NMDA Receptors are not Alone: Dynamic Regulation of NMDA Receptor Structure and Function by Neuregulins and Transient Cholesterol-Rich Membrane Domains Leads to Disease-Specific Nuances of Glutamate-Signalling

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Abstract: Glutamate receptors of the N-methyl-D-asparate (NMDA-) subtype are tetrameric allosteric and ligand-gated calcium channels. They are modulated by a variety of endogenous ligands and ions and play a pivotal role in memory-related signal transduction due to a voltage-dependent block by magnesium, which makes them Hebbian coincidence detectors. On the structural level NMDA receptors have an enormous flexibility due to seven genes (NR1, NR2A-D and NR3A-B), alternative splicing, RNA-editing and extensive posttranslational modifications, like phosphorylation and glycosylation. NMDA receptors are thought to be responsible for excitotoxicity and subsequent downstream events like neuroinflammation and apoptosis and thus have been implicated in many important human pathologies, ranging from amyotrophic lateral sclerosis, Alzheimer's and Parkinson' disease, depression, epilepsy, trauma and stroke to schizophrenia. This fundamental significance of NMDA receptor-related excitotoxicity is discussed in the context of the developing clinical success of Memantine, but moreover set into relation to various proteomic and genetic markers of said diseases. The very complex localisational and functional regulation of NMDA receptors appears to be dependent on neuregulins and receptor tyrosine kinases in cholesterol-rich membrane domains (lipid rafts), calcium-related mitochondrial feedback-loops and subsynaptic structural elements like PSD-95 (post-synaptic density protein of 95 kD). The flexibility and multitude of interaction partners and possibilities of these highly dynamic molecular systems are discussed in terms of drug development strategies, in particular comparing high affinity and sub-type specific ligands to currently successful or promising therapies.

Keywords: NMDA receptor, neuregulin, cholesterol, excitotoxicity, neurodegeneration, dihydropyrimidinase-related protein 2, PARP-1, mitochondrial apoptotic pathway.

MAIN TEXT

L-Glutamate is the major excitatory neurotransmitter in the mammalian central nervous system (CNS), acting through various types of ionotropic and metabotropic receptors. Activation of these receptors is responsible for excitatory synaptic transmission on different time scales and in particular for synaptic plasticity, i.e. long-term structural changes. They have therefore been considered as targets for the treatment of various neurological diseases [1,2]. One important aspect of the pathophysiological roles of glutamatergic neurotransmission relates to these various forms of plasticity, like long-term potentiation (LTP) or long-term depression (LTD), which are initiated by increases of intracellular Ca^{2+} in excitatory synapses. NMDA receptors, which are glutamate-gated calcium channels, are crucial in these processes, which subsequently lead to calmodulin-dependent integration of signalling pathways [3]. The resulting activity-dependent persistent changes of synaptic transmission are accepted to be underlying learning and memory and their impairment being related to neurodegeneration [4,5]. The characteristic features of glutamate-induced effects are crosstalk, feedback loops involving gene transcription and complex interactions between subunits of multimeric allosteric proteins and/or components of higher order protein complexes, which are initially regulated on the relatively fast level of posttranslational modifications. Tyrosine phosphorylation, nitrosylation (NO), calcium-dependent phosphorylation and dephosphorylation and potentially methylation and ADP-ribosylation in this order are the most likely events shaping the underlying networks. Under normal physiological circumstances, the corresponding networks initiate and stabilize neuronal plasticity, but under a variety of stressful conditions they can derail towards increasingly irreversible pathophysiological conditions, all calcium-related [6,7,8,9]. Indeed, obviously "healthy" irreversible processes (LTP/LTD) are very similar in their recruitment of molecular machineries, and probably not so extremely different in their likelihood of occurrence or their degrees of mechanistic freedom as compared to "harmful" irreversible events (apoptosis, DNA-damage) manifesting themselves as neuroinflammation or neurodegeneration [10].

It is not yet absolutely clear how the changes that underlie maintenance of LTP and in particular NMDA receptor-dependent LTP, contribute to memory consolidation [11,12]. However, there is an increasing number of studies providing strong indications that coincidence-detection by NMDA receptors is the most important, if not the only, molecular master-switch fulfilling Hebb's rule: NMDA receptors are the only known molecules which simultaneously are voltage-dependent (because of the voltage-dependent Mg^{2+}-block) and ligand-gated by chemical neuro-

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transmitters, and related work has substantially validated the corresponding theoretical foundations of learning and memory [13]. We would like to note, that the very same molecular components discussed so far, NMDA receptors, calcium, apoptotic markers from oxidative stress etc., have a direct relationship to presinilins, mitochondrial homeostasis and NAD^+-consuming enzymes like PARP-1 and sirtuin, factors considered pivotal in aging and longevity [14,15, 16,17,18,19,20].

The enormous functional flexibility of NMDA receptors is reflected by a corresponding structural diversity: The NMDA family of glutamate receptors (NMDAR) consists of tetrameric cation channels which can selectively be activated by N-methyl-D-aspartate. There are a variety of different subtypes of this receptor due to multiple combinations of individual subunits. To date, seven genes (NR1, NR2A-D and NR3A-B) are known, but in addition numerous splicing variants, in particular for NR1 have been found and RNA-editing further generates diversity. Moreover, posttranslational modifications like phosphorylation, palmitolyation, proteolytic cleavage and glycosylation modulate function, localization, trafficking and clustering of NMDA receptors. They are phosphorylated on serines of NR1 and NR2 subunits and on tyrosines of NR2 subunits [21,22]. In this context phosphorylation of C-terminal PDZ-ligands of NR2 subunits (PDZ-ligands are domains which bind to corresponding PDZ-domains on scaffolding proteins, where PDZ stands for post synaptic density protein, discs large protein, zonula occludens; PDZ domains consist of 80 to 90 amino acids comprising six β-strands and two α-helices, compactly arranged in a globular structure) [23] and palmitolyation affect the calcium-dependent interaction with PSD-95 (post synaptic density protein complex, including guanylate kinase, 95 kD), one of the most important organisational principles of synaptic structural architecture [24,25,26]. PSD-95 in turn differentially modulates NMDA receptor subtypes [27,28].

Physiological function of the receptor is directly related to subunit composition of the oligomer, with NR1 being essential for the formation of functional channels, and NR2 and NR3 playing modulatory roles [29]. Pathophysiological properties on the other hand, e.g. in context of nociception, β-amyloid-induced neurotoxicity or ischemia, are more closely related to posttranslational modifications [30,31, 32,33,34].

The challenge for medicinal chemists in recent years has thus been the synthesis of compounds that can discriminate between these multiple binding sites and sub-types. Next to the glutamate (NMDA-) binding site, the NMDA receptor has allosteric domains modulated by a variety of additional ligands. Efficient NMDA receptor activation requires not only glutamate but also a co-agonist, glycine. Activation can also be modulated by the binding of polyamines. Each of the binding sites (glutamate, glycine, polyamine) has been used as a potential target for the development of both receptor and sub-type selective compounds. Moreover, there is a voltage-dependent block by Mg^{2+}-ions. The most important binding sites are indicated in Fig. **1**, Table **1** provides an overview of ligands.

The NR2 subunits, of which there are four (A_D), determine the pharmacology and functional properties of NMDA receptors, with further contributions from alternative splicing of NR1. Differential assembly of receptor subunits allows for subtype- selective compounds. As a consequence of the fact, that the glutamate binding domain is formed at the cleft between NR1 and NR2 subunits (Fig. **1**), assembly of both subunits is required to form functional receptors. The binding site of the co-agonist glycine, which is essential for glutamate-induced channel opening [35], is part of the NR1 subunit (Fig. **1A** and **C**). NMDA receptors also possess additional binding sites for Zn^{2+}, Mg^{2+}, polyamines and potentially neurosteroids and eicosanoids [1,2,11].

At resting membrane potential, NMDA receptors are blocked by magnesium ions; depolarization, in particular by non-NMDA glutamate receptors (AMPA- and Kainate-receptors) removes this block. Only sustained depolarization of the post-synaptic cell releases the channel inhibition and thus provides the coincidence condition, which is assumed to underlie memory formation. This simultaneous activation of NMDA receptors by independent parameters voltage and neurotransmitters makes them prime candidates for Hebbian coincidence detectors on the molecular level [13]. NMDA receptors are permeable to calcium ions and other cations, like sodium, their activation leads to transient calcium influx into post-synaptic cells, where calcium as a crucial second messenger induces processes leading to plasticity or pathophysiological events [1,2,11].

Apart of the co-agonist glycine, additional drugable target sites on NMDA receptors are located in the extracellular domains which are formed between trans membrane domains TM3 and TM4 (Fig. **1B**) and contribute to agonist and modulatory binding sites. The endogenous ligand for the modulatory domain on NR2A appears to be zinc [11]. Endogenous ligands for NR2B are not known but this is the site of interaction with Ifenprodil-like NR2B noncompetetive antagonists. In addition to its channel blocking action, Mg^{2+} moreover appears to be the endoge-nous ligand for the polyamine modulatory site. Studies of native receptors suggest that polyamines, Ifenprodil and Mg^{2+} share common features in their interactions with the NMDA receptor [36,37].

Taking into account that different NMDA subtypes are expressed in different regions of the brain [38] and at different times in development [39], there is hope that NMDA receptor subtype selective drugs may provide novel and useful physiological effects, without producing unwanted side effects.

NMDA RECEPTOR LIGANDS, A CLOSER LOOK: AGONISTS

Several lines of evidence indicate that potentiating NMDA receptors under certain circumstances should be beneficial for treating anxiety disorders, cognitive disorders and schizophrenia [40]. Enhancing NMDA receptor function can be achieved directly through glutamate site agonists or indirectly through modulatory sites. NMDA receptor agonists have not been explored because of concerns over excitotoxicity. Besides, structural analogues of NMDA cannot discriminate between NMDA receptor subtypes and

