Neurotransmitter 2015; 2: e945. doi: 10.14800/nt.945; © 2015 by Julia Hnat, *et al*.
http://www.smartscitech.com/index.php/nt

BRIEF REPORT

Radioligand kinetics at the NMDA receptor channel revisited: An active part by Na^+ and Tris

Julia Hnat[1], Michael L. Berger[2]

[1]*FH Campus Wien, University of Applied Sciences, A-1100 Vienna, Austria*
[2]*Center for Brain Research, Medical University of Vienna, A-1090 Vienna, Austria*

Correspondence: Michael L. Berger
E-mail: michael.berger@meduniwien.ac.at
Received: August 03, 2015
Published online: August 19, 2015

[^{3}H]MK-801 is widely used as a marker for NMDA receptors (NRs), indicating the opening frequency of the associated ion channel. Association and dissociation of this radioligand in membranes prepared from rat cerebral cortex were accelerated by Na^+ ions (EC_{50} 70 mM, n_H 3). The widely used primary amine buffer tris(hydroxymethyl) aminomethane (Tris) had similar influences, but not the tertiary amine buffer bis(2-hydroxyethyl) amino-tris(hydroxymethyl)methane (BisTris). These results are in agreement with previous physiological data suggesting a site for Na^+ ions increasing the opening frequency of the NR.

Keywords: NMDA receptor; sodium ions; association rate; ion channel; MK-801; Tris buffer; rat brain

To cite this article: Julia Hnat, *et al*. Radioligand kinetics at the NMDA receptor channel revisited: An active part by Na^+ and Tris. Neurotransmitter 2015; 2: e945. doi: 10.14800/nt.945.

Introduction

The NMDA receptor (NR) represents the most frequent receptor for the excitatory neurotransmitter glutamic acid (Glu); as an ionotropic receptor, it controls the conductance of a cationic ion channel [1]. The NR channel ligand [^{3}H]MK-801 was repeatedly invested in kinetic studies under various conditions (see supplementary Table S1). Although this radioligand had been introduced originally in a near-physiological buffer, with 118 mM Na^+ as the main cationic ingredient [2], none of the ensuing investigations kept to this protocol. As often with biological assays, physiological conditions were abandoned as soon as it was realized that much higher signals were possible under more restricted conditions, with only one single cationic species present at rather low concentration (in the following addressed as *low ionic strength conditions*). Under these conditions, the affinity of the radioligand was at its maximum, and the kinetics were comfortably slow, with practically no losses during terminal washing procedures and excellent specific binding (≥90%). At neutral pH, [^{3}H]MK-801 (a secondary amine) carries a positive charge and is in competition with other cations during binding to its target site at the narrow constriction of the NR cation channel; this might explain reduced specific binding in presence of Na^+ or positively charged buffer ions as e.g. the widely used tris(hydroxymethyl)aminomethane (Tris; a primary amine). Here, we describe kinetic experiments with suspensions prepared from rat cerebral cortex and with [^{3}H]MK-801 in presence of a wide range of Na^+ or Tris concentrations, in comparison with results obtained with the less popular buffer bis(2-hydro xyethyl)amino-tris(hydroxy-methyl)methane (BisTris; a tertiary amine).

Methods

Membrane preparation

Tissues were dissected from adult male Wistar rats and disintegrated at 4°C in 50 mM Tris acetate (pH 7.0) with a

