Pergamon

Biochemical Pharmacology, Vol. 50, No. 9, pp. 1373–1377, 1995.

0006-2952/95 $9.50 + 0.00

0006-2952(95)02017-7

SPERMIDINE ATTENUATION OF VOLATILE ANESTHETIC INHIBITION OF GLUTAMATE-STIMULATED [^{3}H](5D,10*S*)-(+)-METHYL-10,11-DIHYDRO-5*H*-DIBENZO[*a,d*] CYCLOHEPTEN-5,10-IMINE ([^{3}H]MK-801) BINDING TO *N*-METHYL-D-ASPARTATE (NMDA) RECEPTORS IN RAT BRAIN

DAN C. MARTIN*† and ROBERT S. ARONSTAM‡

*Department of Anesthesiology, Medical College of Georgia, Augusta, GA 30912; and ‡Guthrie Research Institute, Sayre, PA 18840, U.S.A.

(*Received* 9 *December* 1994; *accepted* 5 *May* 1995)

Abstract—The influence of spermidine, a polyamine agonist, on volatile anesthetic inhibition of *N*-methyl-D-aspartate (NMDA) receptor activation, as indicated by glutamate stimulation of [^{3}H]MK-801 ([^{3}H](5D,10*S*)-(+)-methyl-10,11-dihydro-5*H*-dibenzo[*a,d*]cyclohepten-5,10-imine) binding, was studied in rat brain. Spermidine reserved the inhibition caused by four volatile anesthetics (enflurane, halothane, methoxyflurane and chloroform) at the same concentrations ($EC_{50} \approx 3$ μM) at which it potentiated glutamate opening of the NMDA ion channel. The anesthetics had no effect on the direct stimulation of channel opening by spermidine, which occurred at concentrations of spermidine greater than 30 μM in the absence of receptor agonist. In these actions, spermidine closely resembled the allosteric co-agonist glycine. The present results suggest that anesthetic action on NMDA receptors involves a set of sites on the channel complex that is distinct from the recognition sites for glutamate, glycine, and channel blockers, and are consistent with the idea that blockade of NMDA receptors contributes to the development of the anesthetic state.

Key words: NMDA receptors; MK-801; ion channel; anesthetics; anesthesia; polyamines; spermidine; dizocilpine

NMDA§ receptors are a class of glutamate receptors that mediate excitatory transmission in the central nervous system. Activation of NMDA receptors opens a non-selective cation channel that displays high Ca^{2+} permeability, relatively slow kinetics, a large single channel conductance, and a voltage-dependent blockade by Mg^{2+} [1]. Glycine is an allosteric co-agonist at NMDA receptors, which potentiates glutamate activation of the receptor [2].

Polyamines, such as spermine and spermidine, represent a second class of positive receptor modulators. These polyamines increase NMDA receptor activation as indicated by: (1) an increase in [^{3}H]MK-801 binding to an ion channel site, access to which is dependent upon receptor activation (e.g. [3]), (2) a potentiation of NMDA-induced ionic currents (e.g. [4, 5]), (3) an increase in receptor affinity for the positive modulator glycine (e.g. [5–7]), (4) an increase in agonist-induced $^{45}Ca^{2+}$ influx [8], (5) an attenuation of glycine agonist-induced increases, and a potentiation of antagonist-induced decreases, in the cGMP content of cerebellar cells [9], and (6) a decrease in the development of glycine-sensitive desensitization of the receptor [5]. Moreover, the polyamine antagonist ifenprodil inhibits the glutamate-induced cytotoxicity that is mediated by NMDA receptors [10].

We have shown that volatile anesthetics disrupt transmission at NMDA receptors [11–13]. Volatile anesthetics inhibit glutamate stimulation of [^{3}H]MK-801 binding to NMDA receptors in rat brain. As a consequence of this interaction, $^{45}Ca^{2+}$ influx into brain microvesicles mediated by NMDA receptors is inhibited, and receptor desensitization is mitigated [13]. This antagonism is reversed, or substantially reduced, by glycine.

To further define the mechanism whereby volatile anesthetics disrupt NMDA receptor activation, we examined the influence of spermidine on anesthetic-induced depression of NMDA receptor activation as indicated by glutamate stimulation of [^{3}H]MK-801 binding.