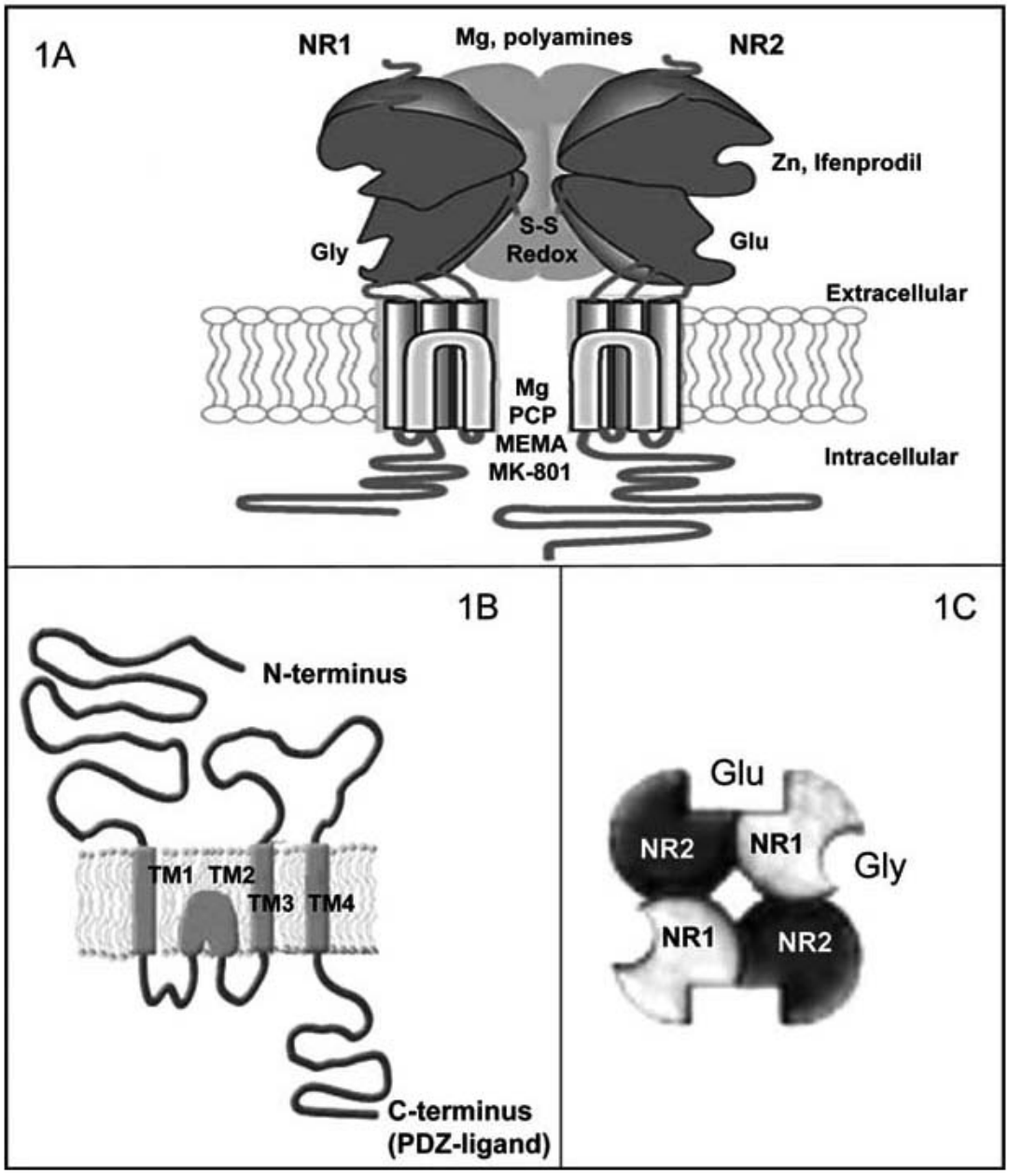

Fig. (1). Structure of NMDA receptors: A schematic presentation of NMDA receptors is shown. They are allosteric proteins with a variety of binding sites for at least four different types of ligands, which are indicated in Fig.1A. Structural investigations indicate four hydrophobic domains in each of the four subunits; a representative subunit is shown in Fig. 1B: There is a huge N-terminal domain and a domain between transmembrane segments 3 and 4, which are extracellular and a huge intracellular C-terminal domain, which includes the PDZ-ligand domain, responsible for interaction with PSD-95 and other synaptic scaffolding proteins. There is evidence that one of these hydrophobic domains does not entirely span the membrane. NMDAR contain two NR1 subunits and two NR2 or more rarely NR3 subunits. The channel pore is lined by all four subunits, and the glutamate and glycine binding site have contributions of adjacent NR1 and NR2 (NR3) subunits, whereas the modulatory sites are mainly on NR2 (NR3) subunits (Fig. 1C). The various ligands are indicated by their names next to supposed binding clefts, Glu is glutamate, Gly is glycine, PCP is for phencyclidine, MEMA is Memantine, TM means transmembrane domain, S-S is a disulfide bridge supposed to be essential for redox state sensing of NMDAR.

therefore have been considered to have limited application. This can potentially be overcome by conformationally constrained analogues like homoquinolinic acid (Fig. **2A**, Table **1**), which shows higher affinity for NR2B containing NMDA receptors than for the other subtypes but the quest to minimize potential excitotoxicity remains [41].

On the other side, NMDA receptor activity can be enhanced through allosteric sites. Activation of the glycine site has shown some promising results: Preclinical evidence suggests that glycine or other glycine site agonists like D-serine and D-cycloserine (Fig. **2B**) are most likely to find clinical use in the treatment of chronic pain, drug abuse, and

Table 1. Compilation of Representative NMDA Receptor Ligands, the Corresponding Chemical Structures are Shown in Figures 2-5. Ligands are Grouped in Agonists (#1-6), Competitive Antagonists (#7-18), Non-Competitive Antagonists (#19-22), Glycine Site Antagonists (#23-28) and Polyamine Site Antagonists (#29-34). The Table is Meant to give an Overview and not Comprehensive

#	Type of ligand	Name of compound	Subunit specifity	References
1	NMDAR agonists	Homoquinolinic acid	NR2B>NR2A>NR2C>NR2D	[41]
2		D-serine		[35,42]
3		D-cycloserine		[35,42]
4	Glycine site (agonists or reuptake inhibitors)	ALX 5407		[43]
5		ORG 24598		[44]
6	Other agonists	Pregnenolone sulfate		[46]
7	NMDAR competitive antagonists	(R)-AP5	NR2B=NR2A>NR2D	[54]
8		(R)-CPP	NR2B=NR2A>NR2D	[54]
9		PBPD	NR1/NR2D	[55]
10		CGS 19755	NR2A>NR2B= NR2C	[56]
11		EAA-090	NR2A>NR2B= NR2C	[56]
12		EAB-318	NR2A>NR2B= NR2C	[56]
13		EAA-090	NR2A> NR2B= NR2C	[56]
14		Felbamate	NR2B	[57]
15		Ro 63-1908	NR2B	[58]
16		Ifenprodil (CP101)	NR2B	[58]
17		Traxoprodil (CP 606)	NR2B	[58]
18		Ro 25-6981	NR2B	[58]
19	NMDAR channel blockers (non-competetive antagonists)	Dizolcipine (MK-801)		[241], [242]
20		Memantine		[60]
21		Ketamine		[60]
22		N20C		[64]
23	Glycine site antagonists	L701,324		[35]
24		L689,560		[35]
25		GV150526A		[35]
26		L683334		[35]
27		ACEA 1416		[35]
28		M241,247		[35]
29	Polyamine site antagonists	Ifenprodil		[73]
30		Eliprodil		[243]
31		Traxoprodil		[244]
32		Ro63-1908		[75]
33		Ro25-6981		[245]
34		Co101314		[246]

as neuroprotective agents [42]. The evidence so far indicates that glycine sites of NMDA receptors are not always saturated *in vivo*, which suggests them as attractive targets for drug development, where both full antagonists and partial agonists might find therapeutic applications. Glycine site ligands also show differences in affinities and intrinsic activities with various subtypes of NMDA receptors [35].

An alternative strategy aims at increasing extracellular levels of glycine by blocking glycine re-uptake into neurons by inhibiting the corresponding glyT-1 transporter (using drugs such as ALX 5407 [43] and ORG 24598 [44], Fig. **2C**). A related approach is the increase of extracellular levels of D-serine or glutamate itself by inhibiting their respective re-uptake into glial cells. Neuroactive steroids may also potentiate or inhibit the NMDA receptor activity; apparently they occupy a site that is distinct from those used by other modulators [45]. For example, the prototype of sulfated steroids, pregnenolone sulfate [46] has a greater (activitation state-dependent) effect on receptors containing NR2A, but also has potentiating properties towards receptors containing NR2B [47,48]. Estimation of binding constants for this type of effect point to low affinities in the μM range [49]. Some authors have reported indications of a distinct steroid domain on NMDA receptor NR2B subunits that is critical for both pregnenolone sulfate enhancement and proton sensitivity, including parts of the glutamate recognition site and the fourth membrane transmembrane region (TM4, see Fig. **1B**) [46]. Other reports show complex inhibitory effects of the endogenous neurosteroid 20-oxo-5 -pregnan-3 -yl sulphate (more pronounced in NR2C-D than NR2A-B), which depend upon kinetic parameters of glutamate exposure, but again appear to be associated with the extracellular loop between transmembrane helices TM3 and TM4 [50].

To our knowledge, a clear proof of a direct interaction of steroids with NMDA receptors has not been shown yet, and thus the authors would like to draw attention to the emerging importance of non-genomic steroid effects which have profound and complex implications for the cytoskeletal organization of affected cells. In particular in view of the interactions of NMDA receptors with PSD-95 via C-terminal PDZ ligand domains (Fig. **1B**) [24,25,26] and the respective regulative effects on the synaptic cytoskeleton [27,28], one

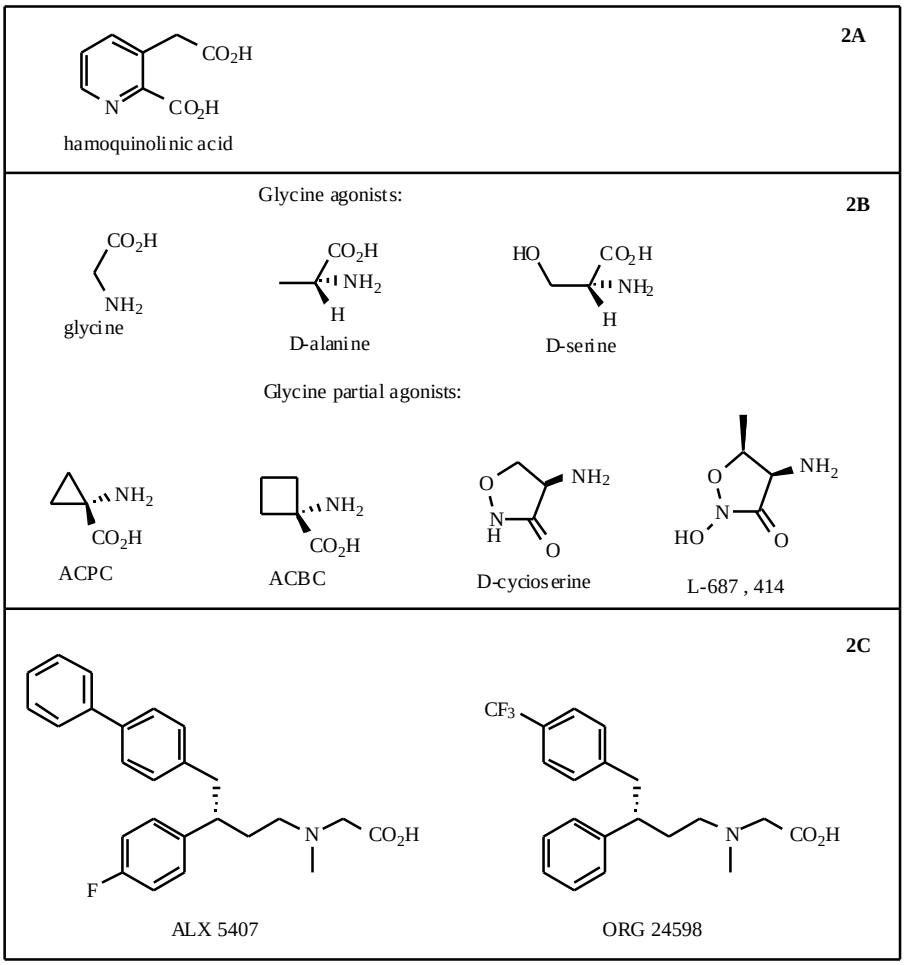

Fig. (2). NMDA receptor agonists, glycine site agonists or partial agonists, glycine reuptake inhibitors: The chemical structures of NMDA receptor agonists listed in Table **1**, are shown; Fig. **2A** is homoquinolinic acid, in Fig. **2B** some glycine site agonists or partial agonists are shown; ALX 5407 and ORG 24598, which are glycine reuptake inhibitors and thus indirect agonists are depicted in Fig. **2C**.

could thus speculate that steroid effects are potentially more indirect via membrane steroid receptors [51].

