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EVIDENCE FOR THE INVOLVEMENT OF A CARBOXYL GROUP IN THE VICINITY OF THE MK801 AND MAGNESIUM ION BINDING SITE OF THE *N*-METHYL-D-ASPARTATE RECEPTOR

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Abstract—A series of protein modifying reagents were tested for their effects on the specific binding of [^{3}H]MK801 to adult rat brain membranes. *N*-Bromosuccinimide, acetyl imidazole, 2,3-butanedione, 5,5′-dithiobis-(2-nitrobenzoic acid) and dithiothreitol all had no significant effect on binding. The carboxylic acid residue modification reagent, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC), inhibited [^{3}H]MK801 specific binding in a dose-dependent manner with an IC_{50} = 1.9 mM. The inhibition by EDAC was due to a decrease in the B_{max} with no change in K_D. The inhibition of [^{3}H]MK801 binding by EDAC was not prevented by prior incubation with competitive antagonists. Protection against EDAC inactivation was obtained, however, in a dose-dependent manner by preincubation with the divalent cations, Ca^{2+} and Mg^{2+}, but not Zn^{2+}. These results suggest that EDAC modifies an important carboxyl group located within the voltage-dependent Mg^{2+} binding site of the *N*-methyl-D-aspartate receptor. This modification yields a decrease in the specific [^{3}H]MK801 binding activity thus demonstrating a close association between the two allosteric regulatory sites.

The *N*-methyl-D-aspartate (NMDA†) pharmacological subclass of the excitatory glutamate receptors is a fast-acting ligand-gated cation channel. NMDA receptor activity is regulated by several classes of compounds which are thought to bind to distinct sites within the receptor protein. These are: (i) the recognition site for the endogenous ligand, glutamate, together with its competitive agonist, NMDA, and antagonist, 2-amino-5-phosphonovalerate; (ii) a strychnine-insensitive allosteric glycine binding site; and (iii) a binding site for the polyamines, spermine and spermidine. Additionally, three sites have been proposed to be associated with the NMDA receptor channel domain namely, (i) the site at which Mg^{2+} exerts a voltage-dependent blockade of the cation flux; (ii) the binding site for the non-competitive antagonists, [(+)-5-methyl-10,11-dihydro-dibenzo[*a*,*d*]cyclohepten-5,10-imine] (MK801), phencyclidine, tenocyclidine (TCP) and ketamine and (iii) a Zn^{2+} binding site which also blocks cation flux (summarized in Ref. 1).

The localization of any one of these regulatory binding sites within the NMDA receptor structure is not known. One approach that has been employed successfully to delineate important amino acids in both enzyme active sites and ligand binding domains within receptors is that of covalent modification [2, 3]. This, together with the respective primary structures deduced from cDNA sequences, provides a powerful approach with which to elucidate binding sites perhaps enabling rational drug design. Thus, we have used this strategy to investigate the importance of particular amino acids in the binding of the channel ligand, [^{3}H]MK801, to the NMDA receptor in adult rat brain membranes. The results are reported below.

MATERIALS AND METHODS

Materials. [^{3}H]MK801 (28.8 Ci/mmol) was from Du Pont (Stevenage, Herts, U.K.), MK801 maleate and ketamine were from Research Biochemicals Inc., (Natick, MA, U.S.A.). TCP was a generous gift from Dr J. F. Collins, City of London Polytechnic, London, U.K. and [^{3}H]spiperone and (+)-butaclamol were generously donated by Dr P. G. Strange, University of Canterbury, Kent, U.K. All other reagents were from commercial sources.

Brain membrane preparation. Forebrains were dissected from adult rats, Wistar strain, snap-frozen in liquid nitrogen and stored at −80° until use. Tissue was homogenized in 50 mM Tris–HCl, pH 7.4, containing 5 mM EDTA, 5 mM EGTA and 200 mM sucrose (Buffer 1; 9 vol.) using a Potter glass/teflon homogenizer (12 strokes). The homogenate was centrifuged for 10 min at 4° at 800 *g* and the supernatant retained on ice. The pellet was rehomogenized in Buffer 1 (4.5 vol.; 6 strokes), centrifuged for 10 min at 4° at 800 *g* and the resultant supernatant pooled with the first one. This was centrifuged for 1 hr at 4° at 126,000 *g* rehomogenized in Buffer 1, frozen at −20° then thawed, rehomogenized, recentrifuged, homogenized and