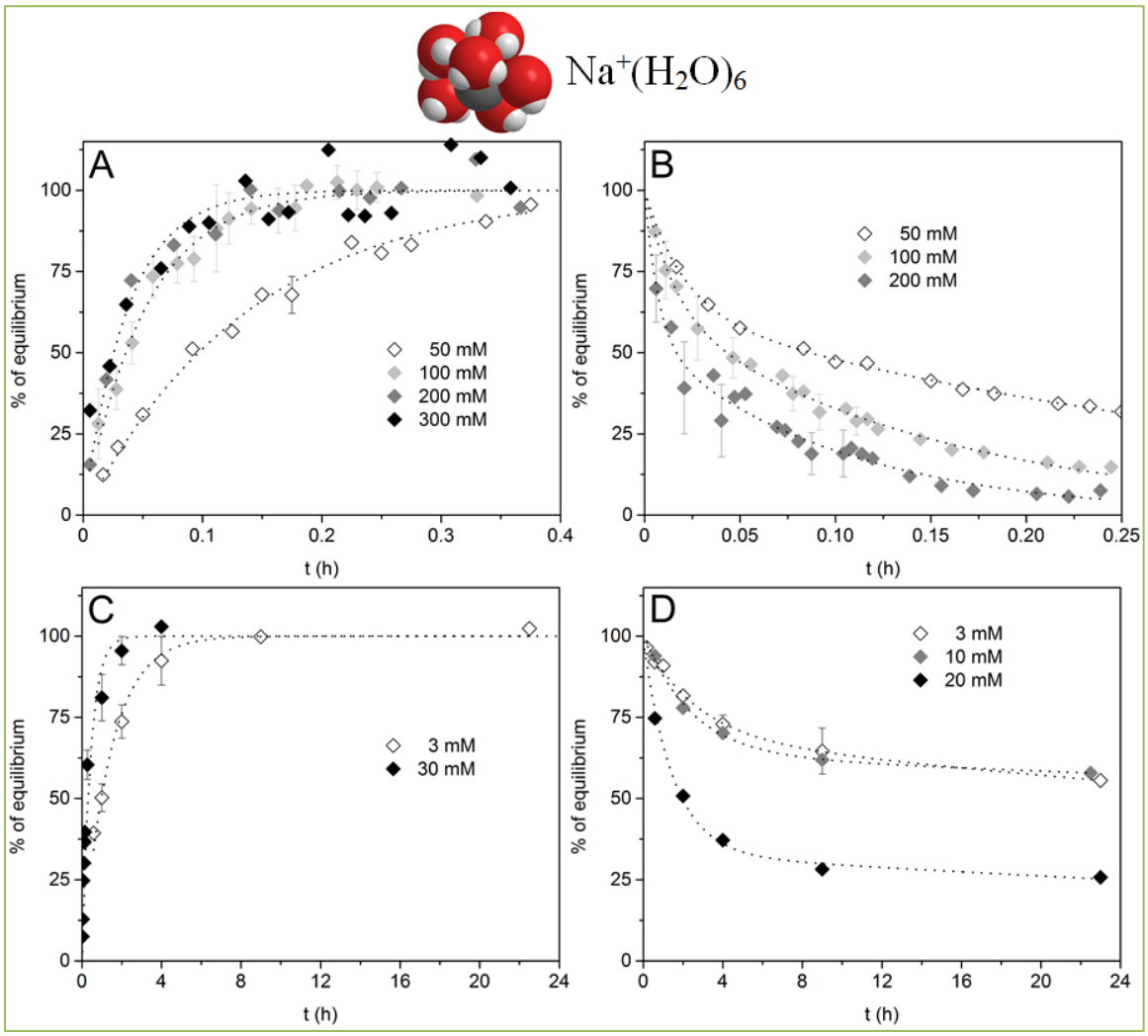

Figure 1. [^{3}H]MK-801 kinetics, dependence on Na^+. Association (A, C) and dissociation (B, D) of [^{3}H]MK-801 to/from rat cortical membranes (mean values, ± SD if n > 2) get progressively faster (A, B) at [Na^+]>50 mM, but get very slow (C, D; note different time scale) at [Na^+]<50 mM. Upper left insert: space-filling model of hydrated Na^+ (ChemBio 3D Ultra).

glass/Teflon homogenizer. Suspensions were supplemented with EDTA to 3 mM and centrifuged at 35.000 x g. The particulate fraction were suspended in fresh buffer (without EDTA), centrifuged a 2nd time, suspended, and left in a 23°C water bath for 2 h for more efficient removal of endogenous compounds. After a 3rd centrifugation, aliquot suspensions were stored at -80°C. For binding experiments, one suspension aliquot was thawed and divided into 2-3 equal portions. Each portion was filled up to 10 ml with the respective incubation buffer, supplemented with Triton X-100 to a final concentration of 100 µM, allowed to stand for 20 min in a 23 °C water bath, and centrifuged a last time. Also this treatment by detergent was intended to improve removal of endogenous interfering compounds [3].

Association time course

Associations were conducted with saturating concentrations of Glu and Gly (each 10 µM, inducing the open conformation of the channel) and 3.9 nM [^{3}H]MK-801 (ARC, 40 Ci/mMol) in a 23 °C water bath, with membranes corresponding to ca. 1 mg original tissue per sample. Influence of Na^+ was studied in 10 mM HEPES buffer (pH 7.0). To bring HEPES to pH 7.0, 3 mM NaOH is necessary; higher Na^+ concentrations were achieved by adding NaCl. For non-specific binding, 100 µM (*S*)-ketamine was added. For kinetic experiments, 2 different strategies were applied. To follow up slow processes from 0.5 to 24 h at low ionic strength conditions, all samples were started together in one volume and collected individually at each time point (0.5 ml, duplicates) on a vacuum filtration manifold (10 places, Hoefer Inc.) equipped with GF-C filters (pre-soaked in 0.3% polyethylene imine). For fast processes, samples were started at individual time points (single values) and collected