NMDA RECEPTOR ANTAGONISTS

Any CNS disorder in which glutamate-induced excitotoxicity occurs has the potential to be treated by blocking NMDA receptors. The list includes cerebral ischemia, stroke, neurodegenerative disorders (for example, Alzheimer's, Parkinson's and Huntington's diseases), epilepsy and neuropathic pain, in which there is an overactivity of excitatory pathways [2]. The underlying mechanistic aspects vary in the different disease indications and will be covered below in more detail. Several studies using models of ischemia and stroke have demonstrated that neurotoxicity is primarily mediated through NMDA receptors and that blocking NMDA receptors during or shortly after an excitotoxic event can prevent much of the neuronal death [52]. Accordingly, both competitive and noncompe-titive NMDA receptor antagonists have been investigated as potential neuroprotective agents for treatments of stroke, Alzheimer's disease and head injuries [2]. Nevertheless, clinical trials in stroke and traumatic brain injury with NMDA antagonists have so far failed [53]. The situation appears to be much more favorable regarding Memantine, a moderate NMDA channel blocker with low affinity, which has shown promising results for the treatment of Alzheimer's dementia and Parkinson disease. Memantine is therefore treated in a special section below.

NMDA receptor competitive antagonists act at the glutamate binding site where a high degree of subtype specificity can be achieved. Some examples of subtype specific NMDA receptor competitive antagonists are given in Fig. **3**. Thus, for example, (R)-AP5 and (R)-CPP show selectivity for NR2A- and NR2B- over NR2D-containing NMDA receptor complexes [54]. In opposite to them PBPD is NR1/NR2D specific antagonist [55]. CGS 19755, EAA-090 and EAB-318 have higher affinities for NR2A- versus NR2B- or NR2C-containing NMDA receptors, with EAA-090 shows even greater selectivity. EAB-318 behaves in a similar fashion, but it also inhibited AMPA and Kainate receptors. The combination of NMDA and AMPA/Kainate block enabled EAB-318 to protect neurons against ischemia induced cell death [56].

On the other hand Felbamate (Fig. **3B**), a novel anticonvulsant, inhibits all NMDA receptors but with a 4-10 fold preference for the NR2B-containing receptors [57]. It is suggested that Felbamate interacts with a unique site on the NR2B subunit (or one formed by NR1 plus NR2B) that interacts allosterically with the NMDA/glutamate binding site. These results suggest that the unique clinical profile of

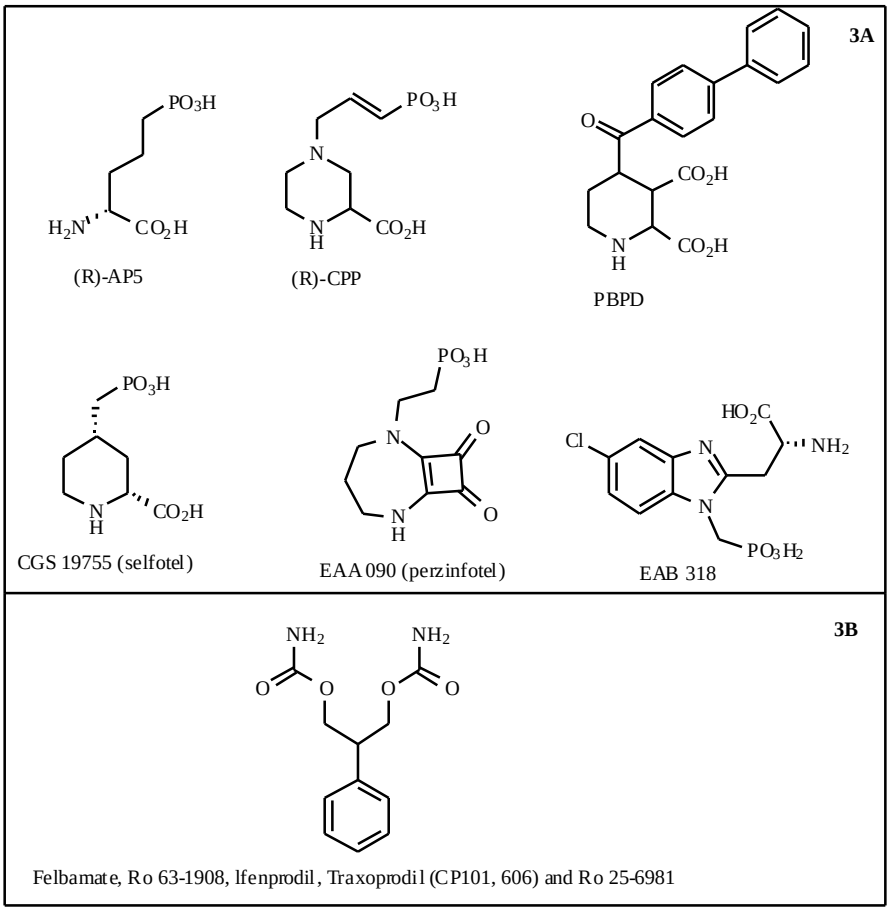

Fig. (3). Competitive antagonists of NMDA receptors: The chemical structures of some sub-type specific competitive antagonists of NMDA receptors are shown in the upper part (Fig. **3A**) and of a non-selective competitive antagonist, Felbamate in the lower part (Fig. **3B**).

Felbamate is due in part to an interaction with the NR1-2B subtype of NMDA receptor. Recent studies support the view that NR2B subunit-selective NMDA receptor antagonists Ro 63-1908, Ifenprodil, Traxoprodil (CP101, 606) and Ro 25-6981 (which also are discussed in a context of polyamine site ligands) produced measurable performance improvements in a number of cognitive tests in rats [58].

NMDA RECEPTOR CHANNEL BLOCKERS

These compounds act by binding to the pore of the NMDA receptor channel and are thus non-competitive antagonists (Fig. **4**), which have no selectivity for particular NMDA receptor subtypes. A clear advantage of this kind of compounds is that they bind preferentially to open states of active receptors which are more important during pathophysiological signaling. Drugs such as Dizolcipine (MK-801) and phencyclidine are open channel blockers at nanomolar affinities that efficiently protect neurons but display significant side effects, in particular with regard to performance in cognitive tasks [59]. Blockers with lower affinities in the submicromolar range, such as Memantine and Ketamine exhibit a better therapeutic profile, although it has been reported that chronic administration of these compounds enhances neuronal death under certain conditions [60]. Nevertheless, Memantine has recently received approval for the treatment of moderately severe to severe Alzheimer's disease and is under investigation for further indications [61].

Thus, the development of novel NMDA receptor open channel blockers of low molecular weight, moderate-to-low receptor affinity, and fast on/off blockade kinetics is actively pursued. These new compounds may be devoid of the adverse *in vivo* effects of well established, high-affinity NMDA antagonists [62,63]. Along this line a new *N*-alkylglycine, N20C (Ki is 5000 nM for open channel blockade) was developed with pronounced *in vitro* and *in vivo* neuroprotective activity [64]. The authors observe an emerging principle here, which aims at finding a balance between outright inhibition of a target with highly specific

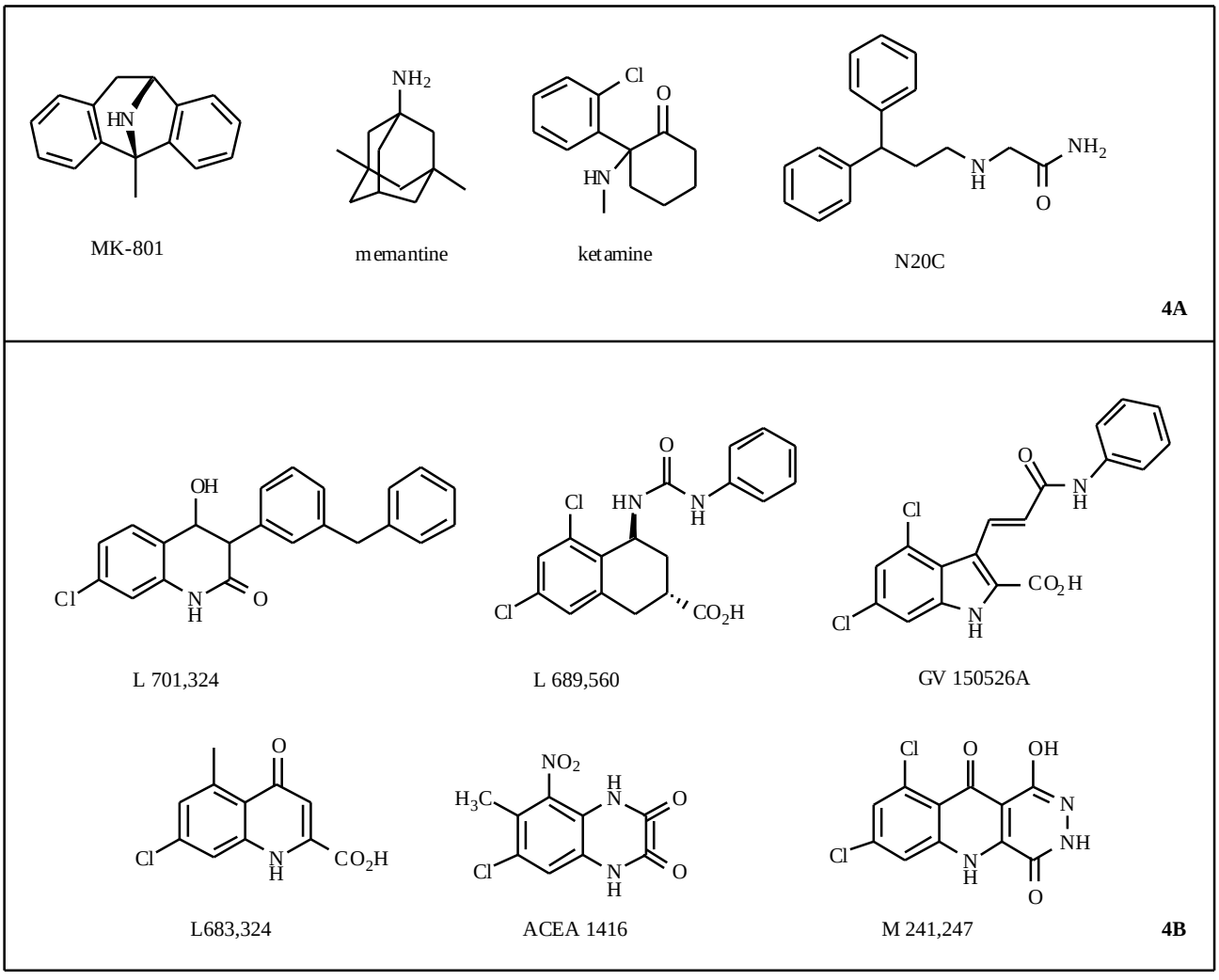

Fig. (4). non-competitive open-channel blockers and glycine site antagonists at NMDA receptors: The chemical structures of some important non-competitive open-channel blockers of NMDA receptors are shown in the upper part (Figure 4A), and glycine site antagonists in the lower part, Figure 4B.

and selective ligands and the otherwise physiological important function of the same target. In the complex protein networks already sketched above, strategies employing low affinity, non-selective substances obviously have a huge unexploited potential. This observation will be supported with further details later in the text.

