# Polyamine regulation of the NMDA receptor complex as a target in drug development

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## Abstract

The NMDA receptor complex (NR) is one of the main targets of the excitatory neurotransmitter glutamic acid. A partial block of the NR by protons even at physiological pH can by removed by the natural polyamines (PAs) spermidine and spermine. Several unbranched diamines as arcaine and 1,12-dodecane-diamine (N12N) seem to block the NR (at least to some extent) via the same mechanism (polyamine inverse agonists). Systematic structural modification led to the discovery of 5-(4-aminobutyl)-2-thiopheneoctanamine (N4T8N), one of the most potent compounds in this family (IC$_{50}$ 0.3 μM). Via allosteric sites, ifenprodil and zinc increase the sensitivity of the NR to proton inhibition, blocking the NR in apparent competition with stimulating PAs. Here, the recent literature is reviewed on compounds interacting directly or indirectly with PA regulation of the NR.

## Introduction

As one of the first organic molecules isolated from a biological source, the polyamine (PA) spermine (SPM) has been discovered by A. V. Leuvenhoeck as early as in 1678 [1]. The major PAs putrescine, spermidine (SPD), and SPM, which contain two, three and four amino groups, respectively, are constituents of all living cells and essentially involved in cell proliferation, differentiation, and apoptosis [2, 3]. Intensive research during the last three decades has revealed pleiotropic actions of PAs at various levels of cell regulation, including aspects of transcription, translation, cell signalling, growth, differentiation and apoptosis (Fig. 1). When cells are stimulated with growth factors, the induction of PA synthesis precedes the increases in DNA-replication and in RNA and protein synthesis [4, 5]. PA formation and catabolism, as well as their relative concentrations, are subject to fine-tuned regulation, involving a series of enzymes. In contrast to this central position of PAs in cell biology, modern textbooks of molecular biology sometimes do not even contain the term "polyamine". The present review will concentrate on a specific target, usually not in the focus of PA-related research: the NMDA-receptor and the role of PAs in modulating its function. This introduction, however, should have reminded the reader, that touching at the metabolism and/or the pharmacology of PAs may have dramatic biological consequences, in both the salutary, but also in the detrimental direction.

## Polyamines and the NMDA Receptor

N-Methyl-D-aspartic acid (NMDA), a synthetic congener of the main excitatory neurotransmitter glutamic acid, is the prototypic agonist for the NMDA receptor (NR). This glutamate receptor subtype is linked to an ion channel with high $Ca^{2+}$ ion permeability, its conductance critically depending on partial depolarization (see a recent review [6]). By virtue of these properties, NRs play a crucial role in synaptic plasticity, but also are at risk to convey a deadly threat to neurons, since prolonged NR stimulation as it may occur in stroke, epilepsy or neurological conditions as Chorea Huntington runs the risk of $Ca^{2+}$ overload. The

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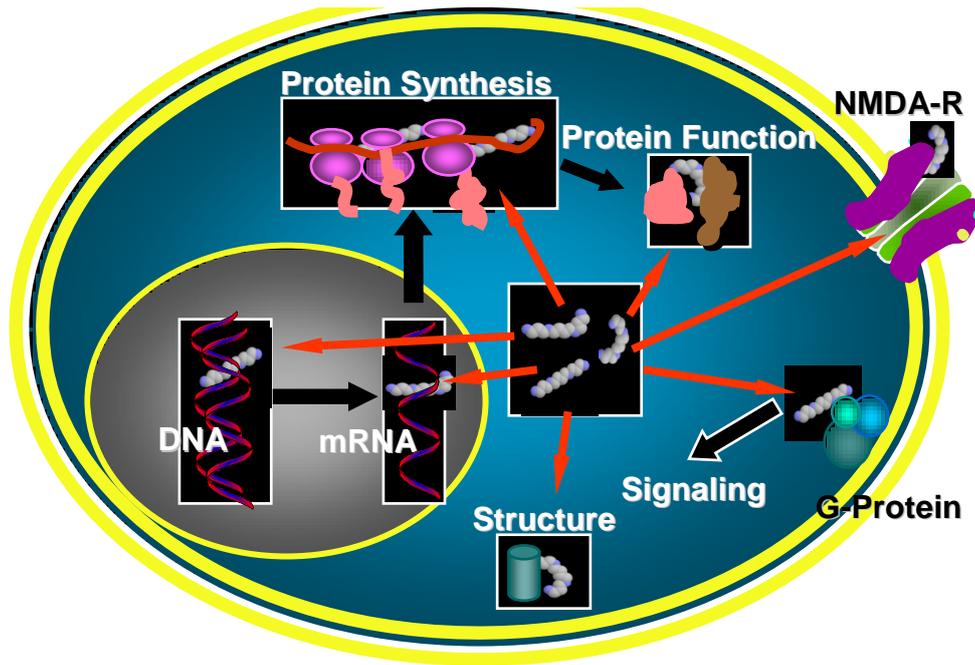e-mail: michael.berger@univie.ac.at