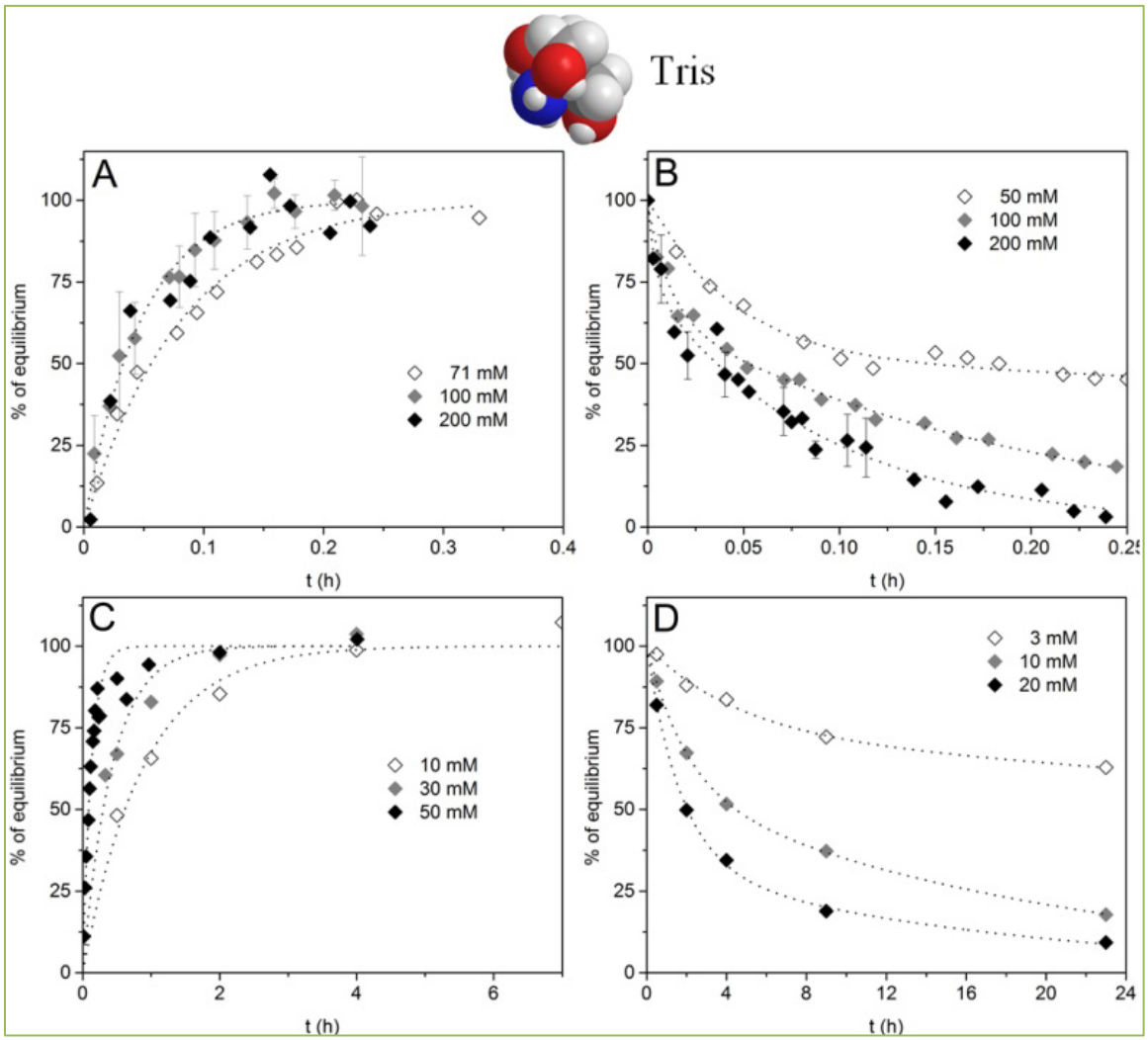

Figure 2. [^{3}H]MK-801 kinetics, dependence on Tris. Association (A, C) and dissociation (B, D) of [^{3}H]MK-801 to/from rat cortical membranes get progressively faster (A, B) at [Tris]>50 mM, but get very slow (C, D; note different time scale) at [Tris]<50 mM. Upper left insert: space-filling models of Tris (ChemBio 3D Ultra).

together 10 s to 30 min later on a Brandel harvester (48 places; GF-C filter).

Dissociation time course

Dissociation was preceded by exhaustive association over 1-16 h (depending on the buffer studied), but with 1 µM instead of 10 µM Glu & Gly (for a better realization of the closed-channel condition after their more efficient removal). Equilibrated membranes were centrifuged at 23°C, the supernatant (with the free radioligand) removed, and pellets rinsed superficially with 1 ml of the respective buffer and then suspended therein to the appropriate volume (by repeated pipetting). Also for dissociation, 2 different strategies were chosen for slow and for fast processes. For both approaches, we added 100 µM (*S*)-ketamine (final concentration) to exclude re-association of released radioligand. In low ionic strength buffers (with slow kinetics), dissociation for all samples was started together in one volume for each buffer condition (3 buffer conditions in parallel) after resuspension of the superficially rinsed 'collective' pellets. The dissociation process was started by adding Glu & Gly to final concentrations of 10 µM each. After 0.5 to 24 h in a 23 °C water bath, 0.5 ml aliquots (duplicates) were removed and filtered individually on the Hoefer filtration manifold (GF-C filters). For fast processes, 2-3 rinsed pellets were suspended separately in the corresponding buffer (2-3 buffer conditions in parallel) and distributed to 48 glass vials (still without any Glu & Gly). Dissociation was started by adding Glu & Gly (final concentration 10 µM). In 8 of the 48 vials, no Glu or Gly was added but the respective antagonists *D*-2-amino-5-phosphono acid (*D*-APV) and 5, 7-dichlorokynurenic acid (DCKA; final concentrations 10 & 1 µM, respectively; closed-channel condition). While we saw substantial release of radioligand

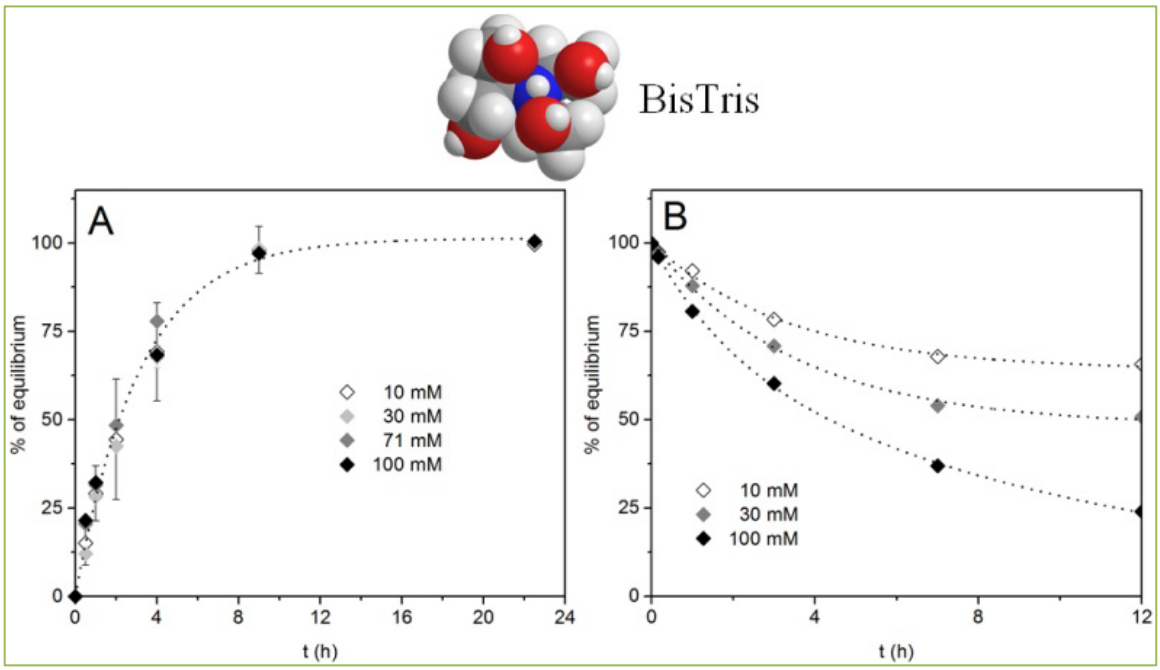

Figure 3. [^{3}H]MK-801 kinetics, dependence on BisTris. Association of [^{3}H]MK-801 to rat cortical membranes was not influenced by BisTris (A); however, the fraction of 'non-dissociable' radioligand was reduced (B). Left insert: space-filling model of BisTris (ChemBio 3D Ultra).