MATERIALS AND METHODS

Adult, male Wistar rats (Harlan Sprague–Dawley, Indianapolis, IN) were killed by decapitation. Their cerebral cortices were homogenized at 2–4° in 5 mM Tris–HCl, pH 7.4. This homogenate was centrifuged at 17,000 *g* for 15 min. The pellet was resuspended in buffer and treated with Triton X-100 (0.04%) for 20 min at 37°, and then washed 4–5 times to remove the Triton and endogenous glutamate and glycine. The final pellet was resuspended in Tris buffer at a protein concentration of 1 mg/mL and used without further treatment.

The binding of 1 nM [^{3}H]MK-801 (22.5 Ci/mmol; Dupont-NEN) to neural membranes (10 μg protein) was measured following a 60-min incubation in 5 mM Tris–HCl, pH 7.4, at 37° in a final volume of 1 mL. Nonspecific binding was determined in the presence of 100 μM unlabeled MK-801. Membranes were collected by vacuum filtration, and their radioactivity content was determined by liquid scintillation counting. The EC_{50} values for spermidine stimulation of [^{3}H]MK-801 binding were

† Corresponding author. Tel. (706) 721-3871; FAX (706) 721-7753.

§ Abbreviations: NMDA, *N*-methyl-D-aspartate; and MK-801, (5D,10*S*)-(+)-methyl-10,11-dihydro-5*H*-dibenzo[*a,d*] cyclohepten-5,10-imine, dizocilpine.

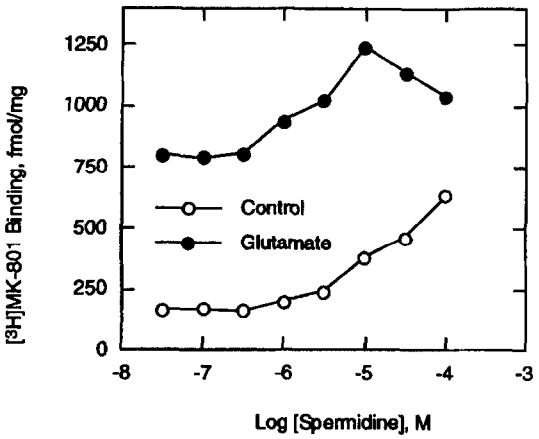

Fig. 1. Influence of spermidine on [^{3}H]MK-801 binding to the NMDA receptor in rat cortical membranes. The binding of 1 nM [^{3}H]MK-801 was measured in the presence of the indicated concentrations of spermidine in the absence (○) and presence (●) of 10 μM glutamate. Each point represents the mean from three experiments, which varied by less than 15%.

calculated by averaging the concentrations at which spermidine increased [^{3}H]MK-801 binding to a point halfway between the binding level in the absence of spermidine and the maximal binding level, as determined in the three independent experiments.

Halothane and enflurane were added to assay tubes as aliquots from saturated stock buffers, and the mixtures were vortexed for 10 sec prior to the addition of membrane protein. Initial and final assay concentrations were determined by gas–liquid chromatography, and were observed to decrease by less than 15% over the course of a 60-min assay. The anesthetic concentrations reported were those measured after 40 min of incubation.

RESULTS

Glutamate increased the binding of 1 nM [^{3}H]MK-801 to cortical membranes after a 1-hr incubation at 37° by up to several hundred percent (Fig. 1). This increase is believed to reflect the fact that MK-801 binds to a site within the NMDA receptor ionophore, and access to this site in the absence of receptor agonists is sterically restricted (e.g. [14, 15]). The magnitude of this fractional stimulation in the different membrane preparations depends on the efficiency of the washing procedures in removing endogenous glutamate and/or glycine; the fractional stimulation was greatest in preparations in which basal [^{3}H]MK-801 binding was lowest (i.e. <200 fmol/mg protein). The EC_{50} for glutamate-induced stimulation of [^{3}H]MK-801 binding was in the range of 1–3 μM [12].

