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

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ABSTRACT

The specific binding of the NMDA receptor (NR) channel ligand [^{3}H]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 Ca^{2+} 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 Ca^{2+} conductance, but is also highly sensitive to inhibition by physiological concentrations of Mg^{2+}.[1] Under resting state conditions NRs are usually silent, with Mg^{2+} sitting at the narrow constriction. This Mg^{2+}, however, quickly leaves the channel and gives way to Na^+ and Ca^{2+}, 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-protectors 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 [^{3}H]MK-801[3] 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.[5] We have demonstrated before that NRs in these 2 regions exhibited similar properties.[6] Binding was conducted with 5 nM [^{3}H]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 [^{3}H]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. IC_{50} values were evaluated by computerized curve fitting to the function $B_x = B_0 \cdot IC_{50}{}^h/(IC_{50}{}^h + x^h) + NB$, where B_x 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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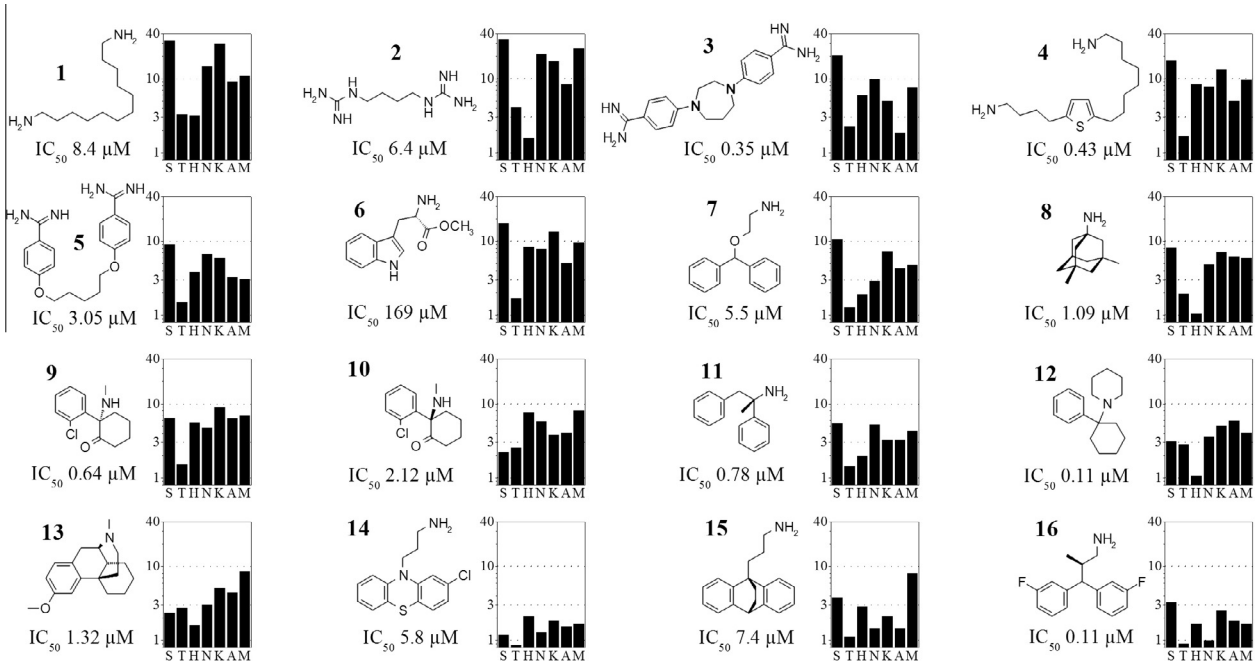
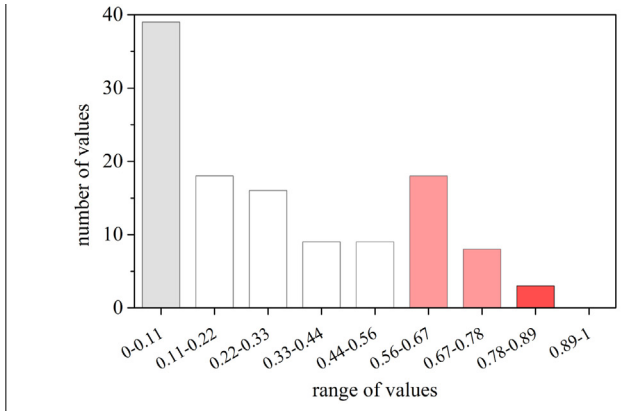