from the membranes starting immediately after addition of 10 µM Glu & Gly during moderate ionic strength conditions, the 8 samples without Glu & Gly but with *D*-APV & DCKA yielded consistently high values without any decay for at least 30 min; the mean of these values was used as binding value at time point zero (starting value) for computer analysis of exponential decay.

Quantification and analysis

Filters were washed 3 times with 5 ml BisTris·HCl (10 mM, pH 7.0) at 23 °C, since this buffer (in contrast to Tris·HCl and HEPES/Na^+) did not accelerate radioligand dissociation. Washed filters were transferred to counting vials, supplied with toluene-based scintillation cocktail, agitated for 20 min, and quantified in a beta-scintillation counter. After subtraction of non-specific binding, specific binding was related to time by non-linear least-squares curve fitting to mono- or bi-exponential functions.

Results

In their original publication introducing the radioligand [^{3}H]MK-801, Wong *et al.* [1] supplied already cursory information on kinetics in presence of 118 mM Na^+: equilibrium was attained in 30 min, and dissociation occurred with a $t_{½}$ of 3 min. Our results obtained in 100 mM Na^+ agree with both numbers (Fig. 1 A & B, light grey diamonds). Association time course was well described by mono-exponential functions (dotted lines); association rate increased sharply from 50 to 100 mM, reaching a plateau after 200 mM (with association $t_{½}$ below 2 min). Also dissociation rate increased with Na^+ concentration (Fig. 1 B & D), but the dissociation time course was at all Na^+ concentrations a composite of faster and slower processes.

Similar results were obtained for the cationic buffer Tris (Fig. 2). Also here, association rate increased sharply from 50 to 100 mM (Fig. 2A), and association time course followed mono-exponential functions (dotted lines). Also with various concentrations of Tris, dissociation time course appeared as a composite of faster and slower processes (B & D). In contrast to Na^+ and to the primary amine buffer Tris, the tertiary amine buffer BisTris had no influence on the association rate of [^{3}H]MK-801 to rat cortical membranes (Fig. 3A). With all BisTris concentrations tested, we observed very slow association, comparable to the slowest values observed with low Na^+ or Tris concentrations ($t_{½}$ of association ca. 2 h). Also in BisTris buffer, the dissociation time course appeared as a composite of 2 processes; BisTris did not accelerate either of these, but did reduce the fraction with very slow kinetics (Fig. 3B).

Non-linear curve fitting of the association time course under various buffer conditions to the mono-exponential function $B(t) = B_{eq} \cdot (1 - e^{-k_{on} \cdot t})$ yielded estimates for the parameters B_{eq} (equilibrium binding at the radioligand concentration 3.9 nM) and k_{on} (observable association rate

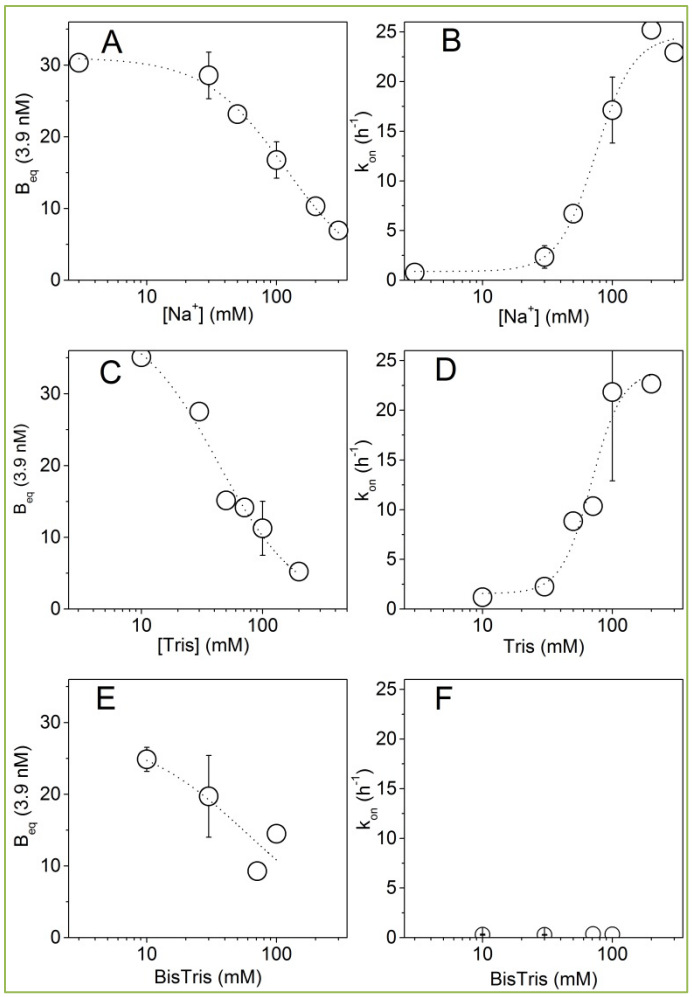

Figure 4. [^{3}H]MK-801 association parameters, summary of influences. All 3 cations had comparable impact on B_{eq} (specific binding at equilibrium; A, C, E); however, only Na^+ and Tris increased the association rate (B, D), not BisTris (F).