Spermidine stimulated the binding of 1 nM [^{3}H]MK-801 by >300% at 100 μM (Fig. 1); significant stimulation was seen with spermidine concentrations as low as 10 μM. Spermidine stimulation was still increasing at 100 μM. Spermidine also stimulated [^{3}H]MK-801 binding in the presence of 10 μM glutamate with an EC_{50} of ≈ 3 μM (Fig. 1). In the presence of glutamate, maximal stimulation was about 75%. Above 10 μM, the degree of spermidine stimulation decreased. This difference in spermidine potency suggests that different mechanisms underlie spermidine potentiation of [^{3}H]MK-801 binding in the absence versus the presence of glutamate. An alternate possibility is that the expression of the inhibitory effect of spermidine (note the biphasic nature of the spermidine curves in Fig. 5) is facilitated in the presence of the agonist; perhaps the inhibitory action of spermidine is dependent on access to an ion channel site.

Several volatile anesthetics, including enflurane, halothane, methoxyflurane and chloroform, inhibited glutamate-stimulated [^{3}H]MK-801 binding, in agreement with our previous reports [11, 12] (Figs. 2 and 3). Anesthetic inhibition of [^{3}H]MK-801 binding was not observed in the absence of glutamate. Maximal inhibition with the different anesthetics ranged from 45 to 60%. In terms of their potency at inhibiting glutamate-stimulated [^{3}H]MK-801 binding, the following series was obtained: methoxyflurane > enflurane ≈ halothane > chloroform. Inclusion of 10 μM spermidine in the incubation me-

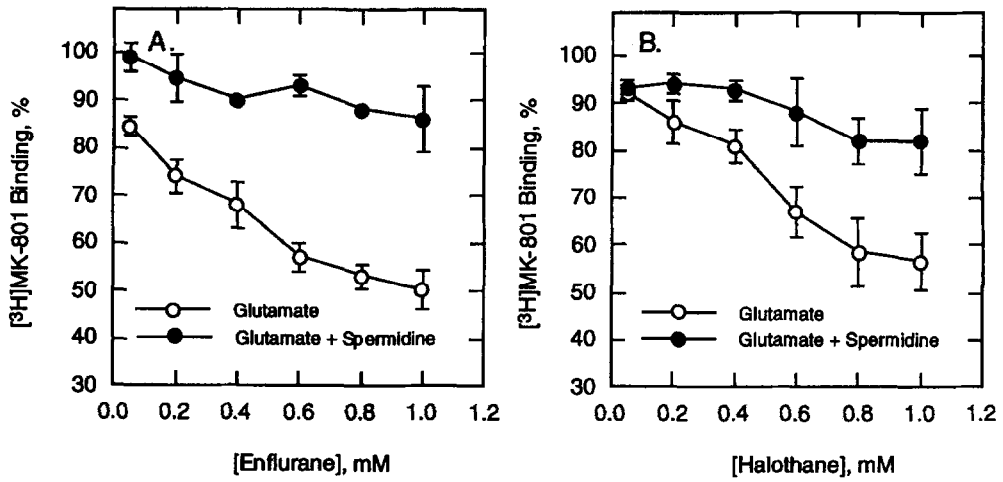

Fig. 2. Influence of spermidine on enflurane and halothane inhibition of glutamate-stimulated [^{3}H]MK-801 binding to the NMDA receptor in rat cortical membranes. The binding of 1 nM [^{3}H]MK-801 was measured in the presence of 100 μM glutamate and the concentration of enflurane (Fig. 2A) or halothane (Fig. 2B) indicated on the abscissa. Binding was determined in the absence (○) and presence (●) of 10 μM spermidine. Control binding was 740 ± 104 fmol/mg protein (glutamate) and 1408 ± 90 fmol/mg protein (glutamate + spermidine). Values are means ± SD from three experiments.