NMDA RECEPTOR GLYCINE SITE ANTAGONISTS

The glycine site is necessary for NMDA receptor activation and the co-agonist increases the affinity of the receptor glutamate [35]. Hence antagonism of this binding site will also antagonize NMDA receptor function. The glycine binding site is a target for an alternative approach to avoid unwanted side effects of complete NMDA receptor blockade. So far, partial blockade of NMDA receptor has been most effectively achieved through this glycine site, for which partial agonists exist (Fig. **2B**). Also, a number of antagonists have been developed for this binding site and chemical structures of some representative are given in Fig. **5**. None of the glycine antagonists developed so far displays sub-type selectivity. The reason for this is that the glycine binding site is exclusively located on the NR1 subunit while the glutamate binding site in addition recruits part of the NR2 subunits. Preclinical evidence suggests that glycine antagonists are most likely to find clinical use in the treatment of chronic pain, drug abuse, and tolerance and as neuroprotective agents [65,66,67].

NMDA RECEPTOR POLYAMINE SITE ANTAGONISTS

Polyamines are positive allosteric modulators of NMDA receptor function in the sense that they increase the affinity for glycine towards NMDA receptors. Antagonists for this site would decrease the affinity of the receptor for glutamate and generate a voltage-dependent inhibition of the channel. The effects of polyamine site antagonists are specific for channels containing the NR2B subunit. Hence, polyamine site antagonists would be very useful as NR2B selective ligands. However, endogenous polyamines may not modulate the NMDA receptor *in vivo* because the site of action on the NMDA receptor is on the extracellular surface of the cell, while polyamines are usually found in the intracellular compartment. Thus, polyamines may not act physiologically on NMDA receptors except under pathological conditions [68]. The endogenous modulator(s) at this site could be protons or magnesium whose effects overlap with those of spermidine [69]. Structures of some high affinity polyamine binding site antagonists are presented in Fig. **6**. It appears that activity-dependent NR2B subtype-selective NMDA antagonists, such as Ifenprodil, show potential for the treatment of stroke, traumatic brain injury, and Alzheimer's and Parkinson's disease without the severe side-effects of earlier drugs [70,71]. By preferentially targeting persistently activated receptors, Ifenprodil-like inhibitors have a reduced affinity and unbind from inacti-

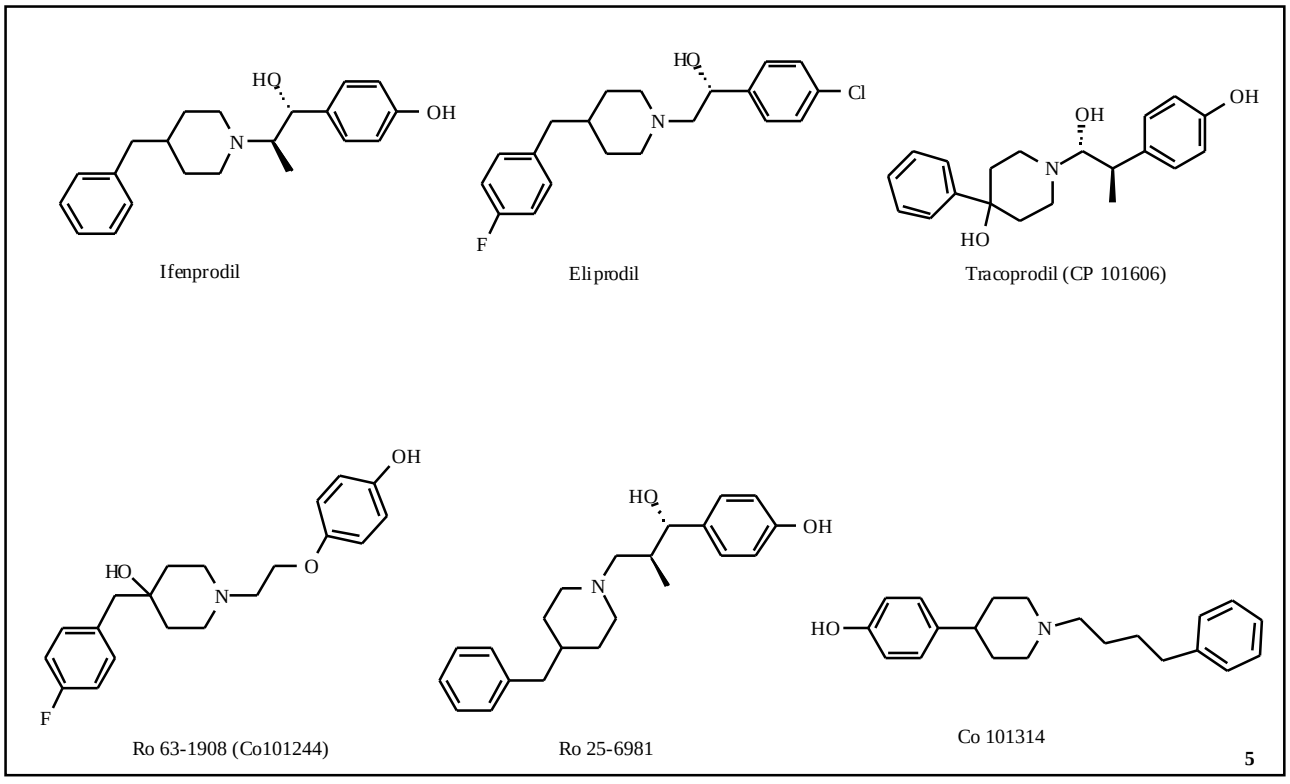

Fig. (5). antagonists of the polyamine site of NMDA receptors: The chemical structures of some polyamine site antagonists are shown, which are also considered to be NR2B-selective ligands.

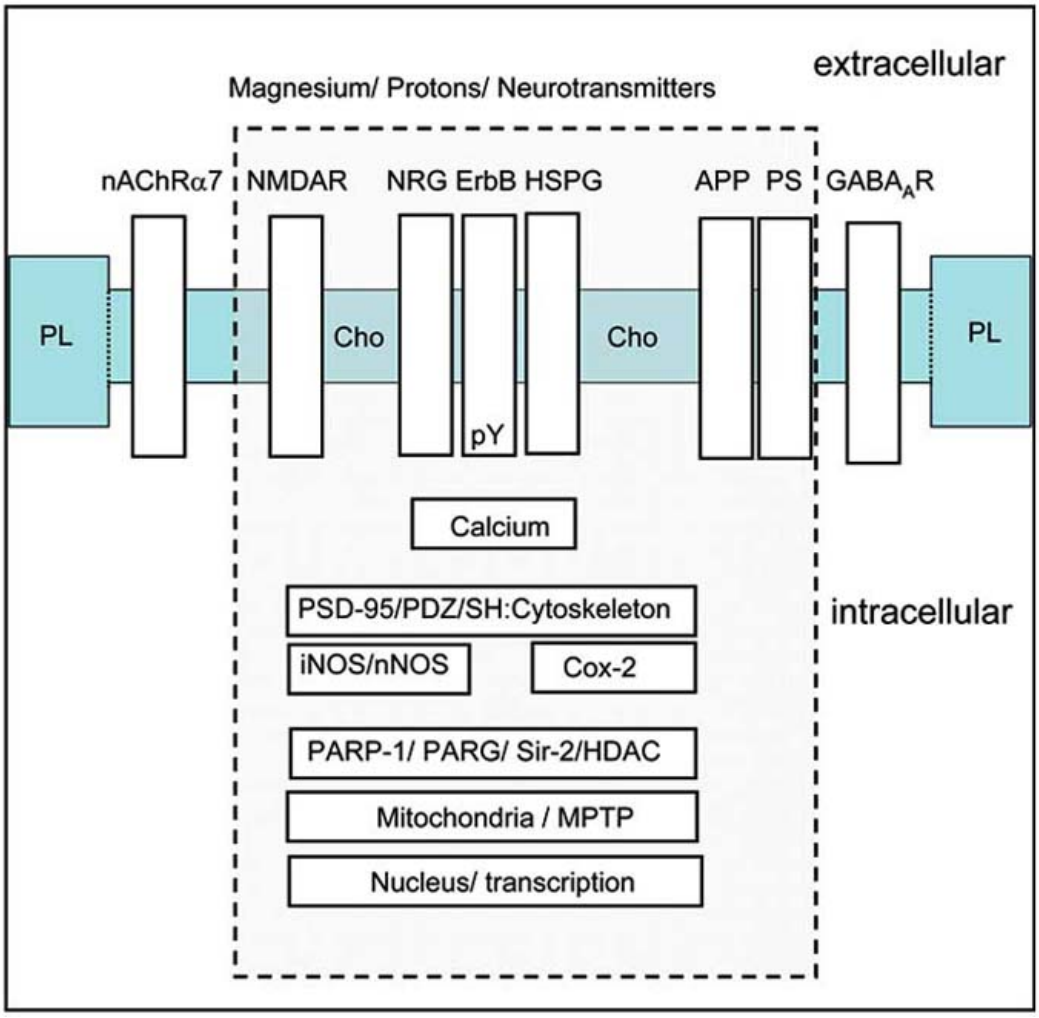

Fig. (6). Potential assembly of NMDA receptors in cholesterol-rich lipid rafts, as part of a dynamic functional supercomplex, including neuregulins, ErbB receptors and further membrane proteins: The figure is an attempt to summarize published facts about these functional complexes, consisting at least of neuregulins (NRG), receptor tyrosine kinases (ErbB receptors), heparansulfate proteoglycans (HSPG) and NMDA receptors (NMDAR), which are assembled together in cholesterol (CHO)-rich membrane microdomains, so-called lipid rafts in an activity-dependent manner; NMDAR signalling is modulated by these interactions and transient associations, thought to be crucial for synaptic architecture. In particular the shaping of calcium signals is important for the interaction with subsynaptic scaffolding proteins by posttranslational modifications (PSD-95, by interaction with certain phosphorylated domains, like PDZ- or SH-domains on partner proteins). The PSD-95 complex directly regulates pro-inflammatory enzymes like nitric oxide synthase (NOS, iNOS is inducible, nNOS is neuronal) and Cox-2 (cyclooxygenase-2), which promote their effects in a complex relationship with related, but not necessarily downstream mechanisms, involving NAD^+-dependent enzymes like PARP-1 (poly-ADP-ribose polymerase-) and Sir-2 (sirtuin-2); PARG is poly(ADP-ribose) glycohydrolase the complementary and antagonistic enzyme to PARP-1, HDAC are histone deacetylases, the general class of enzymes which includes Sir-2. MPTP stands for the mitochondrial permeability transition pore. All these components interact in complex and often recurring feed-back type of interaction and are for this reason underlain with a grey frame (broken line). Also other important membrane proteins, like certain nicotinic acetylcholine receptors (nAChRα7), $GABA_A$ receptors ($GABA_AR$) amyloid precursor protein (APP) and presinilins (PS) are transiently organized in lipid rafts and acquire different functional properties outside the usual phospholipid (PL) environment. On the extracellular side these complexes are modulated by a variety of effectors, like cations, protons, other neurotransmitters or trophic factors, details are provided in the main text.

vated receptors and also leave transiently activated receptors relatively unaffected [72,73], which might be crucial for normal physiological signaling related to plasticity and learning. Moreover, they additional advantages over nonselective competitive and ion channel-blocking NMDA antagonists include a much improved tolerability in animals and in humans [74,75,76]. Interestingly enough some amino-glycoside antibiotics appear to act as spermine-like agents on NR2B subunits [77]. The question of exact structural details defining polyamine-, glutamate- or Mg^{2+}- binding site contributions to the effects of said drugs is not always clear.