Figure 1. Influence of spermine (S, 30 μM), Tris (T, 10 → 50 mM), protons (H, pH 6.4 → 8.2), Na^+ (N, 3 → 50 mM), K^+ (K, 50 mM), ammonium (A, 20 mM), and Mg^{2+} (M, 1.3 mM) on the potency of 16 inhibitors of [^{3}H]MK-801 binding; IC_{50} values inscribed below structures obtained in 10 mM buffer (Tris or HEPES, pH 7.0). Plotted on the ordinates is the attenuation of this IC_{50} under the influence of the respective buffer constituent.



r^2	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	1	0.76	0.34	0.56	0.66	0.12	0.86	0.80	0.44	0.08	0.64	0.35	0.12	0.01	0.17	0.44
2	0.76	1	0.31	0.28	0.33	0.09	0.60	0.85	0.19	0.04	0.73	0.48	0.31	0.02	0.01	0.14
3	0.34	0.31	1	0.60	0.59	0.78	0.31	0.10	0.17	0.00	0.53	0.04	0.02	0.01	0.49	0.19
4	0.56	0.28	0.60	1	0.75	0.57	0.67	0.25	0.77	0.04	0.53	0.00	0.01	0.35	0.62	0.63
5	0.66	0.33	0.59	0.75	1	0.29	0.54	0.30	0.46	0.01	0.63	0.01	0.02	0.08	0.45	0.36
6	0.12	0.09	0.78	0.57	0.29	1	0.22	0.01	0.21	0.06	0.25	0.17	0.01	0.11	0.65	0.30
7	0.86	0.60	0.31	0.67	0.54	0.22	1	0.73	0.60	0.05	0.56	0.25	0.15	0.08	0.33	0.75
8	0.80	0.85	0.10	0.25	0.30	0.01	0.73	1	0.31	0.08	0.61	0.72	0.37	0.00	0.01	0.26
9	0.44	0.19	0.17	0.77	0.46	0.21	0.60	0.31	1	0.13	0.39	0.07	0.14	0.64	0.31	0.58
10	0.08	0.04	0.00	0.04	0.01	0.06	0.05	0.08	0.13	1	0.01	0.07	0.06	0.50	0.00	0.03
11	0.64	0.73	0.53	0.53	0.63	0.25	0.56	0.61	0.39	0.01	1	0.18	0.09	0.02	0.12	0.19
12	0.35	0.48	0.04	0.00	0.01	0.17	0.25	0.72	0.07	0.07	0.18	1	0.55	0.02	0.16	0.02
13	0.12	0.31	0.02	0.01	0.02	0.01	0.15	0.37	0.14	0.06	0.09	0.55	1	0.05	0.14	0.02
14	0.01	0.02	0.01	0.35	0.08	0.11	0.08	0.00	0.64	0.50	0.02	0.02	0.05	1	0.17	0.22
15	0.17	0.01	0.49	0.62	0.45	0.65	0.33	0.01	0.31	0.00	0.12	0.16	0.14	0.17	1	0.65
16	0.44	0.14	0.19	0.63	0.36	0.30	0.75	0.26	0.58	0.03	0.19	0.02	0.02	0.22	0.65	1

Figure 2. Coefficients of determination r^2 obtained by pair-wise comparison of ionic influences on 16 inhibitors of [^{3}H]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[10] to investigate whether it would fit better with the di-cationic or the mono-cationic group. *N*,*N*-Didesmethyl-diphenhydramine **7** was a gift of Parke-Davis (Ann Arbor MI),[11] memantine **8** a gift from Merz (Frankfurt am Main, Germany),[12] and (*S*)- and (*R*)-ketamine **9** and **10** were gifts from Gödecke AG (Freiburg, Germany). (*S*)-1,2-Diphenyl-2-propylamine **11** was provided by Astra Charnwood (Loughborough UK).[13] Phencyclidine **12** and dextromethorphan **13** were from Sigma–Aldrich. Didesmethyl-chlorpromazine **14** (nor^2-chlorpromazine) was prepared as part of the NIMH Chemical Synthesis Program and provided by RBI (Natick MA, contract N01MH30003). Desmethylmaprotiline **15** was a gift from Ciba-Geigy GmbH (Vienna, Austria), and NPS 1392 **16** a gift from NPS Pharmaceuticals (Salt Lake City UT).[14]