Fig. 1. Various targets of polyamines (PAs) in cell biology. Because of their positive charge and their flexibility, the small linear PA molecules are ideally suited to interact as counterions with DNA, RNA, and negatively charged proteins (or protein subunits), adjusting their shape and the spatial distances between the positively charged amino-groups in such a way that they precisely adapt to negatively charged patches on their targets. There is evidence for an essential role of PAs in many biological processes, from transcription and translation to activation of enzymes and modification of signalling pathways.

endogenous PAs SPD and SPM increase frequency and burst length of NMDA-induced currents in rat hippocampal neurons, by relieve from a partial block by protons, even at physiological pH [7]. Diamines like 1,12-dodecanediamine (N12N) and arcaine appear to counteract this effect; thus, they seem to block the NR complex via the same site as protons do. Allosteric inhibitors as e.g. ifenprodil increase the sensitivity of the NR complex to inhibition by protons [8]; they achieve this effect by interaction with a site that is not a direct target of PAs.

The concept of a "binding site" mediating the influence of "protons", often referred to for the sake of simplicity, deserves some cautionary comment.

Depending on the local physicochemical conditions, pH changes influence the degree of protonization of several amino acid residues in a protein. In the concert of pH-dependent conformational adjustments, some of these residues appear to be of greater importance than others, suggesting to constitute topical elements of a "binding site" for "protons" or - in a closer sense - for "hydronium ions". In the case of hydronium ions, however, such a binding concept should not be taken too literally. In the last chapter of this review, we will try to summarize recent data on the molecular mechanism of proton block of the NR and its relief by PAs. If, in the following paragraphs, we continue to use the term "proton site of the NR", this should not suggest the idea of a specific "spot" on the receptor protein.

From a clinical point of view, compounds influencing PA regulation of the NR offer several attractive features: (1) PA analogues acting at the proton/PA site as partial agonists increase NR function up to a certain limited level, and may be envisioned as safe cognition enhancing drugs; e.g dementia in Alzheimer's Disease has been discussed as a condition with NRs not sufficiently involved in neuronal activity (John Olney's NR hypofunction theory) [9]. The use of a positive modulator instead of a direct NR agonist avoids the problem of interference with naturally fluctuating transmitter levels and thus leaves the dynamics of the receptor response intact. (2) Allosteric sensitization of the NR to proton inhibition by ifenprodil type compounds provides a means to reduce the activation of NRs specifically during periods of increased risk of neuronal damage (as e.g. during hypoxia and ensuing acidosis, i.e. increased concentration of protons). Thus, in contrast to traditional NR blockers as phencyclidine and ketamine which have severe psychotic side effects, these compounds offer neuroprotection selectively under pathophysiological circumstances endangering the integrity of neurons, while allowing the continued contribution of the NR to normal neuronal activity in unaffected regions. (3) Drugs structurally related to PAs have the potential to influence, in addition to the NR, other biological processes involving PAs, like cell proliferation, growth, differentiation, and cellular motility. While this represents a considerable source of unwanted side effect, on the other hand it would allow to address synergistically several targets together.

Our search for PA inverse agonists with increased potency started with a study of long chain diamines with increasing chain length [10] and culminated in the discovery of long chain diamines interrupted by an aromatic nucleus; by placing the nucleus at a specific position, the potency was increased by a factor 50 in comparison to N12N [11]. On the other hand, compounds allosterically increasing proton block (like ifenprodil), do not compete for this binding site, but require protons to deploy their blocking effect; thus, their inhibitory potency is increased in an acidic environment. Our experience up to now demonstrates that these two phenomena: (1) attenuation of inhibitory potency by SPM, and (2) increased inhibitory potency at acidic pH, do not correlate with each other [12], suggesting that they reflect separate and independent mechanisms of action, and that both drug targets may lend themselves to the development of different pharmaceutical leads. Our observations are in agreement with molecular biology data indicating that inhibition by protons and sensitization to proton inhibition are conveyed by differing parts of the NR (see [13] for a recent review).

## Methodological Considerations

To study the impact of test compounds on PA regulation of the NR, we use in vitro binding techniques with the radiolabelled open channel blocker [$^3$H]MK-801 [14]. By this method, we can distinguish positive and negative modulators of the ion channel associated with the NR, data otherwise accessible in living cells only. Specific binding of [$^3$H]MK-801 (ARC Inc., St. Louis, USA; ART-661) to rat brain membranes (prepared from hippocampus or from cerebral cortex) has been routine in our laboratory for many years. PAs and PA agonists increase the opening frequency of the ion channel associated with the NR and thereby the accessibility of the channel binding site for the open channel blocker MK-801, if a non-saturating concentration of MK-801 was used. We routinely use 5 nM and observe an affinity constant of 10-15 nM in our membrane preparation. Against widespread habits (non-equilibrium conditions, i.e. incubation time 1 h or shorter, some authors even prefer the nominal absence of glutamate and glycine), we prefer conditions close to equilibrium (3 h incubation time, saturation with glutamate and glycine). Non-specific binding is defined by binding of [$^3$H]MK-801 to closed channels (i.e. without addition of the channel opening co-agonists glutamic acid & glycine, but with addition of their respective antagonists D-APV & 5,7-DCKA). A compound is recognized as PA or full PA agonist, if the specific binding of [$^3$H]MK-801 to neuronal membranes is increased in the presence of saturating concentrations of glutamate and glycine (10 μM each), and if no further increase can be achieved by addition of SPD or SPM (1-30 μM). On the other hand, a compound inhibiting the specific binding of [$^3$H]MK-801 is likely to act as an inverse PA agonist, if its potency is reduced by at least a factor 10 in the presence of 30 μM SPM; attenuation factors lower than 10 indicate that inverse PA agonism is only partly involved in the inhibitory mechanism. Many compounds exhibit mixed inhibitory properties, acting as inverse PA agonist and as direct channel blocker at the same time.