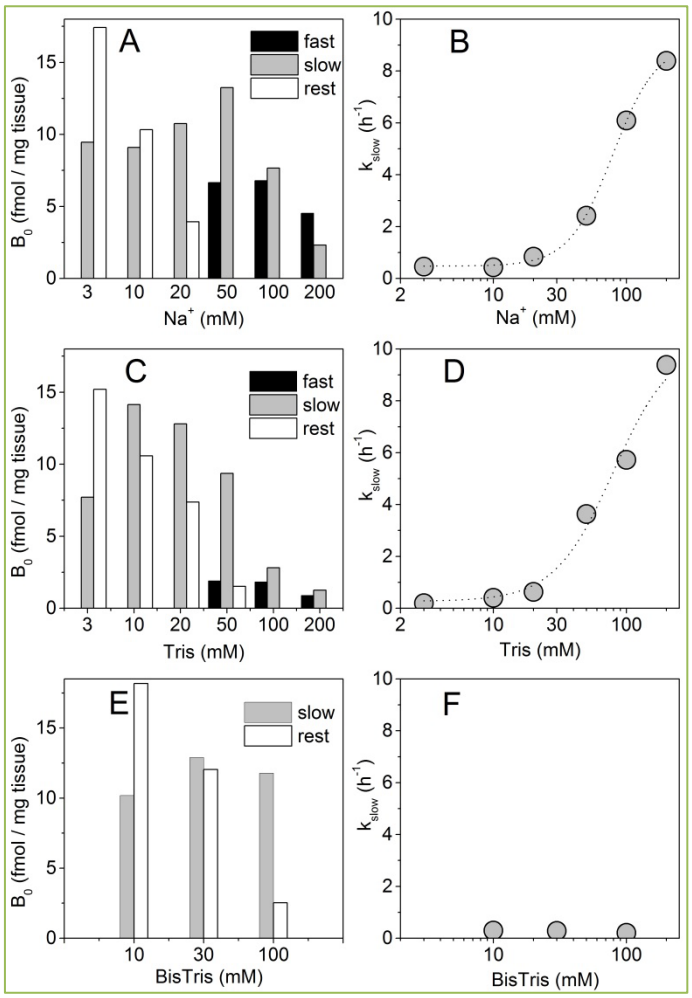

Figure 5. [^{3}H]MK-801 dissociation parameters, summary of influences. Under low ionic strength conditions, most radioligands dissociated very slowly (white columns, 'rest'); with increasing ionic strength, this fraction got smaller and disappeared at 50-100 mM. At higher concentrations, an extremely fast component appeared (black columns, 'fast'), with k_{off} amounting to more than 100 h^{-1}.

constant) presented in Fig. 4. Na^+, Tris and BisTris reduced B_{eq} with comparable potencies (we obtained IC_{50} values of 118 mM for Na^+ and of 43 mM for Tris). Both Na^+ and Tris increased k_{on} with EC_{50}s near 70 mM and steep concentration dependencies (n_Hs close to 3). For BisTris, k_{on} values are presented in Fig. 4F on the same scale as for Na^+ (B) and Tris (D); the numbers amounted to 0.30, 0.28, 0.36, and 0.34 h^{-1} for 10, 30, 71, and 100 mM BisTris, respectively. For Na^+ and Tris, similar lower limiting values had been observed. Apparently, association of the radioligand was not completely dependent on the presence of cations, but markedly facilitated by Na^+ and by Tris, from 0.3 h^{-1} to 24 h^{-1} (corresponding to a reduction in association $t_{½}$ from 2 h to 1.7 min, at radioligand concentration 3.9 nM).

Non-linear curve fitting of the dissociation time course under various buffer conditions required the bi-exponential function $B(t) = B_1 \cdot e^{-k_{fast} \cdot t} + B_2 \cdot e^{-k_{slow} \cdot t}$, with faster and slower dissociation rate constants. Under low ionic strength conditions, k_{slow} often was close to zero, suggesting a non-dissociable fraction of the radioligand ('rest' in Fig. 5). Fractions with very slow dissociation, with $t_{½}$ amounting to several hours, have been reported previously under low ionic strength conditions [4, 5] and related to diffusion of the radioligand along a hydrophobic path bypassing the channel. We observed (Fig. 5, empty columns) that these extremely slow fractions were progressively reduced with increasing concentration of Na^+ (A), Tris (C), and BisTris (E). Nevertheless, also with higher cation concentrations dissociation of [^{3}H]MK-801 stayed biphasic, apparently due to the appearance of a very fast component (black columns in Fig. 5). The component termed 'slow' in Figure 5 was accelerated by Na^+ and Tris (B & D) in a way reminiscent of the acceleration of association, with EC_{50}s near 80 mM and

n_Hs 2-3; however, k_{slow} was as insensitive to BisTris (F) as k_{on} has been.

Discussion

Our observation that the kinetics of [^{3}H]MK-801 were accelerated by the mono-cations Na^+ and Tris, but not by the mono-cation BisTris, excludes non-specific mechanisms as e.g. a general influence of increasing ionic strength on the diffusion of the positively charged radioligand. More likely, the accelerating effect was mediated by specific sites at the NR, accepting some cations but not others. As illustrated in the inserts to Figures 1-3, hydrated Na^+ and Tris may occupy a comparable fraction of space (Na^+ is depicted as surrounded by 6 coordinated H_2O molecules, the most likely state in aqueous solution [6]). BisTris, however, may be too big to fit into the putative site mediating the accelerating influence of Na^+ and Tris.