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† Abbreviations: DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); DTT, dithiothreitol; EDAC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; MK801, (+)-5-methyl-10,11-dihydro-dibenzo[*a*,*d*]cyclohepten-5,10-imine; NBS, *N*-bromosuccinimide; NMDA, *N*-methyl-D-aspartate; TCP, tenocyclidine.

frozen. The freeze–thaw cycle was repeated five times to remove endogenous glutamate [4]. The final pellet was resuspended in 25 mM sodium phosphate, pH 7.4 (2 vol.). A mixed mitochondrial/microsomal membrane preparation with 10–20 mg protein/mL was produced routinely. It was stored at −80° for up to 2 months with no detectable loss of [^{3}H]MK801 specific ligand binding activity.

Membranes from bovine striatum were prepared by the method of Chazot and Strange (1992) [5].

Radioligand binding assays. Membranes (100 μg protein) in 25 mM sodium phosphate, pH 7.4, were incubated with 3 nM [^{3}H]MK801 in the presence of 10 μM *l*-glutamate at 22° for 2 hr. Non-specific binding was measured in the presence of 10 μM MK801, all in a final assay volume of 0.5 mL. Separation of bound and free radioligand was by filtration on polyethyleneimine-treated glass fibre GF/B filters followed by 3 × 10 mL washes with 10 mM sodium phosphate, pH 7.4. For saturation analysis, the range of [^{3}H]MK801 concentrations employed was 0.5–20 nM.

D_2 Dopamine receptor binding to membranes prepared from bovine striatum was carried out as described in Ref. 5.

Chemical modification of brain membrane preparations, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) treatment. Brain membranes (1 mg protein/mL, 0.75 mL) were incubated with 1 μM–30 mM EDAC in either 25 mM sodium phosphate, pH 7.4, or 50 mM Tris–HCl, pH 7.4, as appropriate, for 1 hr at 25°. The EDAC was removed by centrifuging the treated membranes for 5 min at 4° at 40,000 *g*, resuspending the pellet in 25 mM sodium phosphate, pH 7.4, (1 mL), centrifuging as above followed by one more wash cycle. The pellet was finally resuspended in 25 mM sodium phosphate, pH 7.4 to a protein concentration of 1 mg/mL and assayed for specific [^{3}H]MK801 binding activity.

Protection experiments. Brain membranes (1 mg protein/mL; 0.75 mL) were pre-incubated with either MK801, ketamine, TCP, $MgCl_2$, $ZnCl_2$, $CaCl_2$ or KCl at the stated concentrations in 25 mM sodium phosphate, pH 7.4, for drugs and 50 mM Tris–HCl, pH 7.4, for ions for 2 hr at 22°. EDAC (5 mM) was added, the samples incubated for a further 1 hr at 25° and processed as described above for the measurement of [^{3}H]MK801 specific ligand binding activity.

Other modifications. All other modifications were as for the EDAC treatment as above except for 2,3-butanedione where the incubation, membrane washes and the radioligand binding assays were carried out in 0.2 M sodium borate, pH 9.0, containing 0.15 M NaCl, and for 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) where the incubation conditions were 25° and 2 hr. For all treatments parallel experiments were carried out in the absence of the modifying reagent.

Protein determination. Protein concentrations were determined using the method of Lowry *et al.* [6] with bovine serum albumin as the standard protein.

RESULTS

A series of selective amino acid modification reagents at concentrations of the reagents at which they retain their amino acid specificity, were tested for their ability to inhibit [^{3}H]MK801 specific radioligand binding to adult rat brain membranes. The results are summarized in Fig. 1. It can be seen that treatment of the membranes with *N*-bromosuccinimide (NBS), acetyl imidazole, 2,3-butanedione, DTNB and dithiothreitol (DTT) had minimal significant effects (i.e. <15%) on [^{3}H]-MK801 specific binding activity. In contrast, at 5 mM, EDAC inhibited binding by 71 ± 2%. Further investigation showed that this inhibition was dose-dependent with an apparent $IC_{50} = 1.9 \pm 0.5$ mM (Fig. 2). The maximum number of binding sites inhibited was 85 ± 4%.