FUNCTION AND PHYSIOLOGY

We now come back to the point, that glutamate and NMDA receptors are not only crucial in memory-and cognition-related calcium-dependent signal transduction, but

also in pathophysiological conditions where these very same mechanisms derail due to excessive activation under a variety of disease-related conditions [16] essentially always giving rise to excitotoxic intracellular calcium overload, again with a variety of embodiments ranging from sometimes reversible inflammatory conditions, to endoplasmatic reticulum stress (ER-Stress) or autophagy, or to apoptotic and/or necrotic cell death [78]. Moreover, excitotoxicity is dependent upon developmental stages and age [79]. So, NMDA receptor stimulation activates many downstream mechanisms involved in both, cell survival and cell death. [80,81]. Consequently, strategies aimed at counteracting glutamate excitotoxicity, without inhibiting physiological glutamate signalling too severely, appear to be increasingly attractive, albeit challenging. The NR2B polyamine site antagonists discussed above offer a corresponding strategic option.

FUNCTIONAL ASPECTS OF NR2 SUBUNITS

The NR2 subunits of NMDA receptors, which have such an intricate set of different modulatory binding sites and are subject to fast posttranslational modifications like phosphorylation, are moreover regulated by activity-dependent gene transcription. This constitutes a feedback loop between NR2-dependent activity and corresponding subunit composition. The salient feature of NR2-subunits, which is important in this context is, that ectopic expression experiments have shown, that NR2A and NR2B-containing receptors are much more sensitive to the voltage-dependent Mg^{2+}-block than NR2C and NR2D-containing receptors. [82]. This could mean that the initial detection of Hebbian coincident synaptic events is rather mediated by NR2B and NR2A, and more persistent changes, which also are more prone to higher intracellular calcium concentrations (due to a decreased sensitivity towards voltage-dependent Mg^{2+}-block) are associated with NR2C and NR2D.

The situation is further complicated by the fact that other ions, like K^+-ions modulate the affinity for Mg^{2+} and that the co-agonist glycine at high concentrations causes Mg^{2+}-dependent potentiation, (instead of blockade!) of NMDA responses [83,84]. Moreover, the ligand binding niches, especially with contribution of NR2B subunits, have considerable structural flexibility. Agonist efficacy at the NR2B subunit appears to be controlled by the extent of steric clashes between the agonist and the ligand binding domains, and thus by ligand-dependent arrangements of residues within the binding pocket [85]. All this suggests a remarkable flexibility and versatility of interaction between the ligand binding domains within individual NMDA receptors, but as well in immediate exchange with the surrounding solute, as the example of K^+- and Mg^{2+}-ions shows. The study of NR2-chimeric receptors has demonstrated, that differences in Mg^{2+}-block cannot be explained by a single structural determinant, but rather by three domain elements shaping subtype-specific differences of Mg^{2+}-block in heteromeric NMDA receptor channels [86]. Under physiological conditions, protons join: recent data from electrophysiological experiments with transiently expressed NR1/NR2B receptors are consistent with a modest proton inhibition that can describe macroscopic and single-channel properties of the corresponding receptor function over a range of pH values. [87].

On this background, it seems plausible, that the different functional domains of NMDA receptors have a multitude of energy minima, with a lot of functional overlap between neighbouring structural modules, and that this supposedly big number of degrees of freedom is essential for physiological function, but as well responsible for a variety of events leading towards excitotoxicity. Moreover, the majority of interactions with ligands are associated with moderate affinities, physiologically meaningful, because locking NMDA receptors in one state is potentially always detrimental: either in terms of overactivation and excess calcium, or in terms of restricted activation, impeding memory-related signals.

NMDA RECEPTORS ARE NOT ALONE; NEUREGULINS AND TRANSIENT CHOLESTEROL-RICH MEMBRANE DOMAINS SHAPE GLUTAMATE-SIGNALLING VIA CALCIUM AND TYROSINE PHOSPHORYLATION

Next to the "softer" NMDA receptor-intrinsic modulations above, there are two levels of additional regulation: The first is clearly localization in chemically and biophysically distinct parts of the cell membrane, in cholesterol- and sphingolipid-rich patches, called "lipid rafts", which in a very dynamic way recruit further "raft"-proteins, which then in turn and secondly, can cause direct chemical modifications of the receptor. These "hard" molecular changes occur on different time scales ranging from hours via transcription of new NMDA receptor subunits for permanent replacement, relevant for long-term plasticity, to seconds or even less by fast posttranslational modifications like phosphorylations. The fast events tend to be directly activity-dependent as should be expected form a Hebbian coincidence-detector, localization then appears to precede transcription. Independently of membrane environ-ment, the large intracellular C-terminal loops of NR2-subunits (see Fig. **1B**) apparently play a role in the precise synaptic arrangement of NMDA receptors, contain-ing e.g. the PDZ ligands [88,25,23,27,26] (PDZ stands for post synaptic density protein, discs large protein, zonula occlu-dens). NR2B subunits have thus a calcium-dependent interaction with the cytoskeleton and PSD-95 (postsynaptic density protein of 95 kD), via association with the PDZ domain of PSD-95, subsequently affecting the interaction between PSD-95 and calmodulin [89]. NMDA receptors can be located in cholesterol-rich membrane microdomains and their dynamics in the postsynaptic membrane is dependent on functional feedback mechanisms coupling activity and localization [90,91]. Cholesterol-rich rafts provide also an increasingly important principle for regulatory asymmetry of PDZ-proteins, with consequences for clustering and an-choring of membrane proteins. Here the emerging role of non-genomic effects of membrane steroid receptors with regard to cytoskeletal rearrangements, briefly discussed above could play a corresponding role because there is in-creasing evidence, that they are also mediated by cholesterol and rafts [92,93,94].

Related to their localisation in lipid rafts, the emerging relation of NMDA receptors to certain neuregulins (NRG) and ErbB receptor tyrosine kinases is of outstanding impor-

tance [95]. Four genes encode NRG-1, -2, -3 and -4 in vertebrates, and their diversity is further increased by alternate RNA splicing and promoter usage.There are three basic types of NRG-1, which are characterized by different extracellular domains [96]. NRG are ligands of ErbB receptor tyrosine kinases, which constitute a subfamily of four structurally related members of growth factor binding receptors,with EGF (epidermal growth factor) receptor (ErbB-1), ErbB-2, ErbB-3 and ErbB-4. They have very broad significance in many signalling pathways [97]. Very recently it has been shown that NRG-1 β1 has neuroprotective properties *in vivo* [98,99,100]. Previous functional studies moreover show, that NRG-1 β1 induces a specific expression of the NR2C subunits under conditions requiring previous activation of NMDA receptors, related to LTP [101,102]. As discussed above NR2C receptors are much less sensitive to voltage-dependent Mg^{2+}-block, and thus less coincidence detecting, etc.; NRG in the CNS not only integrate several neurotransmitter systems, e.g. by decreasing GABAergic neurotransmission [103], but also have recently been implicated from a genetic level in some diseases of the CNS, which are also intimately linked to excitotoxicity and discussed in more detail below [104,105].

There is evidence that part of the intriguingly integrative properties of NRG in normal and pathophysiological signalling is based on their role in organizing sphingolipid/cholesterol-rich membrane domains [106,107,108]. Briefly, in lipid rafts NRG, heparansulfate binding proteoglycans and ErbB receptors assemble as trimeric complexes in activity- and localisation-dependent mechanisms. This clustering considerably enhances subsequent signalling by tyrosine phosphorylation and calcium [109,110]. The NRG, which are the endogenous ligands of ErbB receptor tyrosine kinases, can exist both as membrane-bound and soluble forms (through alternatively splicing and posttranslational modifications [111]), and not only promote or modulate the local expression and activity of NMDA receptors (via interaction of ErbB with PDZ domain proteins!) [112,113], but also affect in a cholesterol dependent (= raft-dependent) manner NMDA-induced excitotoxic death [114]. Accordingly, in processes relevant for regeneration, heparin-binding forms of NRG accumulate to high levels in the synaptic basal lamina through the developmentally program-med expression of heparan sulfate proteoglycans, thus providing a sustained source of NRG to the most active synapses. Recently, certain NRG isoforms have been discussed in neuroprotective mechanisms and neuroregenera-tion [115,98 ,99,100]. A summary of the findings mentioned above is shown in Fig. **6**. In this context the previous discussion of direct interaction of NMDA receptors with PSD-95/PDZ domain proteins is relevant: association with NRG/ErbB in lipid rafts is supposed to further regulate underlying cytoskeletal architectures [24,25,26,116] and in particular connect to inflammatory mechanisms mediated by tyrosine phosphorylation.

Heparan sulfate proteoglycans (HSPG), the third component of the raft associated NRG/ErbB complex mediating and regulating NMDA receptor activity, interacts further with neural cell adhesion molecule (NCAM) thus indirectly promoting LTP and stabilization of synapses during early synaptogenesis. Interestingly, this type of NCAM-driven synaptogenesis can be blocked by NMDAR antagonists but not by blockers of non-NMDA glutamate receptors and voltage-dependent Na^{+}-channels. So again, like in the case of NRG, there is a mutual influence of the activities of NMDA receptors and HSPG upon each other, with feedback potential [117,118,119]. With regard to the role of NMDA receptor-related excitotoxicity in human diseases it is noteworthy here, that HSPG have also been described as cell-surface binding sites for neurotoxic amyloid-β [120].

EXCITOTOXICITY, NMDA RECEPTORS AND DISEASE AREAS

Excessive stimulation of neurons in the brain and spinal cord by glutamate is one of the best-studied mechanisms for cell death. Normally, nerve cells completely prevent its accumulation through glutamate transporters, which are found both on nerve cells and on astrocytes. In a number of diseases and pathological conditions, however, something goes wrong and glutamate-induced neurotoxicity is core and/or starting point of many problems. In particular, NMDAR cause oxidative stress in neurons, and proceed to cell death involving elements of both necrosis and apoptosis and much of glutamate-induced excitotoxicity is mediated by intracellular calcium increase [121,122]. In the following we will go into some detail, looking at which role NMDA receptors and subsequently calcium-related excitotoxicity are playing in various human diseases, respective neuroprotection and related animal models [123].

ALS

In amyothrophic lateral sclerosis (ALS), little doubt remains that oxidative injury, excitotoxicity, and mitochondrial dysfunction concurrently contribute to the progressive degeneration of both upper and lower motor neurons [124]. Whether mitochondrial dysfunction causes predisposition to vulnerability of motor neurons to calcium-mediated excitotoxicity or vice versa is still under debate, although reactive oxygen species subsequently initiate the intrinsic apoptotic pathway, which is initiated by formation of the mitochondrial permeability transition pore (MPTP, consist-ing of voltage-dependent anion channel VDAC-1, adenine nucleotide translocator ANT-1 and cyclophilin D) and is essentially caspase-independent [125,126]. The only accepted *in vivo* models for ALS are based on the fact, that about 20% of ALS cases are familial and involve mutations in superoxide dismutase gene (SOD-1), which points to an ALS-specific problem in the management of reactive oxygen species and led to the development of corresponding transgenic mouse models [127]. On the other hand, Riluzole, the only agent presently approved for clinical use in ALS patients, is an inhibitor of glutamate release, attenuating excitotoxicity, but unfortunately only extending survival by a few months [128]. A differential proteomic study of protein changes associated with ischemia/hypoxia in neuronal cell culture, showed appearance of several SOD-1-isoforms under this type of neuronal stress, which is moreover associated with transient calcium overload (rather emerging from mitochondria than extracellular space) [129].