For the ease of communication, a symbolic short note for PA structures developed by us [10, 11] is used in this article. Amino groups are symbolized by capital N's, and arabic numbers indicate the number of C atoms

linking these N atoms in unbranched chains. In addition, a capital T is used to symbolize a 2,5-substituted thiophene nucleus. The positions of amino acid residues in NR subunits NR1, NR2A, and NR2B are indicated following the numbering routine of most of the articles cited, counting as number 1 the first amino acid of the nascent chain at the ribosome (methionine). However, the first 18, 19, and 26 residues, respectively, do not become a part of the complete NR complex, since they only function as signal sequences to allow the addressing of the ribosome to the ER as necessary for the synthesis of a membrane protein, and are cleaved off at a later stage of the synthesis process.

**Partial and full Polyamine agonists**

The most parsimonious organic molecules acting at the PA regulatory site of the NR are 1,3-diamino-propane (N3N), 1,4-diaminobutane (N4N, putrescine), and diethylenetriamine (N2N2N, Table 1). Whereas N3N seems to act as a full agonist (though at a concentration of at least 100 μM), N4N and N2N2N are devoid of intrinsic activity and act as antagonists [15]. In 1,4-diaminocyclohexane (DAC), as in N3N, 2 primary amino groups are separated by 3 carbon atoms. However, only the cis isomer (cDAC, Table 1), with a distance of 4.9 Å between the nitrogen atoms, exhibits positive intrinsic activity [16]. In the trans isomer (tDAC), both amino groups apparently are forced to the energetically more favorable equatorial orientation, resulting in a distance of 5.8 Å. Based on these observations, the authors proposed a model of the PA recognition site with at least 2 interaction sites at a distance of 5 Å [16]. Introduction of an oxygen atom in vicinity of one of the terminal nitrogen atoms in SPD (N3N4N) results in aminooxy analogues and in the selective charge elimination from the neighboring nitrogen atom. Thus, the charge pattern in 8-O-SPD (Table 1) reproduces that of N3N ($H_3N^+$-$CH_2$-$CH_2$-$CH_2$-$H_2N^+$-), and that of 1-O-SPD reproduces the charge pattern of N4N. Conveniently, both N3N and 8-O-SPD exhibit positive intrinsic activity, and both N4N and 1-O-SPD act as pure antagonist without intrinsic activity [17]. In cell culture, both aminooxy analogues have been shown to be stable for hours [18]. In vitro, rigid analogues of SPD or SPM, with one or two of the positive charges incorporated into a ring as in DEAP [19], HPZ(3,3) [20], and PIP(3,4,3) [21] (see Table 1), appear to be more specific than the parent compounds, since they produce no inhibitory side effect at concentrations between 30 μM and 1 mM. Several compounds with multiple positive charges have been

shown to exhibit PA-like stimulation of the NR, including the aminoglycoside antibiotics neomycin, kanamycin-A, and apramycin [22, 23, 24, 25] (see Table 1). Excessive stimulation of the NR has been suspected to be responsible for the known ototoxicity of these compounds; however in a structure / activity relationship (SAR) study, high potency as NR stimulator was not found to strictly parallel high ototoxicity [26].

The PA agonists quoted so far share as structural features (1) the structural element $H_3N^+$-$CH_2$-$CH_2$-$CH_2$-$NH_2^+$-; (2) at least 2 primary amino groups; and (3) a high degree of protonization of the amino groups at physiological pH [21, 27]. Exceptions to these "rules" have been described. The dipyridyl compound PYR(5,4,5) [21] (Table 2) violates all these criteria, and nevertheless it acts in vitro as a partial agonist at concentrations down to 0.01 μM (the most potent PA agonist reported so far). N-acylated homopiperazines like HPZ(aa)$_2$ (Table 2) have been synthesized as precursors for alkylated homopiperazines like HPZ(3,3) mentioned above (see Table 1); surprisingly, HPZ(aa)$_2$ was found to stimulate specific binding of [$^3$H]MK-801 by itself (Table 2), with an $EC_{50}$ similar to HPZ(3,3), although the 2 ring nitrogens in HPZ(aa)$_2$ are practically without positive charge. Finally, again in violation of the "rules" mentioned above, stimulation was seen if primary amino groups were separated from each other by more than 4 carbon atoms as in N4T2N-(D)lys-(L)lys (Table 2). All these "unconventional" stimulations have been observed with binding techniques, in buffers of low ionic strength (5 mM Tris/HCl, 5 mM HEPES/Tris, or 10 mM Tris/acetate). With N4T2N-(D)lys-(L)lys, much weaker stimulation is observed in 50 mM than in 10 mM Tris acetate buffer [Berger & Rebernik, unpublished observation]. Furthermore, several of these compounds might behave differently at membranes with intact membrane potential. Thus, the clinical significance of the data in Table 2 remains doubtful, as long as no data are available obtained under more physiologic conditions.

