Differential influence of 7 cations on 16 non-competitive NMDA receptor blockers

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The specific binding of the NMDA receptor (NR) channel ligand [3H]MK-801 to rat brain membranes is sensitive to positively charged buffer ingredients as to tris(hydroxymethyl)aminomethane (Tris), to Na+, or to protons. Here we demonstrate that 16 non-competitive NR antagonists, including 5 long-chain diamines, classical NR channel blockers and several less known compounds, differ widely in their sensitivities to cationic buffer constituents. Although chemically distinguished either as extended di-cationic or as compact mono-cationic, their sensitivities to cationic buffer ingredients did not suggest this grouping. While the di-cationic compounds are known for their sensitivity to spermine (polyamine inverse agonists), also some of the mono-cationic blockers exhibited this feature. They might share as common target a recently described negatively charged extracellular GluN1/GluN2B interface.

The NMDA receptor (NR) mediates Na+ and Ca2+ conductance in response to stimulation by the physiological co-agonists Glu and Gly. The associated ion channel of the NR subtype predominating in mammalian cerebral cortex and hippocampus not only exhibits exceptionally high Ca2+ conductance, but is also highly sensitive to inhibition by physiological concentrations of Mg2+.1 Under resting state conditions NRs are usually silent, with Mg2+ sitting at the narrow constriction. This Mg2+, however, quickly leaves the channel and gives way to Na+ and Ca2+, once the voltage ruling the channel is critically compromised, either during physiological depolarization or during pathological conditions (e.g., exhaustion of energy supply). Non-competitive NR antagonists (not directly competing with the co-agonists) have been proposed as potential neuro-protection in conditions as stroke, cardiac arrest or neurodegenerative disorders; however, in spite of demonstrated efficacy in vitro and in animal models, their clinical use is still limited, due to severe side effects.2 Here, we investigate the inhibitory potencies of 2 groups of these antagonists on the specific binding of the NR channel ligand [3H]MK-801 under various defined ionic conditions: one group consists of mono-cationic classical NR channel blockers and structural analogs; and the other of di-cationic structural analogs of the endogenous polyamines spermidine and spermine (Spm).4 The radioligand binding assay used allows free selection of defined ionic milieus without consideration of physiological consequences (as in living preparations), however with the limitation that one parameter essential for the efficacy of most channel blockers, voltage, is absent.

Membranes were prepared from fronto-parietal cortex and hippocampus of adult male Wistar rats.1 We have demonstrated before that NRs in these 2 regions exhibited similar properties.6 Binding was conducted with 5 nM [3H]MK-801 (Perkin–Elmer or ARC) in 0.5 ml in glass vials (duplicates or triplicates) for 3 h at 23 °C, achieving nearly equilibrium for all ionic conditions, in presence of 10 μM Glu and Gly. For non-specific binding (NB), Glu and Gly were replaced by their antagonists CGP-39.653 (10 μM; Novartis) and 5,7-dichloro kynurenic acid (10 μM; Tocris); all inhibitors tested reduced specific [3H]MK-801 binding down to these NB values at sufficiently high concentrations. Bound radioligand was collected on glass fiber filters with a 48 places Brandel harvester (filters rinsed 3 times at rt with 5 ml 10 mM Tris buffer, pH 7.0). After addition of toluene-based scintillation cocktail, radioactivity was quantified in a scintillation counter. IC50 values were evaluated by computerized curve fitting to the function

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B_x = B_0 \cdot \text{IC}_{50}^{x} + x^2 \times x + \text{NB},
\]

where \(B_0\) is total binding in presence of inhibitor at concentration \(x\), \(B_0\) total binding in absence of inhibitor, an \(h\) the Hill coefficient.