NRs in hippocampus and cerebral cortex are dominated by triheteromeric assemblies of the subunits GluN1, GluN2A and GluN2B [7, 8, 9]; nevertheless, the population of binding sites for [^{3}H]MK-801 was probably not homogenous in our rat cortical membrane preparation, and heterogeneous kinetics might have resulted from various NR subpopulations. Although association time course appeared roughly monophasic under all conditions studied by us, eventual heterogeneity as seen by several authors (see supplementary Table S1) may have turned up with a more detailed range of time points. Biphasic dissociation was obvious under all conditions, even with our rather limited protocol. A very slowly dissociating fraction was seen at low ionic strength conditions (although both agonist sites were fully saturated and the channel, therefore, can be expected to be in the open state); our results with increasing cation concentrations predict that this component will not exist under physiological conditions and thus could be considered as a by-product of artificial low ionic strength conditions. The heterogeneity of [^{3}H]MK-801 dissociation under various buffer conditions may be due to the special location of this binding site in an aqueous pore in close vicinity to a hydrophobic medium. The *open channel blocker* MK-801, once arrived at the channel's narrow constriction, does not impede channel closure and is trapped in the membranous aqueous phase [10] (channel + *vestibule*) once the co-agonists have detached from their sites. Depending on ionic strength, MK-801 may during hours slowly distribute into the inner leaflet of the lipid bilayer and leave the neighborhood of the channel. From there, it will not easily find its way back, giving rise to the 'non-dissociable' fraction. On the other hand, in closed channels specifically bound [^{3}H]MK-801 may detach from and re-associate with its binding site and therefore, from time to time, diffuse freely in the channel and vestibule (especially in absence of any voltage in disintegrated membranes); this transiently free fraction may 'dissociate' first with a disproportionately high rate constant, giving rise to the bi-exponential dissociation seen under higher ionic strength conditions. Only the 'slow' component (grey in Figure 5; between 0.2 and 10 h^{-1}) stood in plausible relation to the k_{on} values observed, compatible with dissociation equilibrium constants (K_D) described for this radioligand (between 2 and 40 nM).

Candidates for Na^+ sites at the NR channel have been described. An intra-cellular site mediating stimulatory effects of Na^+ after influx was reported by Yu and Salter [11]. Influx of Na^+ through voltage-dependent channels has been shown to increase activity of nearby NRs and to promote neurite outgrowth [12]. Since we studied disintegrated membranes, access for cations was possible at the outside as well as at the inside. Another candidate might be a recently described polyanionic site at the interface between the extracellular aminoterminal domains of NR subunits GluN1 and GluN2B [13]. The transition of this interface between open and closed conformations tunes the open probability of the NR channel between low and high. In the absence of cations (or at low ionic strength conditions), the acidic amino acid residues at this interface will repel each other electrostatically and push the conformation of the channel into the low open probability mode. Sodium and Tris cations may be in the position to neutralize these negative charges and thereby relieve the repulsion.

Abbreviations

Tris: tris(hydroxymethyl)aminomethane; BisTris: bis(2-hydroxyethyl)amino-tris(hydroxymethyl)methane; NR: NMDA receptor; *D*-APV: *D*-2-amino-5-phosphonovaleric acid; DCKA: 5,7-dichlorokynurenic acid.

Author's contributions

JH has performed the experiments; MLB has planned and supervised the experiments, analyzed the results and written the article.

References

1. Traynelis SF, Wollmuth LP, McBain CJ, Menniti FS, Vance KM, Ogden KK *et al*. Glutamate receptor ion channels: structure, regulation, and function. Pharmacol Rev 2010; 62:405-496.
2. Wong EHF, Kemp JA, Priestley T, Knight AR, Woodruff GN, Iversen LL. The anticonvulsant MK-801 is a potent NMDA antagonist. Proc Natl Acad Sci USA 1986; 83:7104-7108.
3. Yoneda Y, Ogita K, Ohgaki T, Uchida S, Meguri H. NMDA-sensitive [^{3}H]glutamate binding sites in brain synaptic membranes treated with Triton X-100. Biochim Biophys Acta 1986; 1012:74-80.

4. Javitt DC, Zukin SR. Biexponential kinetics of [^{3}H]MK-801 binding: evidence for access to closed and open NMDA receptor channels. Mol Pharmacol 1989; 35: 387-393.

5. Reynolds IJ. 1,5-(Diethylamino)piperidine, a novel spermidine analogue that more specifically activates the NMDA receptor associated polyamine site. Mol Pharmacol 1992; 41:989-992.

6. Mahler J, Persson I. A study of the hydration of the alkali metal ions in aqueous solution. Inorg Chem 2012; 51:425-438.

7. Luo J, Wang Y, Yasufa RP, Dunah AW, Wolfe BB. The majority of NMDA receptor complexes in adult rat cerebral cortex contain at least 3 different subunits (NR1/NR2A/NR2B). Mol Pharmacol 1997; 51:79-86.

8. Rauner C, Köhr G. Triheteromeric NR1/NR2A/NR2B receptors constitute the major NMDA receptor population in adult hippocampal synapses. J Biol Chem 2011; 286:7558-7566.

9. Tovar KR, McGinley MJ, Westbrook GL. Triheteromeric NMDA receptors at hippocampal synapses. J Neurosci 2013; 33:9150-9160.

10. Huettner JE, Bean BP. Block of NMDA-activated current by the anticonvulsant MK-801: Selective binding to open channels. Proc Natl Acad Sci USA 1988; 85:1307-1311.

11. Yu X-M, Salter MW. Gain control of NMDA-receptor currents by intracellular sodium. Nature 1998; 396:469-474.

12. George J, Dravid SM, Prakash A, Xie J, Peterson J, Jabba SV *et al*. Sodium channel activation augments NMDA receptor function and promotes neurite outgrowth in immature cerebrocortical neurons. J Neurosci 2009; 29:3288-3301.