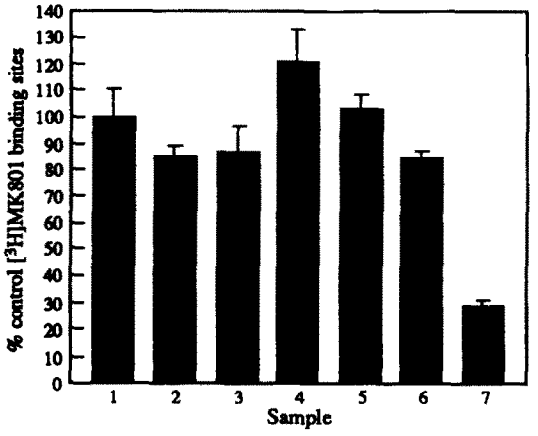

Fig. 1. The effect of a range of amino acid modification reagents on the binding of [^{3}H]MK801 to adult rat brain membranes. Membranes prepared from adult rat brain (750 μg protein) were incubated with a series of different amino acid modification reagents under conditions described in Materials and Methods. Following treatment, the reagent was removed by centrifugation and the membranes assayed for specific [^{3}H]MK801 binding activity. The lanes are: 1, control, untreated membranes; 2, acetyl imidazole (50 mM); 3, NBS (100 μM); 4, 2,3-butanedione (5 mM); 5, DTNB (2.5 mM); 6, DTT (1 mM) and 7, EDAC (5 mM). Values are the mean ± SD for three independent determinations.

To investigate whether the inhibition of [^{3}H]-MK801 binding activity was due to an effect on the dissociation constant, K_D, or the number of sites, B_{max}, saturation binding curves to EDAC-treated and control membranes were carried out. Scatchard analysis of the isotherms showed that for each sample, a best fit was obtained by a single high affinity site with K_D in the range 5–7 nM. However, the B_{max} value for the EDAC-treated samples was significantly decreased (Table 1 and Fig. 3).

The pharmacological specificity of the effect of EDAC on [^{3}H]MK801 binding activity was studied by a series of protection experiments where membranes were preincubated with a range of compounds active at NMDA receptors. Interestingly, the competitive inhibitors of [^{3}H]MK801 binding tested i.e. MK801 (1 μM), TCP (10 μM) and ketamine (100 μM) did not protect the membranes

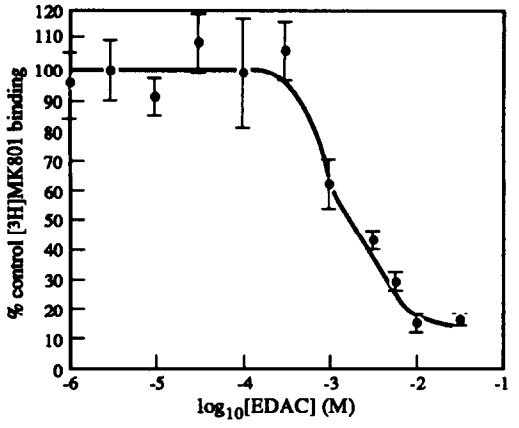

Fig. 2. Concentration-dependence for the inhibition of [^{3}H]MK801 specific binding to adult rat brain membranes by EDAC. Membranes (750 μg protein) were incubated with concentrations of EDAC in the range 1–30,000 μM, for 1 hr at 25°. Membranes were washed by centrifugation and assayed for specific [^{3}H]MK801 binding activity, all as described in Materials and Methods. The results are the mean ± SD for three independent experiments.

against inactivation by EDAC (results not shown). Since the maleate derivative of MK801 was used, it was thought that EDAC may react with its carboxyl moiety thus reducing the effective concentration of MK801 in the protection assay. However, both MK801 and MK801 treated with EDAC gave the same non-specific binding values in radioligand binding assays of EDAC-modified membranes suggesting that the potency of the ligand was not effected by the presence of EDAC.