Alzheimer's Disease

In the treatment of Alzheimer's disease (AD), until recently, only four anticholinesterases, (Tacrine, Donepezil, Rivastigmine and Galantamine: symptomatically beneficial, but not neuroprotective) were approved; but the most recently-launched drug, Memantine, is neuroprotective and a modulator of glutamatergic neurotransmission. The cholinergic hypothesis of AD and the related animal models have produced conflicting evidence and a shift of attention to glutamatergic neurotransmission in AD has occurred [130]. As already outlined above, the concept of intervention in AD targeting excitotoxicity (which is mainly glutamate and NMDA receptor dependent) with non-competitive, low affinity drugs appears to have the potential of true disease-modifying agents, because these compounds apparently do not interfere too much with physiological NMDA receptor function, e.g. coincidence detection of simultaneous signals, important for synaptic plasticity and memory [131].

Memantine has been shown in clinical trials to be effective in the treatment of both mild and moderate-to-severe Alzheimer's disease and possibly vascular dementia. The molecular basis for the drug's use is the at least partial contribution of excitotoxicity in these disease conditions [132]. Given the above mentioned importance of NMDA receptors in normal physiological activity, the role of Memantine as an uncompetitive, low-affinity, open-channel blocker is therapeutically crucial; it enters the receptor-associated ion channel preferentially when it is excessively open, and, most importantly, its off-rate is relatively fast so that it does not substantially accumulate in the channel to interfere with normal synaptic transmission [133]. Clinical use has consequently shown that Memantine is well tolerated. Besides AD, Memantine is currently in trials for additional neurological disorders, including other forms of dementia, depression, glaucoma, and severe neuropathic pain. A series of second-generation Memantine derivatives are currently in development and may prove to have even greater neuroprotective properties than Memantine. These second-generation drugs take advantage of the fact that the NMDA receptor has other modulatory sites in addition to its ion channel that potentially could also be used for safe but effective clinical intervention [134,135].

Depression

Proceeding from AD in an alphabetical order, the next potential indication for NMDA receptor is depression. Very recently, there is increasing evidence, that the glutamatergic system might offer promising targets for novel antidepressant therapies. These findings originate from observations that levels of neuronal nitric oxide synthase (NOS), an intracellular mediator of NMDA receptor activation, are low in locus coerulus neurons from subjects diagnosed with major depression. In particular, increased levels of NR2C indicate altered NMDA/calcium signal transduction in depressive disorders [136]. Interestingly, both functional NMDA receptor antagonists and AMPA receptor potentiators, and moreover, compounds acting at metabotropic glutamate receptors, in particular mGluRI and II antagonists and mGluRIII agonists produce antidepressant-like activity in several preclinical and some clinical studies [137]. Also, although from post mortem samples, with all the caveats involved, there appears to be a glutamatergic dysregulation of cortical-subcortical circuitry in major depression [138]. Taken together, the intricate, feedback-, calcium- and activity-dependent regulation of the subsynaptic cytoskeletal architecture with NMDA receptors appears to be specifically impaired in depression and related disorders (PSD-95, dynamic recruitment of adapter proteins with SH2/3- and PDZ-domains).

Epilepsy

In epilepsy, NMDA receptors also seem to play an important role, generally because excitotoxicity is accepted to be responsible for neuronal death associated with seizures [139], but as well with slightly different nuances as compared to the other disease indications discussed so far. Cortical dysplasia, which is often associated with pharmacoresistant epilepsy, has been linked to increased expression of the NMDA receptor subunit NR2B in dysplastic and epileptic human neocortex. NR2B has thus been suggested as novel antiepileptic target for diagnostic and pharmacotherapeutic approaches [140]. Moreover, dysmorphic large neurons in the subcortical white matter and cerebral cortex, a hallmark of intractable childhood-epilepsy, consistently appear to have changes in NMDA receptors [141]. In this context also brain trauma has to be mentioned, which triggers a rapid excitotoxic process by glutamate release and a related secondary and calcium-driven neuropathology, which is supposed to eventually lead in 25-50 % of all cases to posttraumatic epilepsy. Anticonvulsants, so far had no lasting effect on the incidence of late posttraumatic seizures and consequently early intervention with antiglutamatergic drugs, in particular Memantine is considered as a new strategy [142]. Again, similar to depression-related molecular changes, there is clear evidence of abnormalities of coassembled PSD-95 proteins and NR2B subunits. They are increased in the membrane proteins of brain tissues resected from patients with medically intractable neocortical epilepsy associated with cortical dysplasia as compared with nonepileptic cortex. These findings indicate that this PSD-95/NR2B increase contributes to the *in situ* increased hyperexcitability, which is accepted to cause seizure generation in focal cortical dysplasia [143]. Moreover there is evidence that the underlying pathomechanism is exclusively NMDA receptor-dependent, and that AMPA- and Kainate receptors do not contribute [144].

Multiple Sclerosis

Multiple sclerosis (MS) has a strong autoimmune and inflammatory component, which responds to corresponding treatments to some extent, but currently there is no way of impeding the progression of underlying myelin and axonal injury. The failure of remyelination and depletion of remyelinating cells, quiescence of oligodendrocyte precursor cells and axonal inhibitory signals appear to be at the core of neurodegeneration in MS, but remain elusive to treatment so far. Alternative neuroprotective strategies including statins and other immunemodulatory ligands and neurotrophic factors are emerging, but also modulation of excitotoxicity/hypoxia, NO synthesis, or cationic channels appear to be promising, because oligodendrocytes, the myelinating cells of CNS axons, are so highly vulnerable to excitotoxic signals mediated by glutamate receptors [145,146,147]. It is

interesting that Memantine has shown some effect on certain symptoms of MS [148]. Injury mechanisms via cytokines mediated by adaptive and innate immune system, might share common traits with more classic nitrative and oxidative stress, and excitotoxicity [149].

Pain

Not surprisingly at this stage, glutamate and NMDA receptors have also been considered as targets in nociception and various types of pain treatments, albeit again with a slightly different nuance: in pain/nociception, posttranslational modifications seem to be at the core of the pathophysiology [150]. In particular, in nociception the serine phosphorylation of NR1 subunits of NMDA receptors, which leads to enhanced NMDA receptor activity in the spinal cord following peripheral injury, appears to play an important role. Spinal cord NMDA receptors and in particular NR2B expression are highly dynamic in development, maintenance and recovery from central sensitization following injury. Thus, chronic pain therapies targeted to NMDA receptors are designed for the exact configuration of NMDA receptor subunits and post-translational modifications present during specific stages of inflammation-related pain conditions [30]. This has been confirmed in animal models of neuropathic pain [33]. Moreover, certain effects of morphine and related opiates indicate opioid receptor-independent events and interactions with multiple sites on NMDA receptors, with consequences for subsequent signals by calcium and NO [151]. In an other case, specific NMDA receptor antagonists seem to offer promise in the treatment of painful diabetic neuropathy, which has no effective treatment at present [152]. However, earlier assumptions that NMDA receptor antagonists, and in particular oral Memantine, would be efficacious in the treatment of postherpetic neuralgia, an emerging clinical trial model for chronic neuropathic pain, have not been substantiated by a number of clinical trials [153].

Nevertheless, the role of glutamate as pro-nociceptive agent will further motivate research into mechanisms antagonizing its actions, in particular with regard to the development of new analgesic agents and the cross-talk to opiate effects [151,154]. Since there is no specific therapy against neuropathic pain, it is of interest that glutamate receptor subunits have emerged as new potential targets for this disturbance. In the context of sensitization and hyperalgesia after inflammation an interaction with metabotropic glutamate receptors is probably most important [155].

Parkinson's Disease

Parkinson disease (PD) is a neurodegenerative disease that is characterized by a significant reduction in the number of dopaminergic neurons in the substantia nigra and a dramatic reduction in dopamine levels in the corpus striatum. The special vulnerability of dopaminergic neurons has triggered hypotheses of damage including oxidative stress, growth factor decline, excitotoxicity and inflammation. In the oxidative stress hypothesis of PD, superoxide and NO again are implicated on the already familiar background of NMDA receptor/calcium-related excitotoxicity. Also, the particular aspect of oxidative damage by 4-hydroxy-2-nonenal (HNE), and altered ubiquitination and degradation of proteins have been implicated as key to dopaminergic cell death in PD [156,157,158]. Consequently, in PD general neuroprotective strategies are considered with the aim of preventing the onset of the disease and reducing the severity of levodopa-related adverse effects. Interestingly, the antiexcitotoxic ALS-approved drug Riluzole is under clinical investigation for treatment of PD, but the search for the missing powerful neuroprotective agent active in PD is still in its beginnings [159,160].

Mutations in single genes have been shown to cause PD, and accumulation of α-synuclein seems to be a clue to the pathogenesis of neurodegeneration. However, mutations of single genes account for only a small number of cases (commonplace and not surprising, essentially always true) and so there is agreement that environmental factors play a large role in the majority of cases of sporadic PD. Genetic factors may predispose certain individuals to develop PD if combined with other gene mutations or environmental toxins. Aggregation of insoluble α-synuclein, oxidative stress, mitochondrial dysfunction, excitotoxicity, and glial and inflammatory processes are all thought to contribute to cellular death and agents that interfere with these events may be neuroprotective. The final culmination of harmful events in PD is supposed to be the induction of apoptosis in nigral dopaminergic neurons and this too offers opportunities for providing neuroprotection [161].

There are indications, that altered NMDA receptor localization by changes of the interaction with PSD-95 due to calmodulin-dependent mechanisms play a role in PD, a theme we have already encountered discussing pathomechanisms of nociception, epilepsy and depression; (we will meet it again) [162]. The PD-specific nuance may be an NR1-subunit related decrease of PSD-95 interaction as key of long-term synaptic changes and motor abnormalities of dopamine-denervated striata, with subsequent abnormal α-CaM-kinase II autophosphorylation as a molecular hallmark following dopaminergic denervation [163].

Schizophrenia

Glutamate and NMDA receptor subtypes have drawn intense interest with regard to the pathogenesis of schizophrenia. There is a "glutamate hypothesis" of schizophrenia based on the fact that phencyclidine induces psychotic-like behaviours in rodents, possibly by blocking NMDA receptors. Moreover, there appears to be abnormal transcription of various glutamate receptor gene subtypes in the hippocampus and prefrontal cortex including the NMDA receptor, and related synaptic structural changes, again the (meanwhile familiar) down-regulation of PSD-95 in schizophrenia and bipolar disorder, which may also relate to disease mechanisms of psychosis [164,138]. The prefrontal cortex plays a principal role in higher cognition and particularly in the fast online processing of information to guide forthcoming behaviour. Dysfunction of this process represents a main feature in the pathophysiology of schizophrenia. Both dopamine D1- and NMDA-receptors in the prefrontal cortex play a critical role in synaptic plasticity and cognition. Recent data have shown that D1- and NMDA receptors interact bidirectionally and may greatly influence the output of the prefrontal cortex, which is hypofunctional in schizophrenia [165]. In schizophrenia it is obvious that on

the omnipresent background of excitotoxicity, the interaction of NMDA receptor-invested neurons with other neurotransmitter systems, in particular GABA and dopamine, is crucial. Also neurosteroids may regulate the underlying neuronal excitability, via enhanced responsiveness at NMDA receptors [166,167]. In the light of non-genomic effects of membrane steroid receptors, cytoskeletal implications discussed above could play the key role. Impaired integration and information processing in schizophrenia, not only involves different neurotransmitter systems, but also different types of glutamate receptors and corresponding physiological ligands, like N-acetyl aspartylglutamate (NAAG), glycine, serine, aspartate, etc. NAAG is not only an NMDA receptor antagonist, but as well a highly selective agonist of the type 3 metabotropic glutamate receptor, and its synthesis is intimately linked to synaptic glutamate concentrations/calcium homeostasis [168]. Taken together, in schizophrenia, which affects virtually all brain functions, again calcium-dependent dysregulation of synaptic architecture appears to generate a functional imbalance of metabotropic glutamate, dopamine and NMDA receptors. Inflammatory or excitotoxic implications are speculative or considered adjunctive so far [169,170,171,172].