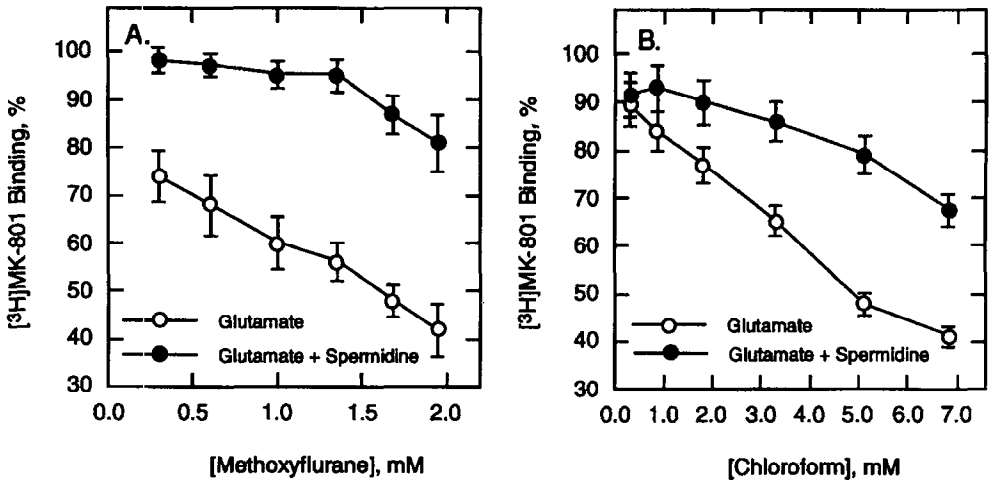

Fig. 3. Influence of spermidine on methoxyflurane and chloroform inhibition of glutamate-stimulated [^{3}H]MK-801 binding to the NMDA receptor in rat cortical membranes. This binding of 1 nM [^{3}H]MK-801 was measured in the presence of 100 μM glutamate and the concentration of methoxyflurane or chloroform indicated on the abscissas. Binding was determined in the absence (○) and presence (●) of 10 μM spermidine. Control binding was 858 ± 154 fmol/mg protein (glutamate) and 1458 ± 131 fmol/mg protein (glutamate + spermidine). Values are means ± SD from three experiments.

dium greatly reduced the inhibitory potency of each of the anesthetics. For example, at the calculated minimum alveolar concentration of enflurane (≈0.4 mM), anesthetic inhibition was reduced from 42 to <10%.

In the absence of glutamate, enflurane and halothane did not affect the low affinity stimulation of 1 nM [^{3}H]MK-801 binding by spermidine (Fig. 4). Methoxyflurane and chloroform were similarly without effect. In the presence of 100 μM glutamate and a concentration of anesthetic that inhibited [^{3}H]MK-801 binding by ≈50%, spermidine reversed the anesthetic-induced inhibition with an EC_{50} of ≈3 μM in every instance (Fig. 5). At concentrations above 10 μM, spermidine decreased [^{3}H]MK-801 binding, as it did in the absence of anesthetic (Fig. 1).

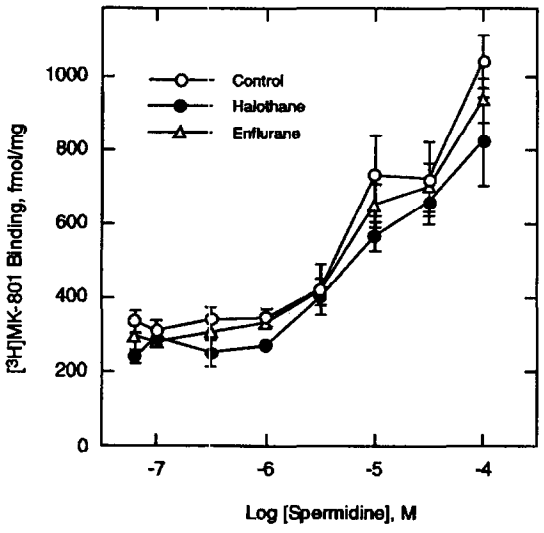

Fig. 4. Influence of volatile anesthetics on spermidine stimulation of [^{3}H]MK-801 binding to the NMDA receptor in the absence of glutamate. The binding of 1 nM [^{3}H]MK-801 was measured in the presence of the indicated concentrations of spermidine in the absence (○) and presence of halothane (●) or enflurane (△). Values are means ± SD from three experiments.

