lond.)* **358**:36–41 (1992).
21. Ikeda, K., M. Nagasawa, H. Mori, K. Araki, K. Sakimura, M. Watanabe, Y. Inoue, and M. Mishina. Cloning and expression of the ε4 subunit of the NMDA receptor channel. *FEBS Lett.* **313**:34–38 (1992).
22. Watanabe, M., Y. Inoue, K. Sakimura, and M. Mishina. Developmental changes in distribution of NMDA receptor channel subunit mRNAs. *NeuroReport* **3**:1138–1140 (1992).
23. Nakanishi, S. Molecular diversity of glutamate receptors and implications for brain function. *Science (Washington D. C.)* **258**:597–603 (1992).
24. Williams, K. Ifenprodil discriminates subtypes of the *N*-methyl-D-aspartate receptor: selectivity and mechanisms at recombinant heteromeric receptors. *Mol. Pharmacol.* **44**:851–859 (1993).
25. Williams, K., S. L. Russell, Y. M. Shen, and P. B. Molinoff. Developmental switch in the expression of NMDA receptors occurs *in vivo* and *in vitro*. *Neuron* **10**:267–278 (1993).
26. Stern, P., P. Béhé, R. Schoepfer, and D. Colquhoun. Single-channel conductances of NMDA receptors expressed from cloned cDNAs: comparison with native receptors. *Proc. R. Soc. Lond. B Biol. Sci.* **250**:271–277 (1992).
27. Durand, G. M., M. V. L. Bennett, and R. S. Zukin. Splice variants of the *N*-methyl-D-aspartate receptor NR1 identify domains involved in regulation by polyamines and protein kinase C. *Proc. Natl. Acad. Sci. USA* **90**:6731–6736 (1993).
28. Leonard, J. P., and S. R. Kelso. Apparent desensitization of NMDA responses in *Xenopus* oocytes involves calcium-dependent chloride current. *Neuron* **2**:53–60 (1990).
29. Boton, R., N. Dascal, B. Gillo, and Y. Lass. Two calcium-dependent chloride conductances in *Xenopus laevis* oocytes permeabilized with the ionophore A23187. *J. Physiol. (Lond.)* **408**:511–534 (1989).
30. Watkins, J. C., P. Krogsgaard-Larsen, and T. Honore. Structure-activity relationships in the development of excitatory amino acid receptor agonists and competitive antagonists. *Trends Pharmacol. Sci.* **11**:25–33 (1990).
31. Carter, C. J., K. G. Lloyd, B. Zivkovic, and B. Scatton. Ifenprodil and SL 82.0715 as cerebral antiischemic agents. III. Evidence for antagonistic effects at the polyamine modulatory site within the *N*-methyl-D-aspartate receptor complex. *J. Pharmacol. Exp. Ther.* **253**:475–482 (1990).
32. Pullan, L. M., and R. J. Powel. Spermine reciprocally changes the affinity of NMDA receptor agonists and antagonists. *Eur. J. Pharmacol.* **207**:173–174 (1991).
33. Pullan, L. M., R. J. Stumpo, R. J. Powel, K. A. Paschetto, and M. Britt. Neomycin is an agonist at a polyamine site on the *N*-methyl-D-aspartate receptor. *J. Neurochem.* **59**:2087–2093 (1992).
34. Williams, K., L. M. Pullan, C. Romano, R. J. Powel, A. I. Salama, and P. B. Molinoff. An antagonist/partial agonist at the polyamine recognition site of the *N*-methyl-D-aspartate receptor that alters the properties of the glutamate recognition site. *J. Pharmacol. Exp. Ther.* **262**:539–544 (1992).
35. Seiler, N. Polyamine metabolism and function in brain. *Neurochem. Int.* **3**:95–110 (1981).
36. Russell, D. H., E. Gfeller, L. J. Marton, and S. M. LeGendre. Distribution of putrescine, spermidine, and spermine in rhesus monkey brain: decrease in spermidine and spermine concentrations in motor cortex after electrical stimulation. *J. Neurobiol.* **5**:349–354 (1974).
37. Harman, R. J., and G. G. Shaw. The spontaneous and evoked release of spermine from rat brain *in vitro*. *Br. J. Pharmacol.* **73**:165–174 (1981).
38. Fage, D., C. Voltz, B. Scatton, and C. Carter. Selective release of spermine and spermidine from the rat striatum by *N*-methyl-D-aspartate receptor activation *in vivo*. *J. Neurochem.* **58**:2170–2175 (1992).
39. Kemp, J. A., and P. D. Leeson. The glycine site of the NMDA receptor: five years on. *Trends Pharmacol. Sci.* **14**:20–25 (1993).
40. Clements, J. D., R. A. J. Lester, G. Tong, C. E. Jahr, and G. L. Westbrook. The time course of glutamate in the synaptic cleft. *Science (Washington D. C.)* **258**:1498–1501 (1992).
41. Lester, R. A. J., J. D. Clements, G. L. Westbrook, and C. E. Jahr. Channel kinetics determine the time course of NMDA receptor-mediated synaptic currents. *Nature (Lond.)* **346**:565–567 (1990).
42. Hestrin, S., P. Sah, and R. A. Nicoll. Mechanisms generating the time course of dual component excitatory synaptic currents recorded in hippocampal slices. *Neuron* **5**:247–253 (1990).
43. Pan, Z. Z., G. Tong, and C. E. Jahr. A false transmitter at excitatory synapses. *Neuron* **11**:85–91 (1993).

Send reprint requests to: Keith Williams, Department of Pharmacology, University of Pennsylvania School of Medicine, Philadelphia, PA 19104–6084.