Di-cationic polyamine analogs (for chemical structures see Fig. 1) were 1,12-diaminododecane 1 (N12N) and arcaine 2 (both from Sigma–Aldrich); homopiperazine-1,4-bisbenzamidine 3 (a gift from Tien Huang, Xavier University, New Orleans LA);7 5-(4-aminobutyl)-2-thiopheneoctanamine 4 (N4T8N);8 and pentamidine 5 (Sigma–Aldrich).9 The methyl ester of (L)-Trp 6

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Figure 1. Influence of spermine (S, 30 μM), Tris (T, 10 → 50 mM), protons (H, pH 6.4 → 8.2), Na⁺ (N, 3 → 50 mM), K⁺ (K, 50 mM), ammonium (A, 20 mM), and Mg²⁺ (M, 1.3 mM) on the potency of 16 inhibitors of [³H]MK-801 binding; IC₅₀ values inscribed below structures obtained in 10 mM buffer (Tris or HEPES, pH 7.0). Plotted on the ordinates is the attenuation of this IC₅₀ under the influence of the respective buffer constituent.

Figure 2. Coefficients of determination $r^2$ obtained by pair-wise comparison of ionic influences on 16 inhibitors of [³H]MK-801 binding as illustrated in Figure 1. The frequency distribution of the 120 $r^2$ values is bi-modal (left panel). In the table below, $r^2$ values above 0.57 ($p<0.05$) have pink and values below 0.11 gray background. None of the apparent similarities remains significant after correction for repeated measurements, or after introduction of normally distributed noise.¹⁵
(Sigma–Aldrich) was included after extensive SAR studies\textsuperscript{10} to investigate whether it would fit better with the di-cationic or the mono-cationic group. \textit{N,N}-Didesmethyl-diphenhydramine 7 was a gift of Parke-Davis (Ann Arbor MI),\textsuperscript{11} memantine 8 a gift from Merz (Frankfurt am Main, Germany),\textsuperscript{12} and \textit{(S)}- and \textit{(R)}-ketamine 9 and 10 were gifts from Gödecke AG (Freiburg, Germany). \textit{(S)}-1,2-Diphenyl-2-propylamine 11 was provided by Astra Charnwood (Loughborough UK).\textsuperscript{13} Phencyclidine 12 and dextromethorphan 13 were from Sigma–Aldrich. Didesmethyl-chlorpromazine 14 (nor\textit{\textsuperscript{2}}-chlorpromazine) was prepared as part of the NIMH Chemical Synthesis Program and provided by RBI (Natick MA, contract N01MH30003). Desmethylnaproxilin 15 was a gift from Ciba-Geigy GmbH (Venna, Austria), and NPS 1392\textsuperscript{16} a gift from NPS Pharmaceuticals (Salt Lake City UT). The ions Tris, H\textsuperscript{+}, Na\textsuperscript{+}, K\textsuperscript{+} and NH\textsubscript{4}\textsuperscript{+} were inhibitory, Spm up to 30 \(\mu\text{M}\) was stimulatory, and Mg\textsuperscript{2+} was stimulatory up to 100 \(\mu\text{M}\) and inhibitory at higher concentrations.\textsuperscript{16} Non-competitive NR antagonists exhibited various degrees of sensitivities to ionic buffer constituents, most of them operating best as inhibitors of [\textsuperscript{3}H]MK-801 binding at low ionic strength conditions (10 mM Tris acetate or 10 mM HEPES + 3 mM Na\textsuperscript{+}, pH 7.0). Figure 1 gives an overview to the influences of 7 milieu constituents carrying

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3}
\caption{Inhibition of \textsuperscript{3}H]MK-801 binding to rat brain membranes by polyamine inverse agonists 1–5 (cyan), by (\textit{\textsuperscript{L}})-Trp methylester 6 (red), and by NMDA channel blockers 7–16 (black symbols): influence of physiological cations and of Tris. All plotted values are ratios of IC\textsubscript{50} (logarithmic scale); the basal condition for evaluating the influence of 30 \(\mu\text{M}\) spermine, 50 mM Tris and pH was 10 mM Tris acetate; for evaluating the influence of 50 mM Na\textsuperscript{+}, 50 mM K\textsuperscript{+}, 20 mM NH\textsubscript{4}\textsuperscript{+} and of 1.3 mM Mg\textsuperscript{2+}, 10 mM HEPES + 3 mM Na\textsuperscript{+} was the reference. Note that the 21 figures a–u are arranged in the form of a correlation table: each of the seven influences is compared to the other six (upper left corner a and lower right corner u have been moved). Many ionic influences were correlated to each other (with the exception of protons);* correlations surviving the introduction of random noise (p <0.05; see Fig. S1 and excel file Table S5, Supporting information).}
\end{figure}
positive charge at physiologic pH (see Supporting information for more detailed data, Table S1). The IC_{50} values at low ionic strength conditions are inscribed below the respective structures. Increasing Tris concentration (column ‘T’) from 10 to 50 mM raised the IC_{50} of 2 by a factor 4, but had no influence on 14, 15 and 16. Relative change was bigger for Na^+ (3 - 50 mM; column ‘N’): some IC_{50}s were shifted by a factor 10 or more (1, 2, 3). As expected, the polyamine spermine (column ‘S’) had a strong influence on long-chain di-cations – (polyamine inverse agonists), but also on 6, 7, 8, 9 and 11 (among them typical channel blockers).