The ions Tris, H^+, Na^+, K^+ and NH_4^+ were inhibitory, Spm up to 30 μM was stimulatory, and Mg^{2+} was stimulatory up to 100 μM and inhibitory at higher concentrations.[16] Non-competitive NR antagonists exhibited various degrees of sensitivities to ionic buffer constituents, most of them operating best as inhibitors of [^{3}H]MK-801 binding at low ionic strength conditions (10 mM Tris acetate or 10 mM HEPES + 3 mM Na^+, pH 7.0). Figure 1 gives an overview to the influences of 7 milieu constituents carrying

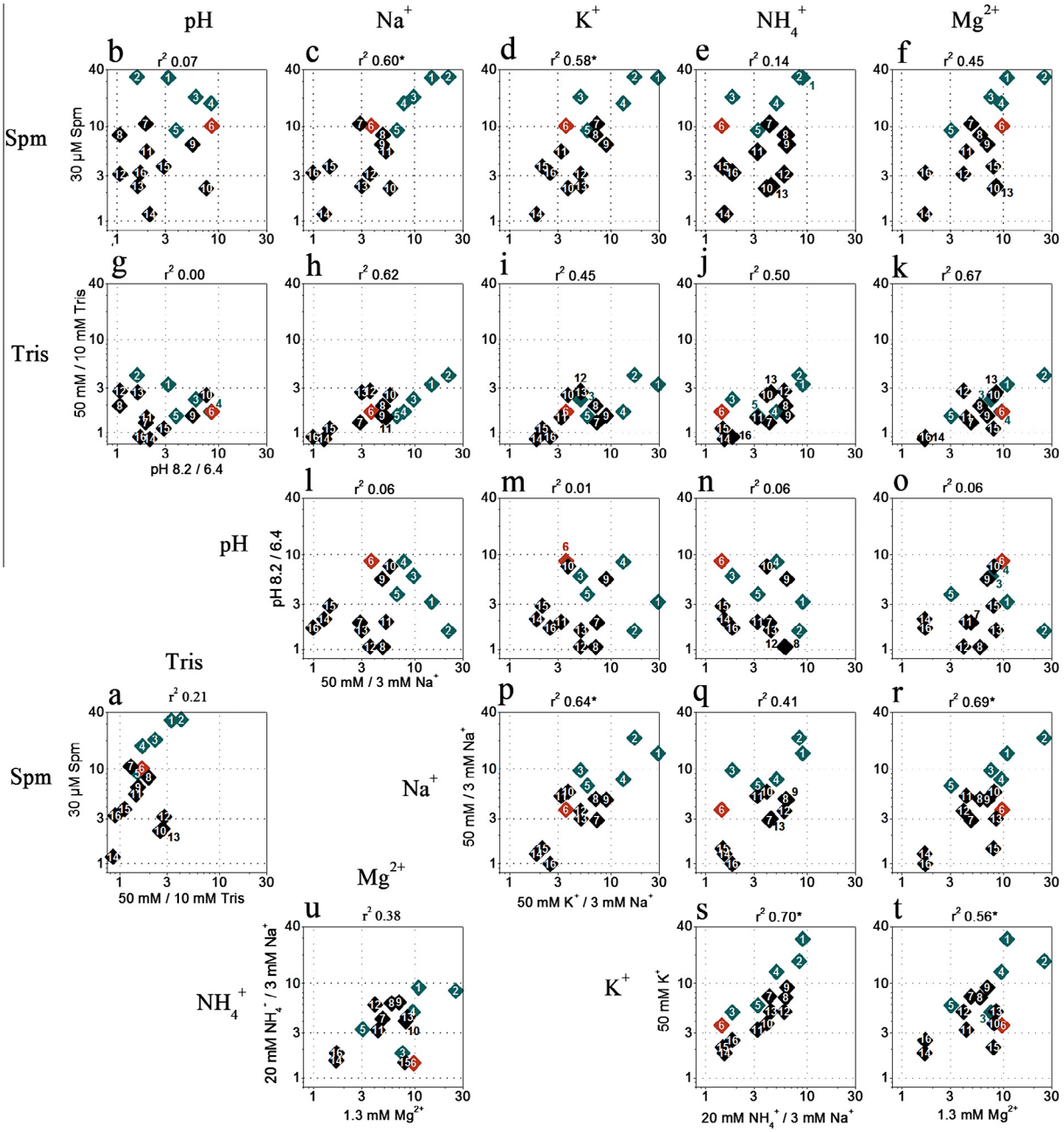


Figure 3. Inhibition of [^{3}H]MK-801 binding to rat brain membranes by polyamine inverse agonists **1**–**5** (cyan), by (L)-Trp methylester **6** (red), and by NMDA channel blockers **7**–**16** (black symbols); influence of physiological cations and of Tris. All plotted values are ratios of IC_{50}s (logarithmic scale); the basal condition for evaluating the influence of 30 μM spermine, 50 mM Tris and pH was 10 mM Tris acetate; for evaluating the influence of 50 mM Na^+, 50 mM K^+, 20 mM NH_4^+ and of 1.3 mM Mg^{2+}, 10 mM HEPES + 3 mM Na^+ 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).

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 **1**–**5** (*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 each compound with each of the other 15, we obtained 120 coefficients of determination (r^2). Frequency distribution suggested heterogeneity, with a third of the values possibly signifying no correlation at all (gray in Fig. 2). Some correlations, however, seem to exist (pink background), although none of them survived random noise superimposed to the data (see Supporting excel file, Table S4). Against our expectations, the obtained coefficients of determination did not reflect the grouping of the antagonists into di-cationic ('polyamine-type', **1**–**5**) and mono-cationic ones ('channel blocker-type', **7**–**16**). The highest r^2 values concerned correlations across group boundaries. The results obtained for the Trp derivative **6** did not facilitate its assignment to one of the groups.[10]