**Long-chain diamines as inverse polyamine agonists**

The first compounds providing NR inhibition highly sensitive to SPD and SPM were the long chain diamines 1,10-diaminodecane (N10N) [28], 1,12-diamino-dodecane (N12N) [10], and the bisguanidino derivative arcaine [29]. Similar properties were reported for the structurally related antimicrobial pentamidine [30] and for analogues of pentamidine [31]. Systematic SAR

Table 1. Polyamine analogues of increasing complexity

| | structure | effect on [$^3$H]MK-801 binding | reference |
|---|---|---|---|
| N3N | | agonist, EC$_{50}$ > 100 μM[a] | [15] |
| N4N | | antagonist, IC$_{50}$ (10 μM SPM) > 100 μM[a] | [15] |
| N2N2N | | antagonist, IC$_{50}$ (10 μM SPM) < 100 μM[b] | [15] |
| cDAC | | partial agonist, EC$_{50}$ 100 μM[c] | [16] |
| tDAC | | inverse agonist, IC$_{50}$ > 1 mM[c] | [16] |
| 8-O-SPD | | partial agonist, EC$_{50}$ 50 μM | [17] |
| 1-O-SPD | | antagonist, K$_i$ (SPM) 165 μM | [17] |
| DEAP | | agonist, EC$_{50}$ 6.7 μM[d] | [19] |
| HPZ (3,3) | | agonist, EC$_{50}$ 24 μM[e] | [20] |
| PIP (3,4,3) | | agonist, EC$_{50}$ 1.0 μM[f] | [21] |
| neomycin | | agonist, EC$_{50}$ 2.4 μM[e] | [24] |
| apramycin | | agonist, EC$_{50}$ 3.9 μM[e] | [25] |

Abbreviations: N3N, 1,3-diaminopropane; N4N, putrescine; N2N2N, diethylene triamine; cDAC, cis-1,4-diaminocyclohexane; tDAC, trans-1,4-diaminocyclohexane; 8-O-SPD, 8-oxaspermidine; 1-O-SPD, 1-oxaspermidine; DEAP, 1,5-(diethylamino) piperidine; HPZ(3,3), *N,N'*-bis(3-aminobutyl)homopiperazine; PIP(3,4,3), $N^1$,$N^4$-bis(piperidinyl)-1,4-diaminobutane

[a] Estimated from Fig. 4 of the reference.
[b] Estimated from Fig. 5 of the reference.
[c] Estimated from Fig. 7 of the reference.
[d] Evaluated from Fig. 2 of the reference.
[e] [$^3$H]MK-801 binding without addition of glutamate and glycine.
[f] Evaluated from Fig. 2b of the reference.

Table 2. Polyamine partial agonists with "unconventional" structures

| structure | EC$_{50}$ ([$^3$H]MK-801 binding) | reference |
|---|---|---|
| PYR(5,4,5) | < 0.01 μM[a] | [21] |
| HPZ(aa)$_2$ | 18 μM | [20] |
| N4T2N-(D)lys-(L)lys | 1.8 μM | [12] |

Abbreviations: PYR(5,4,5), $N^1$,$N^4$-bis(4-ethylpyridyl)-1,4-diaminobutane; HPZ(aa)$_2$, $N$,$N$'-bis(aminoacetyl)homopiperazine; N4T2N-(D)lys-(L)lys, 5-(4-aminobutyl)-2-thiophenethyl-D-lysyl-L-lysine
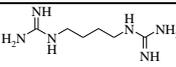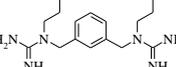
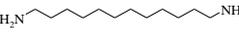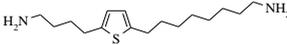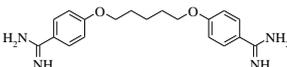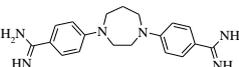[a] Evaluated from Fig. 2c of the reference.