13. Mony L, Zhu S, Carvalho S, Paoletti P. Molecular basis of positive allosteric modulation of GluN2B NMDA receptors by polyamines. EMBO J 2011; 30:3134-3146.

Supplement

Table S1. Published rate constants for [^{3}H]MK-801 binding to rat brain tissue. Association conditions include radioligand concentration, temperature, buffer, and further additions (if any). Dissociation conditions include preceding association time and mode of initiating dissociation; k_{on} is the observable association rate constant (depending on the radioligand concentration). For bi-exponential time courses, both constants are indicated, with the percentage of the faster process in parentheses

		association		dissociation		Ref
tissue	treatment	condition	k_{on} (h^{-1})	condition	k_{off} (h^{-1})	
No extensive treatment of membranes:						
cerebral cortex, 0.32 M sucrose, P2	20 min 23 °C	2 nM 23 °C 5 mM Tris-Cl pH 7.4	26 (81%), 2.1*	1 h association, 100 µM MK-801	12.7 (46%), 3.6*	[1]
whole brain, total membranes	suspension 2 x frozen	1 nM 20-24 °C 20 mM HEPES/Na^+ pH 7.4 100 µM Glu, 30 µM Gly	[0.39]†	1½ h association, 'infinite' dilution	6.12 (19%), 0.53*	[2]
whole brain, total membranes	suspension 2 x frozen	2-3 nM 20-24 °C 20 mM HEPES/Na^+ pH 7.4 100 µM Glu, 30 µM Gly	85 (18%), 1.3	1½ h association, 'infinite' dilution	7.4 (6%), 0.22*	[3]
whole brain, 'buffy coat' membranes	none	5 nM 30 °C 50 mM Tris-Ac pH 7.4	1.91*			[4]
whole brain, 'buffy coat' membranes	none	5 nM 30 °C 50 mM Tris-Ac pH 7.4 10 µM Glu & Gly	61 (38%), 8.9*			[5]
whole brain, 'buffy coat' membranes	suspension 2 x frozen	5 nM 30 °C 50 mM Tris-Ac pH 7.4 10 µM Glu & Gly	89 (36%), 10.0*	16 h association, 100 µM MK-801	45 (43%), 3.6*	[6]
whole brain, total membranes	suspension 2 x frozen	3 nM 20-24 °C 10 mM HEPES/Na^+ pH 7.4 100 µM Glu, 30 µM Gly	52 (23%), 1.56	2 h association, 'infinite' dilution	2.68 (18%), 0.19	[7]
forebrain, total membranes	none	8 nM 25 °C 5 mM HEPES/Tris pH 7.4 10 µM Gly, no Glu added	[0.11]†	18 h association, 200 µM PCP	0.011	[8]
forebrain, P2	none	3-5 nM 25 °C 5 mM HEPES/K^+ pH 7.4 10 µM Glu & Gly	2.64			[9]
cerebral cortex, 0.32 M sucrose, total sediment without P1	30 min 23 °C, frozen in 0.32 sucrose	4 nM 30 °C 50 mM Tris-Cl pH 7.4 10 µM Glu, 10 nM Gly	290	30 min association 100 µM MK-801	19.0	[10]
Prolonged pre-incubation above 30 °C or treatment with Triton X-100:						
cerebral cortex, total membranes	30 min 37 °C	5 nM 25 °C 20 mM HEPES/Na^+ pH 7.4 1 µM Glu & Gly 10 µM Mg^{2+}	9.0	2 h association, 100 µM PCP	6.0	[11]
cerebral cortex & hippocampus, 'buffy coat' membranes	2 x 1 h 37 °C	1 nM 25 °C 5 mM Tris-Ac pH 7.4 10 µM Glu & Gly 30 µM Mg^{2+}	3.0 (93%), 0.16	16 h association, 100 µM TCP	2.88 (72%), 0.012	[12]
whole brain, 'buffy coat' membranes	10 min 2 °C 0.08% Triton X-100	5 nM 30 °C 50 mM Tris-Ac pH 7.4 10 µM Glu & Gly	10.8 (26%), 1.93*			[5]
cerebral cortex & hippocampus, 'buffy coat' membranes	2 x 1 h 37 °C	1 nM 25 °C 5 mM Tris-Ac pH 7.4 10 µM Glu + 50 µM *D*-APV, 10 µM Gly, 30 µM Mg^{2+}	8.4 (50%), 0.53			[13]
whole brain, synaptic plasma membranes	4 x 30 min 32 °C	7 nM 32 °C 20 mM HEPES/K^+ pH 7.0 100 µM Glu & Gly	11 (37%), 0.52*	3 h association, 10 µM MK-801	1.17 (34%), 0.14*	[14]
whole brain, 'buffy coat' membranes	10 min 2 °C 0.08% Triton X-100	5 nM 30 °C 50 mM Tris-Ac pH 7.4 10 µM Glu & Gly	15.4 (49%), 0.88*	16 h association, 100 µM MK-801	5.51 (38%), 0.35	[15]
whole brain, 'buffy coat' membranes	2 x 1 h 37 °C	1 nM 25 °C 5 mM Tris-Ac pH 7.4 10 µM Glu, 50 µM Gly 30 µM Mg^{2+}	9.0 (62%), 1.01			[16]
cerebral cortex, total membranes	suspension 2 x frozen, 30-60 min 37 °C	0.6 nM 21-25 °C 10 mM HEPES/Na^+ pH 7.4 100 µM Glu, 30 µM Gly		2 h association, 'infinite' dilution	0.23	[17]
whole brain, total membranes	suspension 2 x frozen	3 nM 20-22 °C 10 mM HEPES/Na^+ pH 7.4	65 (21%), 0.99	2 h association, 'infinite' dilution	0.23	[18]