DISCUSSION

Anesthetics and NMDA-mediated signal transduction

It is likely that volatile anesthetics produce a generalized depression of synaptic transmission through the alteration of the function of specific proteins involved in synaptic transmission, such as receptors, ion channels and transducer proteins [16, 17]. A number of lines of evidence indicate that volatile anesthetics block NMDA receptors: Volatile anesthetic potency is increased by the receptor antagonists MK-801 [18, 19] and CGS 19755 [20], and volatile anesthetics inhibit NMDA receptor-mediated ion currents [21] and calcium translocation [22]. Ketamine, a dissociative general anesthetic, blocks NMDA neurotransmission at clinically relevant concentrations [23, 24]. As with MK-801, ketamine blockade is voltage dependent and appears to occur at a site within the cation channel. Ethanol, in a manner analogous to the volatile anesthetics, disrupts NMDA transmission by a process that is reversed by glycine [25].

We have shown that volatile anesthetics inhibit glutamate stimulation of [^{3}H]MK-801 binding to NMDA receptors in rat brain [11–13]. This antagonism is reversed, or substantially reduced, by glycine in a manner that suggests an antagonism for the glycine site on the receptor complex. Anesthetics inhibit $^{45}Ca^{2+}$ influx into brain microvesicles mediated by NMDA receptors, and receptor desensitization is mitigated [13].

In the present studies, the polyamine spermidine was found to reverse volatile anesthetic depression of agonist opening of the NMDA ion channel (thus exposing [^{3}H]MK-801 binding sites). Two effects of spermidine were observed: at relatively high concentrations (>10 μM), spermidine directly stimulated [^{3}H]MK-801 bind-

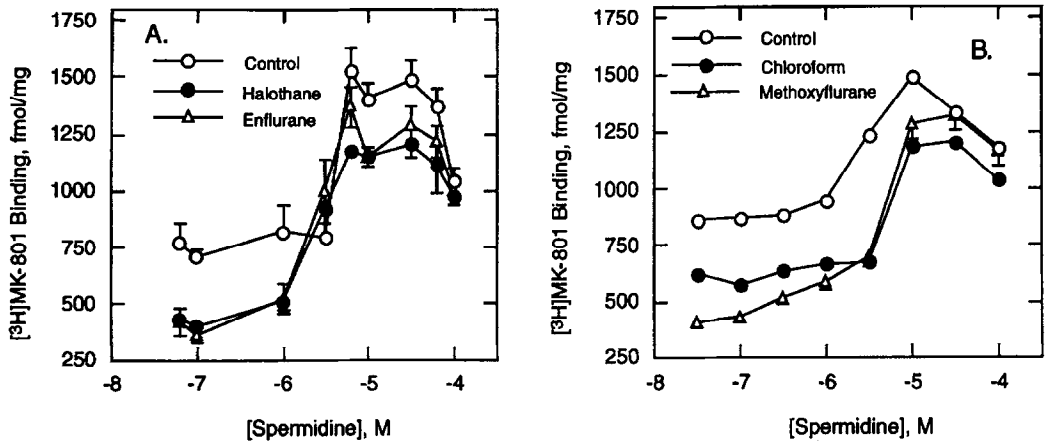

Fig. 5. Influence of volatile anesthetics on spermidine stimulation of [^{3}H]MK-801 binding to the NMDA receptor in the presence of glutamate. The binding of 1 nM [^{3}H]MK-801 was measured in the presence of the indicated concentrations of spermidine in the absence (○) and presence of the indicated anesthetics. Anesthetics were included at concentrations at which they inhibited approximately 50% of the glutamate-stimulated binding. Glutamate (100 μM) was included in the incubation medium. Values are means ± SD from three experiments.

ing in the absence of receptor agonist; at somewhat lower concentrations (1–10 μM), spermidine potentiated glutamate stimulation of [^{3}H]MK-801 binding. Spermidine reversal of volatile anesthetic inhibition of [^{3}H]MK-801 binding occurred at the same concentrations at which spermidine potentiated glutamate channel opening activity, suggesting that the two activities are the consequence of the same molecular interaction. It should be kept in mind that desensitization of the NMDA receptor complex is an inherent complication of equilibrium binding measurements; moreover, volatile anesthetics have been shown to affect the development and extent of NMDA receptor desensitization in a manner similar to glycine [13].