studies resulted in analogues with sub-micromolar potencies. Most effective has been the modification of the spacer in N12N, in arcaine, and in pentamidine. Symmetric insertion of a thiophene nucleus into N12N yielded 2,5-thiophenedihexanamine (N6T6N) and a 7.7-fold increase in potency. The best result, however, was achieved by asymmetric insertion, resulting in 5-(4-aminobutyl)-2-thiopheneoctanamine (N4T8N) with a further 6.5-fold potency increase [11] (see Table 3). Also the potency of arcaine was increased several-fold by insertion of an aromatic nucleus into the spacer [32] (see GUA(prop)$_2$ in Table 3). In pentamidine, the spacer consists in an unbranched pentane chain flanked by 2 phenoxy substituents (Table 3). Here, rigidization of the bridge in a 7-membered ring was successful [33] (HPZ(ba)$_2$, Table 3). Up to now, no asymmetric arcaine or pentamidine congeners have been studied. SAR studies within these 3 families of compounds revealed that those compounds with the highest potency also exhibited the highest sensitivity to SPM or SPD, respectively [11, 32; Berger & Huang, unpublished observation]. Thus, the improvement in potency most likely was due to the optimized interaction with the PA regulatory site at the NR. Only for HPZ(ba)$_2$ data on living cells have been obtained showing that HPZ(ba)$_2$ protects cultured cortical neurons from glutamate toxicity, without exhibiting toxicity by itself [33]. Nothing is known about bio-availability and penetration

into the brain after systemic application of these experimental drugs.

**And ifenprodil?**

For several years, ifenprodil as the prototypic representative of a new class of drugs was supposed to bind with high affinity directly to the PA regulatory site of the NR, until Mott et al. in 1998 convincingly provided proof by experiments including site directed mutagenesis that the amino acid residues responsible for the interaction of ifenprodil with the NR were not the same as those mediating the effects of SPM and SPD [8]. Recently, the N-terminal LIVBP (leucine, isoleucine, valine binding protein) -like domain of the NR2B subunit (see below) was identified as the target of ifenprodil ("Venus-flytrap") [34]. Ifenprodil and congeners are believed to induce, via binding to this domain on the NR2B subunit, a conformational change resulting in the increased sensitivity to proton inhibition. The earlier impression of direct competition between ifenprodil-type compounds on one side and PAs like SPD and SPM an the other was due to the fact that the latter counteract proton inhibition [7], i.e. the same mechanism boosted by ifenprodil. Ifenprodil is a "dirty" drug, exhibiting considerable affinity to several receptors, including $\alpha_1$ and $\sigma$ receptors. In 1996, it was discovered that also the structurally related neuroleptic

Table 3: Unbranched diamines with hydrophobic spacer

| | structure | IC$_{50}$ ([$^{3}$H]MK-801) | ratio |
|---|---|---|---|
| arcaine | | 5.3 ± 3.5 μM (6)[a] | 35, 32[b] |
| GUA(prop)$_2$ | | 0.58 ± 0.15 μM (3)[c] | 21 (3)[c] |
| N12N | | 7.3 ± 3.5 μM (7)[a] | 30 ± 13 (4)[b] |
| N4T8N | | 0.28 ± 0.10 μM (15)[a] | 22 ± 5 (9)[b] |
| pentamidine | | 2.5 ± 1.0 μM (6)[a] | 11 ± 2 (3)[b] |
| HPZ(ba)$_2$ | | 0.23 ± 0.7 μM (12)[a] | 21 ± 9 (4)[b] |

Abbreviations: GUA(prop)$_2$, *N,N'*-bis(propyl)guanidinium; N12N, 1,12-diaminododecane; N4T8N, 5-(4-aminobutyl)-2-thiopheneoctanamine; HPZ(ba)$_2$, *N,N'*-bis(benzamidine)homopiperazine

[a] Inhibition of binding of 5 nM [$^{3}$H]MK-801 to rat hippocampal membranes in 10 mM Tris acetate buffer pH 7.0 [Berger & Rebernik, unpublished].
[b] Attenuation of inhibition by 30 μM spermine
[c] Data from [32], attenuation by 100 μM spermidine

haloperidol (Table 4) inhibited the NR by binding to the NR2B subunit [35]. Occasionally, ifenprodil-type compounds are referred to as phenylethanolamines [8], paraphrasing the common structural element in the more prominent exponents ifenprodil, eliprodil, and CP-101.606 (although, strictly speaking, only eliprodil *is* a phenylethanolamine, see the structures in Table 4). In ifenprodil and in CP-101.606, both carbon atoms of the ethyl chain are substituted, giving rise to 4 stereoisomers. The 4 isomers of ifenprodil - the drug on the market is the racemic (±)erythro diastereomer - differ in their affinities for the NR only by a factor of 4 [36] (the absolute configuration is not known). In eliprodil, only one of the ethyl carbon atoms is substituted, and the NR blocking potency resides almost exclusively in the R isomer [37], whereas in CP-101.606, again with 2 centers of asymmetry, steric conformation like in ifenprodil is of minor relevance [38]. The most potent compound presently known in this family is Ro 25-6981, with an additional carbon atom inserted into the ethyl chain [39]; an oxygen inserted instead of the carbon atom is tolerated nearly as well [40, 41]. Despite the close structural relatedness between all these compounds, it has recently been shown that [$^{3}$H]CP-101.606 did not bind to NRs if, in addition to NR2B, also NR2A subunits were present in the receptor oligomer, whereas [$^{3}$H]Ro 25-6981 bound equally well to NR2B in the presence and in the absence of NR2A [42]. Deviating from the main structural lead, the phenol moiety can be replaced, without prominent loss in potency, by imidazole [43] or by aminotriazole [44] (see Table 4). Also the piperidine ring of the molecule can be reoriented [45] or resolved into an open chain, even with gain of function [46] (Table 4). These open chain compounds are reminiscent of nylidrin, a β-sympatomimetic developed in the 1960ies and described in 1997 as NR2B specific ligand [47]. Finally, without much disadvantage, the central nitrogen can be part of