In contrast, preincubation with the non-competitive antagonist of the MK801 site, Mg^{2+}, prevented EDAC inactivation. Further characterization showed that the protection by Mg^{2+} was dose-dependent with an apparent EC_{50} = 1.3 ± 0.6 mM. Dose-dependent protection was also found for Ca^{2+} ions (Fig. 4). No apparent protection was elicited by Zn^{2+} or K^+. These results are summarized in Table 2. The EC_{50} value for protection of inactivation of [^{3}H]MK801 binding sites by EDAC for Mg^{2+} was the same as the IC_{50} value for the inhibition of [^{3}H]MK801 binding activity by Mg^{2+} in contrast to the respective values for Ca^{2+} which showed no similarity (Fig. 4, Table 2, [7]). Furthermore, although Zn^{2+}, like Mg^{2+} is a non-competitive inhibitor of [^{3}H]MK801 specific binding activity, it does not elicit protection against inactivation of these sites by EDAC. This is in agreement with previous observations where it was concluded that the two cations act at different sites [8]. To exclude the possibility that the effective concentration of EDAC in the protection assays was reduced following reaction with Mg^{2+}, control experiments with the D_2 dopamine receptor were carried out. It is established that the modification of a carboxyl group in the D_2 dopamine receptor results in the loss of specific [^{3}H]spiperone binding activity [3]. Thus, parallel experiments were carried out in which the modification of [^{3}H]spiperone binding sites by EDAC was compared in the presence and absence of Mg^{2+}. No specific differences were found between the different conditions of inactivation (Fig. 5) thus showing that the effective concentration of EDAC is not changed in the presence of Mg^{2+}.

DISCUSSION

Chemical modification of proteins can yield important information about both the amino acids involved in substrate and ligand binding as well as providing evidence for receptor heterogeneity. Examples of the latter include the use of diethylpyrocarbonate to distinguish between benzodiazepine agonist and partial inverse agonist binding to the $GABA_A$ receptor [19] and the differential effects of DTNB treatment on [^{3}H]spiperone binding to the D_2 dopamine receptors of the pituitary gland and brain [5].

In this paper, we have described the first detailed report of the study of amino acids involved in [^{3}H]MK801 binding to the NMDA receptor of adult rat brain. We have shown that the residues tyrosine, arginine, tryptophan, cysteine and cystine do not have a significant role in the binding of [^{3}H]MK801 to the receptor since the modification reagents specific for these amino acids namely, acetyl imidazole, 2,3-butanedione, NBS, DTNB and DTT, respectively, had no significant effect on ligand binding. In contrast, EDAC caused a dose-dependent inhibition of binding which was a result of a specific decrease in the B_{max} value. EDAC specifically covalently modifies carboxyl groups although it can modify both cysteine and tyrosine residues [2]. Since DTNB and acetyl imidazole, reagents specific for

Table 1. A comparison of the [^{3}H]MK801 binding parameters to control and EDAC-treated adult rat brain membranes

	K_D (nM)	B_{max} (fmol [^{3}H]MK801 binding sites/mg protein)
Control membranes	5.0 ± 0.8	1820 ± 192
EDAC-treated membranes*	6.8 ± 0.9	538 ± 26

* The concentration of EDAC used was 5 mM.
Values are the means ± SD for three independent determinations.

