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REVIEW

Allosteric modulators of NR2B-containing NMDA receptors: molecular mechanisms and therapeutic potential

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N-methyl-D-aspartate receptors (NMDARs) are ion channels gated by glutamate, the major excitatory neurotransmitter in the mammalian central nervous system (CNS). They are widespread in the CNS and are involved in numerous physiological and pathological processes including synaptic plasticity, chronic pain and psychosis. Aberrant NMDAR activity also plays an important role in the neuronal loss associated with ischaemic insults and major degenerative disorders including Parkinson's and Alzheimer's disease. Agents that target and alter NMDAR function may, thus, have therapeutic benefit. Interestingly, NMDARs are endowed with multiple extracellular regulatory sites that recognize ions or small molecule ligands, some of which are likely to regulate receptor function *in vivo*. These allosteric sites, which differ from agonist-binding and channel-permeation sites, provide means to modulate, either positively or negatively, NMDAR activity. The present review focuses on allosteric modulation of NMDARs containing the NR2B subunit. Indeed, the NR2B subunit confers a particularly rich pharmacology with distinct recognition sites for exogenous and endogenous allosteric ligands. Moreover, NR2B-containing receptors, compared with other NMDAR subtypes, appear to contribute preferentially to pathological processes linked to overexcitation of glutamatergic pathways. The actions of extracellular H^+, Mg^{2+}, Zn^{2+}, of polyamines and neurosteroids, and of the synthetic compounds ifenprodil and derivatives ('prodils') are presented. Particular emphasis is put upon the structural determinants and molecular mechanisms that underlie the effects exerted by these agents. A better understanding of how NR2B-containing NMDARs (and NMDARs in general) operate and how they can be modulated should help define new strategies to counteract the deleterious effects of dysregulated NMDAR activity.
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Abbreviations: 3α5βS, 3α-hydroxy-5β-pregnan-20-one sulphate; NMDAR, N-methyl-D-aspartate receptors; NTD, N-terminal domain; PS, pregnenolone sulphate; QBP, glutamine-binding protein

Molecular architecture of NMDA receptors

N-methyl-D-aspartate receptors (NMDARs) are multisubunit complexes associating NR1, NR2 and, more rarely, NR3 subunits (also named GluN1, GluN2 and GluN3, respectively; Alexander *et al*. 2008). NR2 and NR3 subunits exist as four and two subtypes, respectively (NR2A-D and NR3A-B), each subtype being encoded by a distinct gene. NR1 exists as seven subtypes (NR1a–g), which are generated by alternative splicing from a single gene (Dingledine *et al*., 1999). Most NMDARs in the central nervous system (CNS) are comprised of NR1 and NR2 subunits forming a tetrameric complex of two NR1 and two NR2 subunits. Similarly to other glutamate-receptor ion channels (α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate (AMPA) and kainate receptors), NMDARs are thought to arrange and operate as dimer of dimers (Furukawa *et al*., 2005). The NR2 subunits that have differential developmental and anatomical profile are the major determinants of functional diversity by conferring on NMDARs' distinct biophysical and pharmacological properties (Cull-Candy and Leszkiewicz, 2004; Paoletti and Neyton, 2007).

Structurally, NMDAR subunits, like AMPA and kainate receptor subunits, share a common design and are organized into four different modules or units (Mayer, 2006; Paoletti and Neyton, 2007; Figure 1A): two large domains in the extracellular region, the N-terminal domain (NTD) and the agonist-binding domain (ABD, binding glycine in NR1 and

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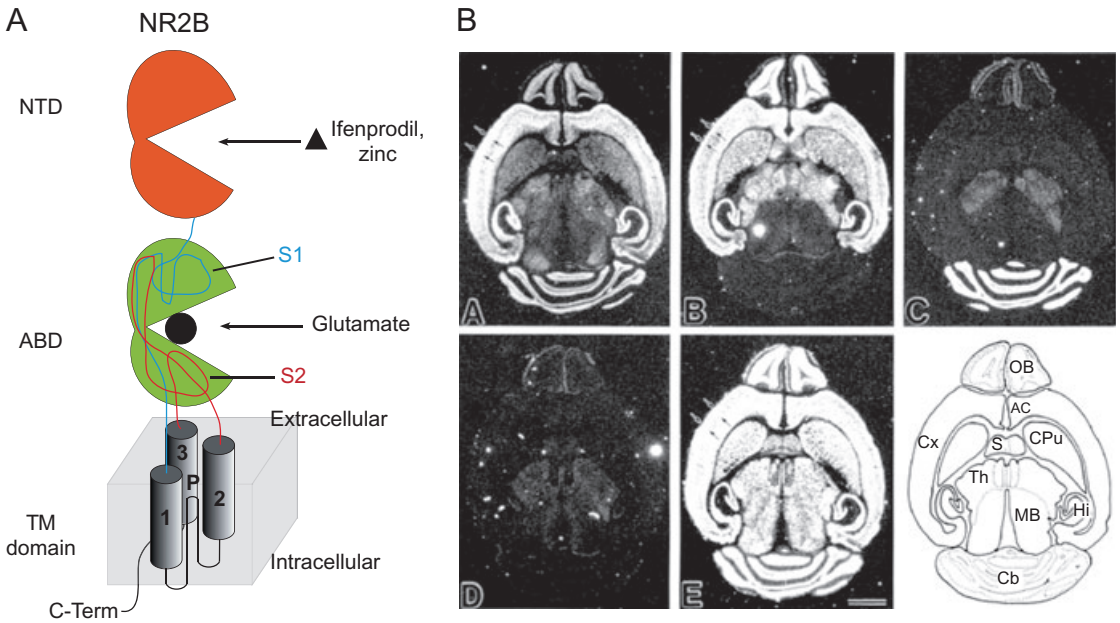