Our data also allowed for investigating similarities between the 7 different milieu conditions (Fig. 3). The most impressive result was a negative one: None of the investigated ionic influences bore any resemblance to the influence of H_3O^+ ions ('protons'; pH in the figure). The highest correlation was observed between the influences of K^+ and NH_4^+ (Fig. 3s). These cations have similar ionic radii and enthalpies of hydration;[17] they may interact with the same target. The influences of the poly-cation Spm exhibited some parallels to the influences of Na^+ and K^+ cations (c and d); also influences by Mg^{2+} seemed to correlate with influences by these cations (r and t). The buffer cation Tris seemed to exert influences similar to the influences of Na^+ (h); also with Mg^{2+} (k) Tris might share similar targets. These data are preliminary and await confirmation by other approaches.

Both 30 μM Spm and 1.3 mM Mg^{2+} may exert stimulatory influences via a poly-anionic site at the extracellular GluN1/2B interface.[18] At least in the rat hippocampus, the majority of NRs seems to harbor such an interface in triheteromeric GluN1/2A/2B receptors.[19] At this N-terminal interface, a specific pattern of acidic amino acid residues keeps the subunits apart from each other due to electrostatic repulsion. This disconnected conformation imparts a low open probability to the channel (*low* P_o state); it depends on the nature of the neutralizing cations to which extent this repulsion is relieved. Polyamines like spermidine with 3 and spermine with 4 precisely spaced positive charges are well suited for this exercise, but also Mg^{2+} and, under artificial conditions in the absence of both, other cations may at least in part step in. We have recently shown that the di-cationic compound **5** and several of its structural analogs bring about inhibition of the NR in part by interaction with this acidic domain on GluN1 but not on GluN2B;[20] by this unilateral attachment they seem to keep the interface in the *low* P_o state. Ionic influences on NR inhibition by 5 di-cationic and 11 mono-cationic compounds as presented here revealed several analogies between these 2 structural classes. These similarities may indicate that the N-terminal GluN1/2B interface is not only the target of most di-cationic NR antagonists, but also of some of the mono-cationic ones.