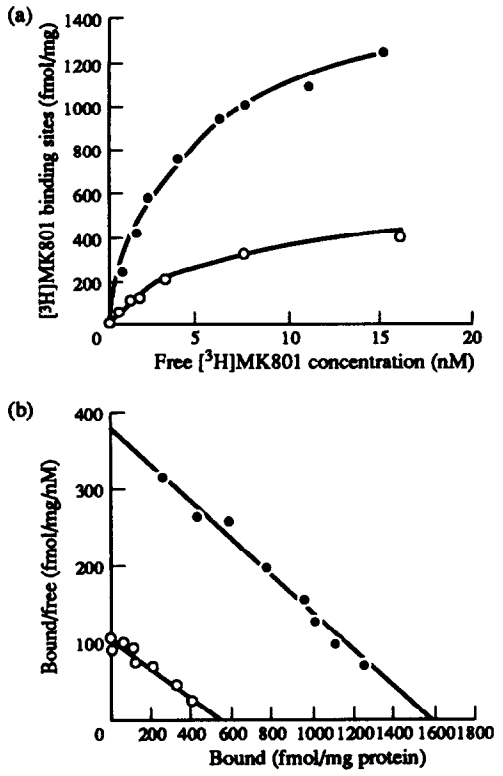

Fig. 3. Saturation analysis of [^{3}H]MK801 binding to control and EDAC-treated adult rat brain membranes. Adult rat brain membranes (750 μg protein/mL) were incubated in the presence and absence of 5 mM EDAC for 1 hr at 25°. Membranes were washed by centrifugation and assayed for [^{3}H]MK801 specific ligand binding activity with a range of radioactive ligand concentrations using 10 μM MK801 for the measurement of non-specific binding. The figure is representative of three independent experiments. Each data point is the mean of triplicate determinations. (a) is the saturation curve and (b) the respective Scatchard transformation for (●) control and (○) EDAC-treated membranes. Values are summarized in Table 1.

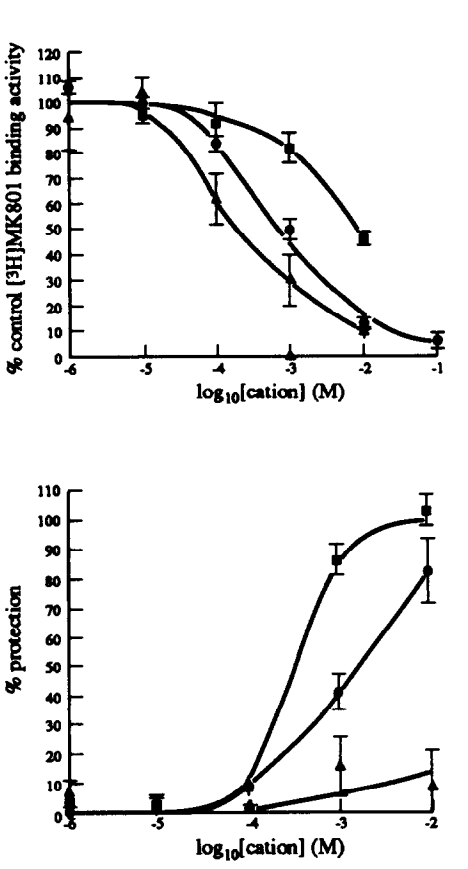

Fig. 4. The effect of cations on [^{3}H]MK801 binding to rat brain membranes and the protection against the inhibition of [^{3}H]MK801 binding by EDAC. (a) Adult rat brain membranes were assayed for [^{3}H]MK801 specific binding activity in the presence of a range of concentrations of cations. Assay incubation time was 2 hr at 22°, using a final concentration of 3 nM [^{3}H]MK801 and 10 μM MK801 for the measurement of non-specific binding. (b) Membranes were preincubated for 2 hr at 22° with a range of cation concentrations prior to their treatment with 5 mM EDAC for 1 hr at 25°. Membranes were washed by centrifugation and assayed for specific [^{3}H]MK801 binding activity. All appropriate control experiments were always carried out in parallel. These included the preincubation of non-EDAC-treated membranes in the presence or absence of the respective cations. For both (a) and (b), the results shown are from a representative experiment that was repeated at least three times with similar results and is summarized in Table 2. (▲) $ZnCl_2$; (●) $MgCl_2$ and (■) $CaCl_2$.

cysteine and tyrosine amino acids respectively as described above, had no effect on specific [^{3}H]-MK801 binding activity, it was concluded that indeed a carboxyl group(s) of either glutamate or aspartate is implicated in binding.

The inactivation or inhibition of [^{3}H]MK801 binding sites by EDAC was not prevented by prior incubation of membranes with MK801 or other non-competitive antagonists of glutamatergic transmission active at this site. However, the effect of EDAC was abolished by the prior incubation of membranes with Mg^{2+}. Further characterization of this phenomenon using other cations showed that the rank order of potency for protection was $Ca^{2+} > Mg^{2+} > Zn^{2+} > Na^+$ and K^+. This profile is consistent with that for the pharmacological specificity of the high affinity voltage-dependent Mg^{2+} binding site of the NMDA receptor channel [7]. This demonstrates that EDAC directly modifies carboxyl groups located within the Mg^{2+} binding site which indirectly attenuates the MK801 binding. It further suggests the close proximity of the two binding sites. Note that the EC_{50} value for protection of inactivation of [^{3}H]MK801 binding sites by EDAC for Ca^{2+} was not similar to the IC_{50} value for direct inhibition of binding thus suggesting that the Mg^{2+}-block and the Ca^{2+} binding sites are at distinct loci within the protein.