Table 4. Ifenprodil-type drugs

| | structure | IC$_{50}$ (µM) | reference |
|---|---|---|---|
| haloperidol | | 4.3[a] | [35] |
| (-)(threo)-ifenprodil | | 0.22[a] | [36] |
| (R)-eliprodil | | 0.050[b] | [37] |
| (+) CP-101.606 | | 0.011[c] | [38] |
| Ro 25-6981 | | 0.006[d] | [39] |
| Co 101071 | | 0.025[a] | [40] |
| Ro 63-1908 | | 0.010[e] | [41] |
| 4-benzyl-1-[4-(1H-imidazol-4-yl)but-3-ynyl]piperidine | | 0.037[a] | [43] |
| 1-(triazolylthioethyl)-4-benzylpiperidine | | 0.035[a] | [44] |
| N-(4-phenylbutyl)-4-(4-hydroxyphenyl)piperidine | | 0.022[a] | [45] |
| nylidrin | | 0.18[a] | [47] |
| Co 101677 | | 0.008[a] | [46] |
| N-(4-phenylbutyl)-4-hydroxycinnamide | | 0.077[a] | [48] |

[a] block of conductance in NR1a/2B expressed in Xenopus oocytes
[b] block of [³H]ifenprodil binding
[c] inhibition of glutamate induced neuron death in primary rat hippocampal cultures
[d] K$_D$ of [³H]Ro 25-6981
[e] inhibition of [³H]Ro 25-6981 binding to rat brain membranes

an amide group [48] (Table 4), indicating that no positive charge is required for interaction with NR2B. Nevertheless, the latter authors proposed a pharmacophore with a central site of electrostatic interaction, connecting 2 open chain spacers with hydrophobic nuclei at their ends, one of them with a small hydrophilic substituent for interaction with a hydrophilic spot embedded into one of the 2 hydrophobic binding pockets [47]. The distance between the 2 pockets was estimated to be 17.5 Å.

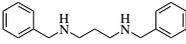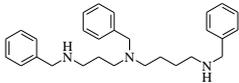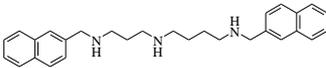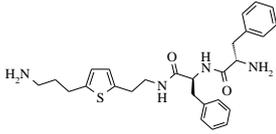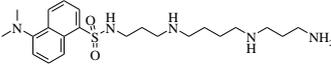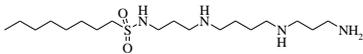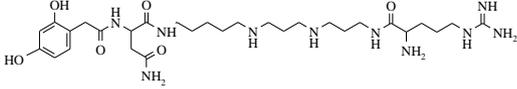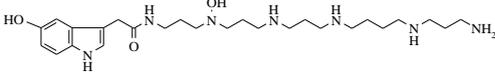**Block of NMDA channels by polyamine analogues**

Since the ion channel associated with the NR is ready, in its activated state, to conduct positively charged ions ($Ca^{2+}$, $Na^+$), it is not surprising that polycations like SPD and SPM, at sufficiently high concentrations, directly interfere with the conductance of the NR channel. Such a channel blocking effect has been demonstrated for SPM and SPD at concentrations above 30 μM [49, 50], and also for the inverse PA agonists N10N, N12N [51] and arcaine [52]. As a rule of thumb, introduction of hydrophobic substituents into N3N, SPD or SPM eliminates their NR stimulatory properties, leaving behind only the channel blocking character of polycations with increased lipophilicity as e.g. benz$_2$-N3N (Table 5) [53]. SPD-1,3-naph (Table 5) inhibits [$^3$H]MK-801 binding essentially independent of PAs or pH [54], whereas N4T2N-(L)phe-(L)phe (Table 5, [12]) is weaker and more sensitive to SPM. N$^1$-dansyl-SPM [55] and N$^1$,N$^4$,N$^8$-tribenzyl-SPD (benz$_3$-SPD) [56] have been characterized as voltage-dependent channel blockers with sub-micromolar potencies (Table 5). In judging the significance of IC$_{50}$ values, it must be taken into consideration that, as a principle, voltage-dependent channel blockers yield lower IC$_{50}$-values in preparations with intact membrane potential than in suspensions of disrupted membranes. Mutation of the aspartic acid residue D669, located on NR1 just outside the channel's mouth (see Fig. 2), to uncharged asparagine (D669N) or alanine (D669A) abolished both stimulation and inhibition by SPM [57], and also reduced the potency of the channel blocker benz$_3$-SPD [58], indicating that also stimulatory SPM operates very close to the channel's entrance. Interestingly, mutation of D669 to charged glutamic acid (D669E) abolished only SPM stimulation, not SPM inhibition. N$^1$-dansyl-SPM and benz$_3$-SPD exhibit similar inhibitory potencies at NR1/NR2A and NR1/NR2B heteromers, whereas SPM stimulation is much more pronounced at NR1/NR2B than at NR1/NR2A combinations [59]. It must be concluded, that stimulatory PAs and