	30-60 min 37 °C	100 µM Glu, 30 µM Gly				
Solubilization:						
whole brain, total membranes	deoxycholate / glycerol	10 nM 30 °C 50 mM Tris-Ac pH 7.4 10 µM Glu & Gly	1.34			[19]
cerebral cortex & hippocampus, P2	deoxycholate / glycerol	7 nM 23 °C 20 mM HEPES/K^+ pH 7.0 100 µM Glu & Gly 1 mM EDTA	0.19	5 h association, 10 µM MK-801	0.052	[20]
Tissue slices:						
cerebral cortex, striatum & hippocampus	tissues combined, mashed & frozen	8 nM 22 °C 30 mM HEPPS/Na^+ pH 7.45 100 µM Glu & Gly 1 mM EDTA	0.70	2½ h association, 200 µM ketamine	0.31	[21]
frontal cortex	30 min 4 °C	5 nM 23 °C 50 mM Tris-Ac pH 7.4 no additions (no enhancement with NMDA)	2.6*	2 h association, 'infinite' dilution	0.32*	[22]

*, data presented graphically in the reference; individual data points were measured directly from the presented figures und subjected to non-linear curve fitting; †, true association rate constant ($h^{-1} \cdot nM^{-1}$)

References

1. Wong EHF, Knight AR, Woodruff GN. [^{3}H]MK-801 labels a site on the NMDA receptor channel complex in rat brain membranes. J Neurochem 1988; 50:274-281.
2. Reynolds IJ, Miller RJ. Multiple sites for regulation of the NMDA receptor. Mol Pharmacol 1988; 33:581-584.
3. Reynolds IJ, Miller RJ. Ifenprodil is a novel type of NMDA receptor antagonist: interaction with polyamines. Mol Pharmacol 1989; 36:758-765.
4. Yoneda Y, Ogita K. Labeling of NMDA receptor channels by [^{3}H]MK-801 in brain synaptic membranes treated with Triton X-100. Brain Res 1989; 499:305-314.
5. Yoneda Y, Ogita K, Suzuki T. Interaction of strychnine-insensitive glycine binding with MK-801 binding in brain synaptic membranes. J Neurochem 1990; 55:237-244.
6. Enomoto R, Ogita K, Han D, Yoneda Y. Differential modulation by divalent cations of [^{3}H]MK-801 binding in brain synaptic membranes. J Neurochem 1992; 59:473-481.
7. Reynolds IJ. 1,5-(Diethylamino)piperidine, a novel spermidine analogue that more specifically activates the NMDA receptor-associated polyamine site. Mol Pharmacol 1992; 41:989-992.
8. Boje KM, Skolnick P. Ontogeny of glycine-enhanced [^{3}H]MK-801 binding to NMDA receptor-coupled ion channels. Developm Brain Res 1992; 65:51-56.
9. Marvizón J-C, Baudry M. [^{3}H]Dizocilpine association kinetics distinguish stimulatory and inhibitory polyamine sites of NMDA receptors. J Neurochem 1994; 63:963-971.
10. Reinés A, Peña C, Rodriguez de Lores Arnaiz G. Allosteric modulation of [^{3}H]dizocilpine binding to NMDA receptor by an endogenous Na+,K+-ATPase inhibitor: dependence on receptor activation. Brain Res 2004; 996:117-125.
11. Kloog Y, Nadler V, Sokolovsky M. Mode of binding of [^{3}H]dibenzocycloalkenimine (MK-801) to the NMDA receptor and its therapeutic implications. FEBS Lett 1988; 230:167-170.
12. Javitt DC, Zukin SR. Biexponential kinetics of [^{3}H]MK-801 binding: evidence for access to closed and open NMDA receptor channels. Mol Pharmacol 1989; 35:387-393.
13. Javitt DC, Frusciante MJ, Zukin SR. Rat brain NMDA receptors require multiple molecules of agonist for activation, Mol Pharmacol 1990; 37:603-607.
14. Williams K, Dawson VL, Romano C, Dichter MA, Molinoff PB. Characterization of polyamines having agonist, antagonist, and inverse agonist effects at the polyamine recognition site of the NMDA receptor. Neuron 1990; 5:199-208.
15. Yoneda Y, Ogita K, Enomoto R. Characterization of spermidine-dependent [^{3}H]MK-801 binding in brain synaptic membranes treated with triton X-100. J Pharmacol Exp Therap 1991; 256:1161-1172.
16. Sircar R, Zukin SR. Kinetic mechanisms of glycine requirement of NMDA channel activation. Brain Res 1991; 556:280-284.
17. Reynolds IJ, Palmer AM. Regional variations in [^{3}H]MK-801 binding to rat brain NMDA receptors. J Neurochem 1991; 56:1731-1740.
18. Rajdev S, Reynolds IJ. Effects of pH on the actions of dizocilpine at the NMDA receptor complex, Brit J Pharmacol 1993; 109:107-112.
19. Ogita K, Yoneda Y. Solubilization of spermidine-sensitive [^{3}H]MK-801 binding activity from rat brain. J Neurochem 1990; 55:1515-1520.
20. Romano C, Williams K, Molinoff PB. Polyamines modulate the binding of [^{3}H]MK-801 to the solubilized NMDA receptor. J Neurochem 1991; 57:811-818.
21. Subramanian S, McGonigle P. Quantitative autoradiographic characterization of the binding of [^{3}H]MK-801 in rat brain: regional effects of polyamines. J Pharmacol Exp Therap 1991; 256:811-819.
22. Sakurai SY, Cha J-HJ, Penney JB, Young AB. Regional distribution and properties of [^{3}H]MK-801 binding sites determined by quantitative autoradiography in rat brain. Neurosci 1991; 40:533-543.