Stroke

Despite a similar background of glutamate/calcium-related excitotoxicity in stroke [123,173,174,139], clinical trials with NMDA receptor antagonists failed to show neuroprotective efficacy in stroke-related human clinical trials or produced intolerable CNS adverse effects [175,176]. However, more specific pharmacological strategies treating excitotoxicity as a downstream consequence of arterial occlusion and distinguishing acute ischemic stroke and acute coronary syndromes, aim at improving effectiveness and versatility of available agents in narrow therapeutic windows [177]. Targeting more than one mechanism in the excitotoxicity cascade, including Memantine, AMPA receptor antagonists and blockers of voltage-gated channels might be a fruitful approach for the development of neuroprotective drugs [178,133]. Recently, the NO/PARP-1/mitochondrial apoptosis-related signal cascade emerging from excitotoxicity has attracted new interest using experimental models of stroke and PD [179]. This might indicate a combination of NMDA-related with antiapoptotic strategies, like PARP-1 inhibition in stroke [9]. Also the role of astrocytes, which secrete a variety of neurotrophic factors and modulate glutamate and calcium homeostasis, appears to be crucial in ischemic stroke[180]. In light of the interaction of NMDA receptors with NRG, potentially in cholesterol-rich domains, very recent findings of neuroprotective *in vivo* experiments with soluble fragments of NRG1β in stroke-related models appear to offer an interesting lead which should not be treated independently of excitotoxicity [98,99,100].

Trauma

The goal of inducing functional regeneration after traumatic brain or spinal chord injury (TBI and SCI) has unfortunately not yet been achieved despite the many strategies that have been developed [181]. Following the initial impact, there is a cascade of downstream events termed 'secondary injury', culminating in progressive degenerative events which include ischemia, inflammation, free radical-induced cell death, glutamate excitotoxicity, cytoskeletal degradation and induction of extrinsic and intrinsic apoptotic pathways. The central role of glutamate receptors in mediating excitotoxic neuronal death in traumatic injuries has been well established [182,142]. There are parallels between ischemic and traumatic neuronal death in the triggering of elevated extracellular glutamate, the activation of NMDA receptors and subsequent excitotoxic signalling [174,183]. Although calcium ions are again considered as key excitotoxic regulators, specific second messenger pathways rather than total Ca^{2+}-load, are suggested to be responsible for trauma-related secondary neurodegeneration. Here localization of NMDA receptors at the postsynaptic density (PSD) and the interaction with cytoskeletal proteins and PDZ domain-containing proteins facilitating downstream neurotoxic signals during glutamate overactivity could play the decisive role [139].

Severe head trauma and the risk of late seizures are intimately related, head trauma accounts for 5% of all epilepsy cases and 20% of symptomatic epilepsy. In particular late posttraumatic seizures appear to be a consequence of the rapid excitotoxic process triggered by glutamate release, which is very similar to that seen with ischemia. So there is a mechanistic overlap between conditions underlying, epilepsy, trauma and stroke [142]. But albeit underlying mechanistic similarities, so far anti-excitotoxic therapeutic approaches for TBI and SCI have remained controversial.

SO MANY DISEASES, BUT DIFFERENT ROUTES TO NMDA-RECEPTOR MEDIATED CALCIUM TOXICITY

At this stage it is evident, that NMDA receptor-related calcium toxicity and/or calcium-dependent disarrangement of NMDA receptor/PSD-95-cytoskeletal architectures are major common features of all disease indications discussed so far. It is fair to say: they include nearly all major diseases and pathological conditions of the CNS. Obviously a variety of abnormal conditions can deviate the NMDA receptor-calcium signal, which is of absolutely unique importance for synaptic plasticity and neural development (glutamate as major excitatory neurotransmitter, NMDA receptor as its most abundant receptor and only coincidence detector), to toxic overreaction or functional/localisational imbalance [184,185]. We have seen during the course of this review that the nuances of NMDA receptor contribution to the various diseases are always slightly different and it might be useful at this stage to compile the known genetic risk factors or aberrant proteins associated with these diseases and try to relate them to corresponding disease-specific NMDA receptor and/or glutamatergic abnormalities. We find amyloid precursor protein, presinilins and NRG, ApoE4 (cholesterol-transport) and τ-hyperphosphorylation for AD, SOD-1 for ALS; NMDA receptor, NRG and dihydropyrimidinase-related protein 2 (DRP-2) for schizophrenia, inflammatory autoimmune reactions in MS, calmodulin/ PSD-95, α-synuclein and proteasome-related proteins (parkin) for PD. Many of these proteins can be integrated in the cholesterol-rich raft model presented in Fig. **6**, indeed considerations on this background have recently led to novel neuroprotective strategies [186] and those few of the risk factors not yet

accommodated potentially can be investigated in a new light from this perspective. Even some other forms of dementia, like e.g. HIV-associated dementia would fit into this scheme, because the neurotoxic HIV-1 envelope glycoprotein 120 (gp120) has been found to precipitate proapoptotic neuronal cascades [187] On top of perturbed calcium homeostasis, excitotoxicity and mitochondrial alterations [188], there are parallels between the strong, microglia-activating inflammatory processes in MS and AIDS, converging towards similar neuroprotective strategies [189,190]. Also, as in other cases of infections the cholesterol-rich rafts are apparently the actin-dependent entry (and exit) points for neurotoxic HIV proteins or even whole viruses [191,192,193] and dysregulation of sphingolipid and cholesterol metabolism by ApoE4 (one of the major genetic risk factors of AD), not only plays a significant role in AD but also determines the severity of HIV dementia [194]. Moreover, there are striking reported cases of ALS or ALS-like disease in patients with HIV infection, although the causal relationship remains uncertain [195].

NMDAR-calcium signals moreover appear to intimately linked to age-related mechanisms (PARP-1, Sir-2), which in turn directly respond to calcium-events associated with oxidative stress and hypoxia, with toxic β-amyloid fragments and toxic HIV-gp120. This confirms hypotheses around PARP-1 of representing an early evolutionary type of stress response, later adapted in developmental and plasticity/ memory-related signalling with a close communication between these mechanisms and mitochondrial/apoptotic events on the other hand [18,20,179,8,7,10]. NRG and ErbB and the transient formation of cholesterol-rich membrane microdomains make good candidates integrating all facts compiled so far, offering intervention points at various stages, ranging from NRG-isoforms acting directly at the raft, protease-inhibitors for raft-associated proteolytic processing, a special subset of NMDA receptor ligands, down to level of PARP-1 inhibitors or factors inhibiting intrinsic mitochondrial apoptosis. The model would also assign lower priorities to components more in the middle of the scheme: anti-inflammatory drugs (NSAID's, Cox-2- and NOS-inhibitors and steroids) would rather be palliative or even detrimental [196]. In the following we will focus on the NMDA receptor associated implications, looking on the proposed background in some more detail at Memantine, an emerging successful principle with integrative potential.

Memantine

As already discussed in some detail above, being an uncompetitive, moderate affinity antagonist of NMDA receptors, Memantine appears to be able to provide inhibition of pathological functions in AD while leaving physiological processes in learning and memory unaffected. Consequently, Memantine is also reported to have beneficial effects in most of the other CNS disorders with underlying excitotoxic mechanisms or dysregulation of NMDA receptor localization: PD, stroke, epilepsy, CNS trauma, ALS, drug dependence and chronic pain. Memantine has been investigated extensively in animal studies and following this, its relative efficacy and safety has been established and confirmed by clinical experience in humans. It exhibits none of the undesirable effects associated with competitive NMDA antagonists [197]. Moreover, neurotoxicity caused by HIV-1 proteins can be blocked completely by Memantine and disease-specific combination therapies (e.g. with anticholinesterases in AD, antiviral HAART regimens in HIV-dementia or Riluzol in ALS) should provide considerable advantages [198].

CONCLUSION: ARE NMDA RECEPTORS ALWAYS EMBEDDED IN COMPLEX FUNCTIONAL NETWORKS AND DO RELATED DISEASES REQUIRE LOW AFFINITY NON-SELECTIVE COMPOUNDS OR COMBINATION THERAPIES?

From the previous considerations it has become clear, that NMDA receptors are pivotal part of a flexible and highly dynamic molecular system responsible for the architectural modelling of specific synaptic requirements, with functional consequences on time scales between seconds and years: A typical "systems biology" phenomenon. Nearly half of the literature which can be found in the medline under keywords "systems biology" is from 2005! The field is expanding, concepts are maturing at very fast pace and the relevance to drug discovery, and in particular in neurodegeneration becomes more and more convincing: biology-driven approaches involving compound screening by automated response profiling in disease models based on complex human-cell systems promise to significantly reduce time and costs of new developments [199,200,201,202]. The development of technologies for the characterization of global patterns of protein expression in tissue and biofluids delivering accurate protein biomarker signatures and integrating protein expression data with clinical data such as histopathology, clinical functional measurements, medical imaging scores, patient demographics and clinical outcome can provide the tools for biomolecular disease profiling [203,204]. Proteomic technologies are core, simply because biological information is far too condensed on the level of nucleic acids, and because disease-related events, cellular stress, infections etc. are essentially epigenetic, very often only visible and understandable on the level of posttranslational modifications of proteins, and moreover defined kinetically [205,206]. The same is true of age-related processes and corresponding pathologies. Most of the complex diseases which we have discussed so far have as most important risk factor simply age. What does that mean? In these conditions, the molecular changes associated with age must play a key role: they are epigenetic in a broader sense, beyond DNA-methylation: e.g. poly-(ADP-)-ribosylation, N-formyl kynurenine from dioxidized tryptophanes or advanced glycosylation products, etc. [207,208].

In systems biology, also the considerable freedom of binding sites by ligand-dependent arrangements of residues within the binding pocket [85] defines networks of interactions. Certain domain modules contribute cooperatively to the stability of the dynamic protein complexes, with additive effects of pre-organized protein states. Binding and folding determine the chemical and biophysical properties of active biological surfaces [209]. First attempts of computational biology to develop unified theories for these complex systems are still naïve, but some features are emerging [210]. Memantine and excitotoxicity in the context

of memory-related neurodegenerative diseases however provide some unifying traits, and the relative clinical success of Memantine adds a flavour which by no means is naïve. In developmental toxicology, exposure of the developing rat to NMDA antagonists has been established as a systemic model for NMDA antagonist-induced brain cell death [211].

In the following, we will concentrate on recent proteomic studies which show a systematic relationship between some of the parameters responsible in neurodegenerative diseases and NMDA receptor-related signal transduction. Neurotoxic amyloid-β isoforms ($A\beta_{1-40}$ and $A\beta_{1-42}$) not only can induce calcium transients, which look very similar to excitotoxic conditions, but moreover are believed to oxidize specific proteins in AD brain, which in turn have been shown to be risk factors for schizophrenia or PD, like DRP-2 [212], or ubiquitin carboxy-terminal hydrolase L-1[213]. In another case, proteins modified by 4-hydroxy-2-nonenal (HNE), a reactive aldehyde generated in oxidative stress by peroxidation of membrane lipids have been investigated in spinal cord motor neurons of ALS patients and the major model, G93A-SOD1 transgenic mice. Among a few supposed house keeping proteins again DRP-2 was found as a differentially relevant surrogate biomarker [214]. The same protein was differentially affected by excitotoxic conditions, associated with calcium overload induced by an NMDA agonist [95].