structurally related channel blockers share recognition sites at the NR, although these interactions result in quite opposite effects. Structurally related to these synthetic compounds are the spider toxins Argiotoxin-636 and Agel-505 (Table 5). Both act as highly voltage dependent NR-channel blocker [60, 61, 62] and have been reported, at 100-300 μM, not to interfere with LTP in rat hippocampal slices [63]. Although argiotoxin-636 blocks non-NMDA receptor channels with comparable potency [64], it protects cultured cerebellar granule cells preferentially against NMDA toxicity [65]. Given the considerable potency of NR channel blocking PA analogues like N$^1$-dansyl-SPM, benz$_3$-SPD, and some spider toxins, these compounds might represent a promising structural lead for the development of new NR channel blockers.

**A complex network of targets**

The systematic review of stimulatory and blocking influences of PAs and PA analogues at the NR reveals a complex network of separate targets interrelated to each other, reflecting the cooperative interaction between various types of NR subunits. The conductance of the NR channel depends on the accessibility to cations of the channel's mouth, and on the conformation of the channels gate in the depth of the membrane. As identified in extensive point mutation experiments, the ionic milieu at the channel's mouth critically depends on several acidic amino acid residues in the extracellular N-terminus (E181 [8], E185 [66] and E339 & E342 [67] on NR1; E201 [68] and E191 [66] on NR2B; see Fig. 2, white circles) and in the extracellular loop between the $3^{rd}$ and the $4^{th}$ membrane domain of NR1 (D669 [57]). Thus, NR1 is the main target for PAs: Although robust stimulation by PAs is only seen in the simultaneous presence of the NR2B subunit [59], most mutations eliminating PA stimulation in NR1 are ineffective if introduced analogously into NR2B. If exon 5 is spliced in between lysine 190 and alanine 191 of NR1, some of its basic amino acid residues interact with these critical acidic amino acid residues and allow for free access of $Na^+$ and $Ca^{2+}$ ions even at acidic pH (relieve of proton block [7]). In splice variants without exon 5 (the more common situation), the same result is brought about by extracellular PAs like SPD and SPM. Conformational changes induced by acidification allosterically couple to corresponding changes at the channel's gate. The neutral amino acid residues W608 & N616 in the pore loop region, Y647 in M3 [69], and C744 & C798 in the glycine binding domain between M3 and M4 [70] have been found essential for this pH-dependent coupling.

Table 5. Polyamines as NMDA receptor channel blockers

| | structure | effect | ref. |
|---|---|---|---|
| benz$_2$-N3N |  | complete block at 1 mM[a] | [53] |
| benz$_3$-SPD |  | IC$_{50}$ 0.2 µM[b] | [56] |
| SPD-1,3-naph |  | IC$_{50}$ 4.4 µM[a] | [54] |
| N4T2N-(L)phe-(L)phe |  | IC$_{50}$ 28 µM[a] | [12] |
| N$^1$-dansyl-SPM |  | IC$_{50}$ 0.3 µM[b] | [55] |
| N$^1$-OS-SPM |  | IC$_{50}$ 0.4 µM[b] | [55] |
| argiotoxin$_{636}$ |  | IC$_{50}$ 3.0 µM[a] | [60] |
| | | IC$_{50}$ 0.01 µM[b] | [62] |
| agel-505 |  | stimulation at 10 & 100 µM[a] | [61] |
| | | IC$_{50}$ 0.013 µM[b] | [61] |

Abbreviations: benz$_2$-N3N, N,N'-dibenzyl-1,3-diaminopropane; benz$_3$-SPD, $N^1,N^4,N^8$-tribenzylspermidine; SPD-1,3-naph , $N^1,N^8$-bis(2-naphthylmethyl)-spermidine; N4T2N-(L)phe-(L)phe, 5-(4-aminobutyl)-2-thiophenethyl-L-phenylalanyl-L-phenylalanine; N$^1$-dansyl-SPM, $N^1$-dansylspermine; N$^1$-OS-SPM, $N^1$-(n-octanesulfonyl)-spermine

[a] [$^3$H]MK-801 binding to rat brain membranes
[b] inhibition of glutamate induced currents in Xenopus oocytes expressing NR1/NR2

The cysteine residues also are responsible for redox modulation of the NR [71]: The enhancement of NR currents by sulfhydryl-reducing reagents is eliminated of one of these residues is replaced by alanine, in parallel with the elimination of proton block and stimulation by SPM [69]. NRs containing any of the 4 NR2 subunits are redox sensitive, in contrast to the preferential stimulation of NRs containing NR2B by PAs.