Site of anesthetic action on the NMDA receptor complex

The NMDA receptor is a heteromeric complex with multiple sites of drug interaction. Discrete sites on the NMDA receptor complex have been identified for (1) agonist recognition (i.e. the glutamate binding site), (2) co-agonist (glycine) recognition, (3) ion translocation (i.e. the ion channel), (4) divalent cation modulator (e.g. Zn^{2+}) binding, (5) Mg^{2+} inhibition (within the ion channel), and (6) polyamine and polyamine antagonist binding. Occupancy of each of these sites has multiple and complex effects on both receptor activation and ligand activity at the other sites. The picture is further complicated by the fact that multiple polyamine sites and actions have been described. Moreover, it has become clear that polyamine antagonists such as ifenprodil are allosteric regulators of polyamine action. Thus, it may be reasonable to discuss anesthetic action on NMDA receptors in terms of their functional effects on the NMDA receptor rather than specific sites of interaction.

Spermidine closely resembles glycine in its mitigating effects on anesthetic inhibition. Glycine also possesses discrete direct and potentiating activities on NMDA receptor activation. Moreover, glycine reversal of volatile anesthetic inhibition also occurred in the concentration range at which glycine potentiated rather than directly stimulated channel opening. This led to our suggestion [12] that anesthetic actions at obligatory allosteric sites (such as the glycine site) underlies this activity. Glycine and the polyamines act at different sites on the NMDA receptor and have complex allosteric effects on each other's activities (e.g. [5, 7]). The fact that two distinct classes of allosteric NMDA receptor modulators reverse volatile anesthetic inhibition raises the possibility that anesthetic action involves a downstream effect on the receptor activation process rather than a direct action at either one of these two sites.

The present results are consistent with the notion that volatile anesthetics block transmission at NMDA receptors at clinically relevant concentrations and in a manner that is consistent with production of an anesthetic state. This inhibition appears to be specific for the ion channel activation process (i.e. the glutamate-stimulated conformational change that increases the accessibility of [^{3}H]MK-801 for its binding site within the ion channel). Multiple classes of positive allosteric regulators (i.e. polyamine and glycine) reverse anesthetic inhibition with extremely similar properties, suggesting that the anesthetic action involves a discrete set of sites on the channel complex.

Acknowledgements—This work was supported by GM46408, NS31518, NS25296 and the Research Service of the Veterans Administration.

REFERENCES

1. Seeburg PH, The molecular biology of mammalian glutamate receptor channels. *Trends Pharmacol Sci* **14:** 297–303, 1993.
2. Kaplita PV and Ferkany JW, Evidence for direct interactions between NMDA and glycine recognition sites in brain. *Eur J Pharmacol* **188:** 175–179, 1990.
3. Steele JE, Bowen DM, Francis PT, Green AR and Cross AJ, Spermidine enhancement of [^{3}H]MK-801 binding to the NMDA receptor complex in human cortical membranes. *Eur J Pharmacol* **189:** 195–200, 1990.
4. McGurk JF, Bennett MV and Zukin RS, Polyamines potentiate responses of *N*-methyl-D-aspartate receptors ex-

pressed in *Xenopus* oocytes. *Proc Natl Acad Sci USA* **87:** 9971–9974, 1990.