While the pattern of 7 columns with differing heights in Figure 1 might suggest similarities between some compounds, we tried to quantify this apparent correspondence by applying a systematic correlation analysis. By comparing the pattern of sensitivities of the target of most di-cationic NR antagonists, but also of some of the mono-cationic ones.

Since inhibition of [H]MK-801 binding by the well tolerated NR antagonist 8 (memantine) was influenced by various cations in a way similar to the classical long-chain diamines 1 and 2, a closer investigation of compounds with this kind of signature may be warranted. In our collection, the lesser known compound 7 came close to this signature (Fig. 2). Didesmethyl-diphenhydramine is a metabolite of the antihistaminic diphenhydramine. We are not aware of any investigation into the neuropharmacological properties of this metabolite. The di-methylated parent compound inhibited [H]MK-801 binding with lower potency (IC_{50} 93 µM), as did the mono-methylated metabolite (IC_{50} 22 µM). Also 2 other primary amine metabolites of well-established psychoactive drugs were tested: 14 and 15. Their potencies were comparable to the potency of 7, and again the parent compounds chlorpromazine (IC_{50} 21 µM) and maprotiline (IC_{50} 38 µM) were weaker. In contrast to 7 and 8, 14 and 15 were only weakly influenced by cations (Fig. 1), suggesting differing modes of interaction with the target. The considerable potency at the NR of metabolites of widely used drugs is remarkable. On the one hand, this may explain for one or the other unwanted side-effect of the parent compound; on the other hand, desired therapeutic effects as, for example, neuroprotection could be afforded by direct application of these metabolites, benefiting from increasing drug safety after a long history of medical use.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2015.08.017.

References and notes

5. Tissues were disintegrated in ice-cold 50 mM Tris acetate buffer (pH 7.0) with a glass/Teflon homogenizer (Potter). After addition of 3 mM EDTA and centrifugation (38,000 g), pellets were suspended in fresh buffer (without EDTA) and centrifuged a second time. Suspended membranes then were treated with 0.023 Triton X-100 in a 37 °C water bath for 10 min for more efficient removal of endogenous compounds. After a third centrifugation, membrane suspensions were stored as aliquots at –80 °C until use. Before investing them into radioligand binding experiments (at ca. 1 mg tissue per vial), they were centrifuged a fourth time from the final incubation buffer.
11. Parke–Davis also provided the parent compound and the desmethyl metabolite.
15. Normally distributed noise was superimposed to the logarithmic ratios with excel by the function NORMINV, using random numbers and SD. By this random modification, 100 new data sets were created and analyzed. See excel file ‘Supporting information’.
16. [H]MK-801 at 5 mM specifically labeled 39.1 Mol/mg tissue on cortical and 72.7 Mol/mg tissue on hippocampal membranes. These basal levels were influenced by the 7 ionic conditions as follows: 30 µM Smp: +20%; 50 mM Tris: +28%; pH 6.4: –20%, pH 8.2: +17%; 50 mM NaCl: –28%; 50 mM KCl: –33%;
20 mM NH₄Cl: –43%; 1.3 mM Mg²⁺: –44%. Influence of Spm and pH was tested in 10 mM Tris acetate, influence of the other cations in 10 mM HEPES; pH of HEPES was adjusted to 7.0 by 3 mM NaOH or by 1.3 mM Mg(OH)₂; NH₄Cl was adjusted to pH 7.0.