Recently, the cDNA sequences of two NMDA receptor subunit types have been reported [10–12]. In NMDAR1, it was noted that there are six

Table 2. A comparison between the EC_{50} values for the protection of [^{3}H]MK801 binding site inactivation by EDAC and the IC_{50} values for the direct inhibition of [^{3}H]-MK801 binding by cations

Cation	EC_{50} (μM)	IC_{50} (μM)
Mg^{2+}	1344 ± 607 (3)	1700 ± 656 (3)
Ca^{2+}	500 ± 344 (4)	8500 ± 500 (3)
Zn^{2+}	>10,000 (3)	400 ± 160 (3)
K^+	>10,000 (2)	>10,000 (2)

Values are the mean ± SD for (N) independent determinations.

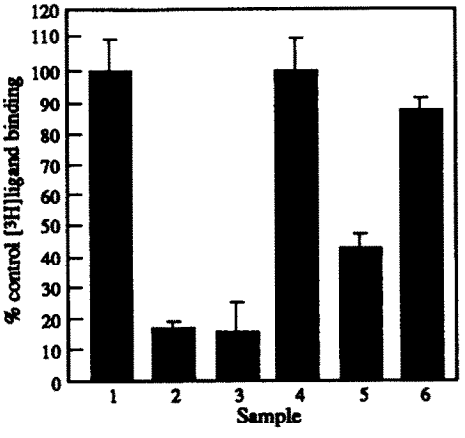

Fig. 5. The effect of Mg^{2+} preincubation on the inhibition of EDAC of [^{3}H]spiperone binding to the D_2 dopamine receptor of mammalian brain. Membranes were prepared from either bovine striatum or rat forebrain and treated with EDAC in the absence or presence of 10 mM Mg^{2+}. EDAC was removed by centrifugation and membranes were assayed for either specific [^{3}H]spiperone (lanes 1–3) or [^{3}H]MK801 (lanes 4–6) binding activities all as described in Materials and Methods. Lanes are: 1, control bovine striatal membranes; 2, bovine striatal membranes treated with 3 mM EDAC; 3, bovine striatal membranes treated with 3 mM EDAC in the presence of 10 mM $MgCl_2$; 4, control rat forebrain membranes; 5, rat forebrain membranes treated with 3 mM EDAC; 6, rat forebrain membranes treated with 3 mM EDAC in the presence of 10 mM $MgCl_2$. The results are the mean ± SD for three independent experiments.

contiguous acidic amino acids at the base of the putative channel domain, TM2. It was proposed that this region may be a potential Mg^{2+} binding site which would be in agreement with the important class of amino acids identified here. For the other subunit type, three isoforms have been described which when each is expressed in combination with NMDAR1 in mammalian cells showed differences in Mg^{2+} sensitivity [12]. Comparison between the three deduced amino acid sequences revealed the existence of two candidate positions where acidic amino acids are conserved between NMDA2A and NMDA2B but not with NMDA2C which does indeed exhibit the least sensitivity to Mg^{2+} block [12]. The first of these is predicted to be extracellular at the end of TM3 and the second is in the putative cytoplasmic loop region adjacent to TM4 (residues 655 and 788, NMDA2C numbering, respectively). It has been proposed that the site of the high affinity Mg^{2+} block is intracellular [13].

Further investigation of these candidate acidic amino acids by site-directed mutagenesis may demonstrate unequivocally their role or otherwise in Mg^{2+} regulation of the NMDA receptor channel. Note that a recent report from the group of Mishina [14] showed, as described here, a close association between the MK801 binding site and the Mg^{2+}-block site. Additionally, mutagenesis of the above-described contiguous acidic amino acids of NMDAζ1 (i.e. the mouse homologue of NMDAR1) had only a small effect on the Mg^{2+}-block site compared to mutagenesis of asparagine 589 in TM2 [14].

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