5. Benveniste M and Mayer ML, Multiple effects of spermine on *N*-methyl-D-aspartate receptor responses of rat cultured hippocampal neurones. *J Physiol (Lond)* **464:** 131–163, 1993.
6. Grimwood S, Struthers L and Foster AC, Polyamines modulate [^{3}H]L-689,560 binding to the glycine site of the NMDA receptor from rat brain. *Eur J Pharmacol* **266:** 43–50, 1994.
7. Williams K, Zappia AM, Pritchett DB, Shen YM and Molinoff PB, Sensitivity of the *N*-methyl-D-aspartate receptor to polyamines is controlled by NR2 subunits. *Mol Pharmacol* **45:** 803–809, 1994.
8. Ohkuma S, Katsura M, Chen D-Z, Chen S-H and Kuriyama K, Presence of *N*-methyl-D-aspartate (NMDA) receptors in neuroblastoma × glioma hybrid NG108-15 cells—Analysis using [$^{45}Ca^{2+}$] influx and [^{3}H]MK-801 binding as functional measures. *Brain Res Mol Brain Res* **22:** 166–172, 1994.
9. Rao TS, Cler JA, Mick SJ, Iyengar S and Wood PL, Polyamines modulate events mediated by the *N*-methyl-D-aspartate (NMDA) receptor complex through an ifenprodil-insensitive pathway: *In vivo* measurements of cyclic GMP in the cerebellum. *Neuropharmacology* **30:** 567–573, 1991.
10. Tamura Y, Sato Y, Yokota T, Akaike A and Takaori S, Ifenprodil prevents glutamate cytotoxicity via polyamine modulatory sites of *N*-methyl-D-aspartate receptors in cultured cortical neurons. *J Pharmacol Exp Ther* **265:** 1017–1025, 1993.
11. Martin DC, Abraham JE, Plagenhoef M and Aronstam RS, Volatile anesthetics and NMDA receptors. Enflurane inhibition of glutamate-stimulated [^{3}H]MK-801 binding and reversal by glycine. *Neurosci Lett* **132:** 73–76, 1991.
12. Martin DC, Plagenhoef M, Abraham J, Dennison RL and Aronstam RS, Volatile anesthetics and glutamate activation of *N*-methyl-D-aspartate receptors. *Biochem Pharmacol* **49:** 809–817, 1995.
13. Aronstam RS, Martin DC and Dennison RL, Volatile anesthetics inhibit NMDA-stimulated ^{45}Ca uptake by rat brain microvesicles. *Neurochem Res* **19:** 1515–1520, 1994.
14. Foster AC and Wong EHF, The novel anticonvulsant MK-801 binds to the activated state of the *N*-methyl-D-aspartate receptor in rat brain. *Br J Pharmacol* **91:** 403–409, 1987.
15. Bonhaus DW and McNamara JO, Uncompetitive antagonist binding: A biochemical index of activation of the NMDA receptor-coupled ion channel. In: *Neurotransmitters in Epilepsy* (Eds. Avanzini G, Engel J Jr, Fariello R and Heinemann V), pp. 181–188. Elsevier, New York, 1992.
16. Franks NP and Leib WB, What is the molecular nature of general anaesthetic target sites? *Trends Pharmacol Sci* **8:** 169–174, 1987.
17. Aronstam RS and Dennison RL, Anesthetic effects on muscarinic signal transduction. In *International Anesthesiology Clinics* (Ed. Lebowitz PW), pp. 265–272. Little Brown, Boston, 1989.
18. Scheller MS, Zornow MH, Fleischer JE, Shearman GT and Greber TF, The noncompetitive *N*-methyl-D-aspartate receptor antagonist, MK-801 profoundly reduces volatile anesthetic requirements in rabbits. *Neuropharmacology* **28:** 677–681, 1989.
19. Daniell LC, The noncompetitive *N*-methyl-D-aspartate antagonists, MK-801, phencyclidine, and ketamine, increase the potency of general anesthetics. *Pharmacol Biochem Behav* **36:** 111–115, 1990.
20. Daniell LC, Effect of CGS 19755, a competitive *N*-methyl-D-aspartate antagonist, on general anesthetic potency. *Pharmacol Biochem Behav* **40:** 767–769, 1991.
21. Yang J and Zorumski CF, Effects of isoflurane on *N*-methyl-D-aspartate gated ion channels in cultured rat hippocampal neurons. *Ann NY Acad Sci* **625:** 287–289, 1991.
22. Puil E, El-Beheiry H and Baimbridge KG, Anesthetic effects on glutamate-stimulated increase in intraneuronal calcium. *J Pharmacol Exp Ther* **255:** 955–961, 1990.
23. Davies SN, Alford ST, Coan EJ, Lester RAJ and Collinridge GL, Ketamine blocks an NMDA receptor-mediated component of synaptive transmission in rat hippocampus in a voltage-dependent manner. *Neurosci Lett* **92:** 213–217, 1988.
24. Yamamura T, Harada K, Okamura A and Kemmotsu O, Is the site of action of ketamine anesthesia the *N*-methyl-D-aspartate receptor? *Anesthesiology* **72:** 704–710, 1990.
25. Rabe CS and Tabakoff B, Glycine site-directed agonists reverse the actions of ethanol at the *N*-methyl-D-aspartate receptor. *Mol Pharmacol* **38:** 753–757, 1990.