The long extracellular N-terminal parts of the different NR subunits are composed of modules

Fig. 2. Tentative model of the NR complex [73, 8, 66, 76, 74, 13]. Four NR subunits are arranged around the ion channel pore, 2 NR1 and 2 NR2 subunits. The long extracellular N-termini form outer (LIVBP domain) and inner (LAOBP domain) modules for the binding of small molecules. In the case of NR2A, the small molecule bound by the outer module is known to be zinc. Ifenprodil interacts with the outer module of NR2B. The outer module of NR1 contains a fatty acid binding domain (FABP) and a region for splicing in exon 5. Still no ligand has been proposes for the LIVBP domain of NR1. The inner modules bind glutamate (glu, NR2) and glycine (gly, NR1), respectively. The NR chains penetrate into the cell's interior via the first membrane domain (M1), make a short return into the membrane by forming the pore loop selectivity filter (M2), leave the interior via M3 giving rise to long extracellular loops, which contribute to the inner binding modules, and finally reenter the cell via M4. Question marks denote hypothetical low molecular weight endogenous interaction partners. Individual amino acid residues marked by small white circles are crucial for polyamine stimulation: acidic residues on NR1 (E181 [8], E185, E297, D303 [66]; E339, E342 [67]; D669 [57]) and on NR2B (E191 [66]; E201 [68]) are direct targets for polyamines; other residues on NR1 (W608, N616, Y647 [69]; C744, C798 [70]) contribute indirectly. C744 & C798 are allocated in analogy to the crystal structure of an AMPA receptor fragment [72] and are also involved in redox modulation of the NR [70]. No individual residues are shown in the 2$^{nd}$ of the 2 NR1 subunits.

structurally related to bacterial proteins specialized in the capture of small molecules (Fig. 2; for a recent review, see [13]): In analogy to data obtained with a crystallized AMPA receptor fragment [72], a LAOBP (lysine, arginine, ornithine binding protein) domain on NR1 is supposed to bind the co-agonist glycine, and on NR2 an equivalent domain the main agonist glutamate [73]. On NR2, an additional LIVBP (leucine, isoleucine, valine binding protein) domain is on the 2B subtype the target of ifenprodil-type drugs ([33], see above); yet, no endogenous ligand is known. An equivalent domain binds zinc on the 2A subtype [74]; still no drugs have been described to interact with this latter site. The functional role of a similar domain (C22-N375) on NR1

[66], partly overlapping with a fatty acid binding domain (P263-Q393; FABP in Fig. 2) [75], is unclear; but data obtained with AMPA receptor fragments point to a general role of LIVBP domains in subunit assembly [13]. In a manner analogues to ifenprodil-type drugs at the NR2B, zinc by binding to the LIVBP domain of NR2A also enhances proton inhibition [76], explaining apparent competitive interaction between zinc inhibition and SPM stimulation in the same way as for ifenprodil. Inhibition of the binding of the channel ligand [$^3$H]MK-801 to rat brain membranes by ifenprodil and by zinc is either additive, super-additive, or less than additive [77], depending on the source of the membranes, and reflecting the regional distribution of NR2A and NR2B and various degrees of cooperativity between these subunits. Neither ifenprodil and PAs, nor zinc and PAs compete for common binding sites at the NR complex; they just affect - by different mechanisms - a common target in mutually antagonizing ways: on one side the PAs by direct charge screening, on the other ifenprodil and zinc by inducing a conformational change in NR2B and NR2A, respectively, shifting the critical acidic amino acid residues in less influential positions.

**Outlook**

Although much has been learned about the molecular mechanisms involved in PA's actions at the NR complex, the practical output of this knowledge is still very limited. In essence, only drugs targeting the LIVBP domain of NR2B are in clinical use, and this is not the merit of NR related research; some of these drugs have been in use for over 30 years, without clinicians being aware of their NR blocking effect. Presently, drug companies invest some efforts to reduce unwanted interactions of these drugs with other pharmacological targets as β, α$_1$ and σ receptors [78,79]. Since the interaction of drugs with the LIVBP domain is not mainly based on charge neutralization, compounds can be designed with favorable physicochemical properties, with a good chance to reach targets inside the blood brain barrier (BBB). Therefore, another promising target for drug development is the LIVBP domain of NR2A mediating zinc inhibition of the NR. Compounds intervening at the level of charge neutralization of acidic amino acid residues by PAs in the channel's vestibule appear to suffer from physicochemical constraints dictated by their genuine mechanism of action: They need to bear charges and, thus, represent a problem for the BBB. But do they? PA analogues with unconventional structure (see Table 2) suggest that

charge may be dispensable, at least at some positions. And after all, also polycations like SPD and SPM can be transported into the brain by specific transporters; SAR studies at these transporters should be taken into consideration in developing PA-type drugs, if neurologic or psychiatric diseases are the target. Furthermore, NRs are distributed widely in the whole body [80], and still very little is known about NRs in peripheral tissues as potential drug targets. Finally, in the light of dramatic advances in recent years of our knowledge concerning the molecular identity of the targets involved, a systematic approach employing rational drug design appears promising, including in silico and molecular biology techniques.

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