In a differential proteomic study comparing molecular signatures of performance in several behavioural models, next to NAD^+-dependent deacetylase sirtuin 2 (Sir-2) and some other proteins, DRP-2 showed elevated levels [215]. Yet another proteomic approach, looking at alterations in protein levels in the thalamus of rats treated with MK-801, as compared to saline-treated animals, again found DRP-2 and some additional heat shock and metabolic proteins validating an animal model by linking it on the molecular level with genetic risk factors thought to be underlying pathophysiological mechanisms of schizophrenia [216]. This protein, which is without known function so far, turned up in quite a number of proteomic studies, which differentially addressed neuronal stress, temperature effects, in a variety of models and tissues [217,95].

NAD^+-dependent histone deacetylases like Sir-2 are interesting, because Sir-2 is not only thought to be neuroprotective in AD and essential for normal aging, and even longevity [218], but is moreover intimately connected to PARP-1, because the two enzymes crosstalk via competition for the same substrate: NAD^+[219]. PARP-1 is directly related to mitochondrial apoptotic mechanisms [220,221] and plasticity and memory [222,223]. The human sirtuin SIRT1 moreover promotes neuronal cell survival during acute anoxic injury through PARP-1 and mitochondrial associated "anti-apoptotic" pathways [224].

In PD, there is a connection of PARP-1 to certain polymorphic microsatellite repeats associated with the α-synuclein gene, which has been implicated in familial PD. Apparently inhibition of PARP-1 is able to increase endogenous levels of α-synuclein under certain conditions, which could point to a specific role of mitochondrial dysfunction in PD [225]. Deficits in mitochondrial function and oxidative stress, contributing to the accumulation of aberrant or misfolded proteins, and ubiquitin-proteasome system dysfunction not only play a principal role in sporadic and familial forms of PD [226], but could also connect excitotoxicity in other diseases [227,228].There are findings suggesting that a close association of impairments in both, mitochondrial and ubiquitin-proteasome systems, contribute together to the pathogenesis of PD [229,230].

Taken together, from all which was discussed above, NMDA receptor- mediated signal transduction, in particular calcium excitotoxicity is common to most of major human diseases of the CNS. In all these diseases there are specific and unique components, but the considerations around the Memantine experience appear to suggest a strategy, which employs low affinity drugs, preferentially with multiple modes of action, or disease specific combination therapies. The main reason is that the mechanisms involved are truly complex in a systems biology sense: certain nuances are essential in normal functioning of the CNS (memory and synaptic plasticity), with very peculiar embodiments in specific parts of the brain (hippocampus in AD, substantia nigra in PD, motor neurons in ALS, oligodendrocytes in MS): May be, that high affinity ligands, with high specificity for one target have a tendency to perturb subtle balances of calcium homeostasis, mitochondrial redox-state, and ER-protein folding conditions, all in equilibrium with apoptotic signals emerging from control mechanisms of DNA-integrity in nucleus and mitochondria. Similar to NMDA receptor-evoked calcium transients, downstream poly ADP-ribosylation provides a very well balanced signal on the level of a posttranslational modification integrating energy-status, ROS and redox-state (NAD/NADP) of neurons feedbacking to gene regulation. It's noteworthy, that this energy-aspect also involves some of the supposed house keeping proteins of energy-related metabolism, like GAPDH [231] and aconitase[232], which according to very recent data play a much more sophisticated role, than previously thought, by integrating redox state and apoptotic signals with gene transcription and maintainance of mtDNA integrity [233,232] in the context of the diseases discussed. We assume that all substances maintaining or stabilizing a "normal" calcium/mitochondrial status will be beneficial/ neuroprotective in neurodegenerative pathologies ranging from stroke to AD and PD. As the example of schizophrenia shows, not only amplitudes of NMDA receptor-triggered pathways can derail, but also localization, concentration, recruitment of interacting proteins, in particular pro-inflammatory Cox-2 and NOS via PSD-95 are part of the functional network modulated by NMDA receptor activity. A summary of these relations and the respective pathologies is provided in Fig. **7**.

Some intriguing questions arise from here: what precise role play so far enigmatic, but nevertheless omnipresent proteins like DRP-2? How contribute membrane-bound proteasomal proteins to these mechanisms? Returning to the extracellular side: is the tenacious resistance of NMDA receptors to crystallisation or solution structure (with some recent success for recombinant ligand binding pockets [234]) merely reflecting the enormous flexibility of overlapping NMDA receptor binding sites with many energy minima? Redox state is directly sensed by a disulfide bridge between NMDAR subunits, is the close interaction of NMDAR with

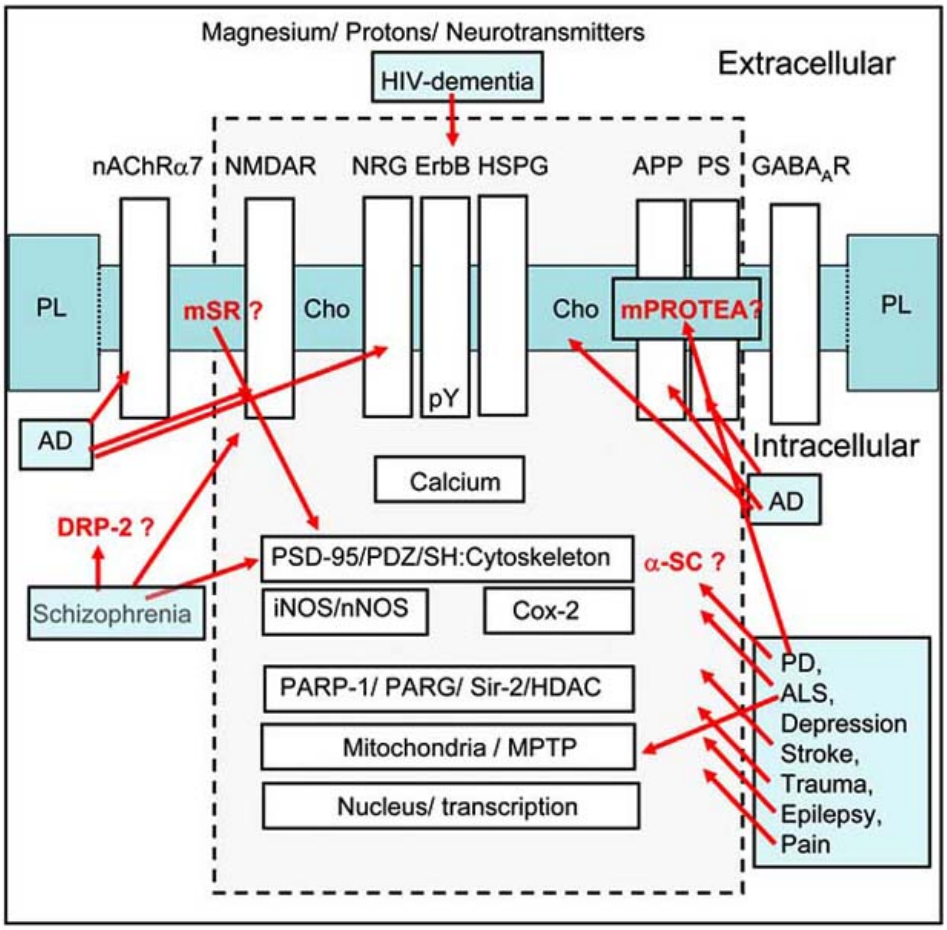

Fig. (7). Disease specific marker proteins or genes relate at various levels to NMDA receptors and other components of the "lipid-raft" associated functional supercomplex: On the background of the molecular complexes shown in Fig. **6**, human diseases of the CNS are linked with red arrows with supposed disease-specific involvement of respective components. AD is Alzheimer's disease, ALS is amyotrophic lateral sclerosis, PD is Parkinson's disease, HIV-dementia is AIDS-related dementia; the protein names in red indicate known genetic risk factors or disease markers, whose role is unclear or enigmatic so far: DRP-2 is dihydropyrimidinase-related protein-2, α-SC, is α-synuclein, mPROTEA is for supposed membrane associated proteasomal proteolytic complexes, mSR is for membrane steroid receptors; details in main text.

purinergic systems related to "energy"-status, extracellular adenosine nucleotides or even temperature [235,236]?

Some related topics could neither be treated within the scope of this article: The interaction of neurons and astrocytes maintaining calcium and glutamate homeostasis in normal and pathological brain function, the interaction with microglia in neuroinflammation and the respective role of NMDA receptors; numerous interactions with other neuro-transmitter systems, adenosine nucleotides [235], acetyl-choline [237,238,239], opioid systems [240], and the different time scales addressed by metabotropic glutamate receptors, which all have impact upon NMDA receptor localization and function, but at the same time modulate downstream mechanisms, in a truly systems biology sense.

At the end can we have any hope to escape this terrible complexity and come up with some suggestions for drug development in this difficult field? We think, that the Memantine experience reveals a potential general strategy: a low-affinity, non-specific drug, with suggested side activities at nicotinic receptors (nAChR) (α7−and α9 subtypes of nAChR-are supposed to be blocked [237,238,239]), is successfully used in or suggested for very broad applications in a variety of important human diseases and disorders. To our knowledge so far no high through-put screening for low affinity compounds takes place, combination therapies are currently mainly pursued in treatments of AIDS-related HIV-dementia, why not promoting this strategy in AD, PD or stroke? Combining moderate antiexcitotoxic drugs, like NMDAR open-channel blockers, with moderate PARP-1 or MPTP-inhibitors on a general level and with additional and more disease specific compounds in certain cases, on a background of cell-based biological screening could provide a novel route to related drug development.

ABBREVIATIONS

AD	=	Alzheimer's disease
ALS	=	Amyothrophic lateral sclerosis
AMPA	=	α-amino-3-hydroxy-5-methyl-4-isoxazole propionate

ANT-1	=	Adenine nucleotide translocator-1
AP-5	=	2-Amino-5-phosphonopentanoic acid
CNS	=	Central nervous system
Cox-2	=	Cyclooxygenase-2
DRP-2	=	Dihydropyrimidinase-related protein 2
EGF	=	Epidermal growth factor;
GABA	=	γ-Amino-butyric acid
HDAC	=	Histone deacetylases
HIV	=	Human immunodeficiency virus
HNE	=	4-Hydroxy-2-nonenal
HSPG	=	Heparane sulphate proteoglycans
LTD	=	Long term depression
LTP	=	Long term potentiation
MPTP	=	Mitochondrial permeability transition pore
MS	=	Multiple sclerosis
NAAG	=	N-acetyl aspartylglutamate
nAChR	=	Nicotinic acetylcholine receptor
NCAM	=	Neural cell adhesion molecule
NMDA	=	N-methyl-D-asparate
NOS	=	Nitric oxide synthase
NSAID	=	Non-steroidal anti-inflammatory drug
PARP-1	=	Poly-(ADP-ribose-) polymerase
PARG	=	Poly(ADP-ribose) glycohydrolase
PBPD	=	(2R,3S)-(1-biphenylyl-4-carbonyl)-piperazine-2,3-dicarboxylic acid
PD	=	Parkinson's disease
PDZ	=	Post synaptic density protein, discs large protein, zonula occludens
PSD-95	=	Post-synaptic density protein of 95 kD
SCI	=	Spinal chord injury
SH	=	src homology
Sir-2	=	Sirtuin-2
SOD	=	Superoxide dismutase
TM	=	Trans membrane
TBI	=	Traumatic brain injury
VDAC-1	=	Voltage-dependent anion channel-1

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