Since inhibition of [^{3}H]MK-801 binding by the well tolerated NR antagonist **8** (memantine)[21] 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 [^{3}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

1. Paoletti, P. *Eur. J. Neurosci.* **2011**, *33*, 1351.
2. Carter, A. J. *J. Pharmacol. Exp. Ther.* **1994**, *269*, 573.
3. Wong, E. H. F.; Kemp, J. A.; Priestley, T.; Knight, A. R.; Woodruff, G. N.; Iversen, L. L. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 7104.
4. Williams, K.; Dawson, V. L.; Romano, C.; Dichter, M. A.; Molinoff, P. B. *Neuron* **1990**, *5*, 199.
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 2nd time. Suspended membranes then were treated with 0.02% Triton X-100 in a 37 °C water bath for 10 min for more efficient removal of endogenous compounds. After a 3rd 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 4th time from the final incubation buffer.
6. Berger, M. L.; Rebernik, P. *J. Pharmacol. Exp. Ther.* **1999**, *289*, 1584.
7. Tao, B.; Huang, T. L.; Sharma, T. A.; Reynolds, I. J.; Donkor, I. O. *Bioorg. Med. Chem. Lett.* **1999**, *9*, 1299.
8. Berger, M. L.; Schödl, C.; Noe, C. R. *Eur. J. Med. Chem.* **1998**, *33*, 3.
9. Reynolds, I. J.; Aizenman, E. *J. Neurosci.* **1992**, *12*, 970.
10. (a) Berger, M. L. *Neurosci. Lett.* **2000**, *296*, 29; (b) Berger, M. L.; Palangsuntikul, R.; Rebernik, P.; Wolschann, P.; Berner, H. *Curr. Med. Chem.* **2012**, *19*, 3044.
11. Parke–Davis also provided the parent compound and the desmethyl metabolite.
12. Kornhuber, J.; Bormann, J.; Retz, W.; Hübers, M.; Riederer, P. *Eur. J. Pharmacol.* **1989**, *166*, 589.
13. (a) Subramaniam, S.; Donevan, S. D.; Rogawski, M. A. *J. Pharmacol. Exp. Ther.* **1996**, *276*, 161; (b) Berger, M. L.; Schweifer, A.; Rebernik, P.; Hammerschmidt, F. *Bioorg. Med. Chem.* **2009**, *17*, 3456.
14. Moe, S. T.; Shimizu, S. M.; Smith, D. L.; Van Wagenen, B. C.; DelMar, E. G.; Balandrin, M. F.; Chien, Y.; Raszkiewicz, J. L.; Artman, L. D.; Mueller, A. L.; Lobkovsky, E.; Clardy, J. *Bioorg. Med. Chem. Lett.* **1999**, *9*, 1915.
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. [^{3}H]MK-801 at 5 nM specifically labeled 39.1 fMol/mg tissue on cortical and 72.7 fMol/mg tissue on hippocampal membranes. These basal levels were influenced by the 7 ionic conditions as follows: 30 μM Spm: +26%; 50 mM Tris: −28%; pH 6.4: −29%, pH 8.2: +17%; 50 mM NaCl: −28%; 50 mM KCl: −33%;

20 mM NH_4Cl: −43%; 1.3 mM Mg^{2+}: −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 $Mg(OH)_2$; NH_4Cl was adjusted to pH 7.0.

17. Holleman, A. F.; Wiberg, E.; Wiberg, N. *Lehrbuch der Anorganischen Chemie*, 101. Auflage; de Gruyter: Berlin, 1995. p 654.
18. Mony, L.; Zhu, S.; Carvalho, S.; Paoletti, P. *EMBO J.* **2011**, *30*, 3134.
19. Tovar, K. R.; McGinley, M. J.; Westbrook, G. L. *J. Neurosci.* **2013**, *33*, 9150.
20. Berger, M. L.; Maciejewska, D.; Vanden Eynde, J. J.; Mottamal, M.; Zabinski, J.; Kazmierczak, P.; Resler, M.; Jarak, I.; Piantanida, I.; Karminski-Zamola, G.; Mayence, A.; Rebernik, P.; Kumar, A.; Ismail, M. A.; Boykin, D. W.; Huang, T. L. *Bioorg. Med. Chem.* **2015**, *23*, 4489.
21. Johnson, J. W.; Kotermanski, S. E. *Curr. Opin. Pharmacol.* **2006**, *6*, 61.