Figure 1 Organization and expression of NMDAR subunits. (A) Schematic representation of the NR2B subunit. It is composed of an NTD that binds allosteric inhibitors such as zinc and ifenprodil, an S1–S2 ABD (binds glutamate), a TM region (TM 1, 2, 3 and a re-entrant loop) that forms the ion channel and a C-terminal cytoplasmic region. (B) Distribution of NMDAR subunit mRNAs in the mouse brain at post-natal day 21 (figure reproduced with permission from Watanabe *et al.*, 1993). NR2A (panel A), NR2B (panel B), NR2C (panel C), NR2D (panel D) and NR1 (panel E). Note the restricted expression of the NR2B subunit in the forebrain. ABD, agonist-binding domain; AC, anterior cingulate cortex; Cx, cerebral cortex; Cb, cerebellum; CPu, caudate-putamen; Hi, hippocampal formation; MB, midbrain; NMDAR, N-methyl-D-aspartate; NTD, N-terminal domain; OB, olfactory bulb; S, septum; Th, thalamus; TM, transmembrane.

glutamate in NR2); the membrane region, comprising three transmembrane segments and a re-entrant loop that forms the ion channel and resembles an inverted K^+ channel; and the cytoplasmic region, comprising a C-terminal tail involved in cellular trafficking of the receptor and coupling to various intracellular signalling pathways.

The two extracellular domains, the NTD and the ABD, share homology with bacterial periplasmic proteins. The NTD, composed of the first 380 amino acids, is related to the leucine isoleucine valine binding protein (LIVBP; O'Hara *et al.*, 1993; Paoletti *et al.*, 2000) and plays an important role in subunit assembly (Herin and Aizenman, 2004). In NR2A and NR2B subunits, it also forms a regulatory domain, binding allosteric inhibitors (see below). Its structure has not been determined yet, but by homology to LIVBP, it is thought to exhibit a clamshell-like structure (Masuko *et al.*, 1999; Paoletti *et al.*, 2000). The ABD, which is related to the glutamine-binding protein, is also a bilobate domain. It is split in two discontinuous segments, S1 and S2, by insertion of the ion channel (Figure 1A). Several X-ray crystal structures of ABD of different ionotropic glutamate receptor (iGluR) subunits are now available, including those of NR1 and NR2A subunits complexed with glycine and glutamate respectively (Furukawa *et al.*, 2005). In all these structures, the agonist binds in the cleft of the clamshell, stabilizing a closed conformation of the two lobes. Competitive antagonists bind the same cleft but, in contrast to agonists, impede cleft closure, thus, preventing channel activation (Furukawa and Gouaux, 2003).

The mechanism that couples agonist binding to channel gate opening is partly known. ABD dimerization plays a key role in this process. Indeed, because two neighbouring ABDs are 'attached' one to the other through back-to-back apposition of their lobe 1, ABD closure induced by agonist binding can be transduced into tension onto the linkers connecting the ABDs to the channel. This in turn leads to channel opening (Mayer, 2006; see Figure 4B later). Breaking of the ABD dimer interface provides a means to relax this tension, and hence, close the channel. This process occurs during AMPA and kainate receptor desensitization (Sun *et al.*, 2002; Mayer, 2006) and during allosteric inhibition of NMDARs by protons and NTD ligands (Gielen *et al.*, 2008; see also, below and Figure 2B).

The NR2B subunit: expression and subcellular localization

While the NR1 subunit is expressed in virtually all neurons and at all developmental stages in the brain, NR2 subunit genes display different regional and developmental expression patterns. Thus, in the embryonic brain, NR2B and NR2D subunits predominate, while NR2A and NR2C are absent; in contrast, in the adult brain, NR2A predominates, being ubiquitously expressed, while NR2B expression is restricted to forebrain areas, and NR2C is highly enriched in the cerebellum (Watanabe *et al.*, 1992; Akazawa *et al.*, 1994; Monyer *et al.*, 1994, Figure 1B). Closer inspection of NR2B subunit gene expression in the rodent brain indicates that at E17, high levels of NR2B are present in the cortex (especially layer I), thalamus and spinal cord. In this latter region, NR2D is also strongly expressed. Around birth, NR2B expression is wide-

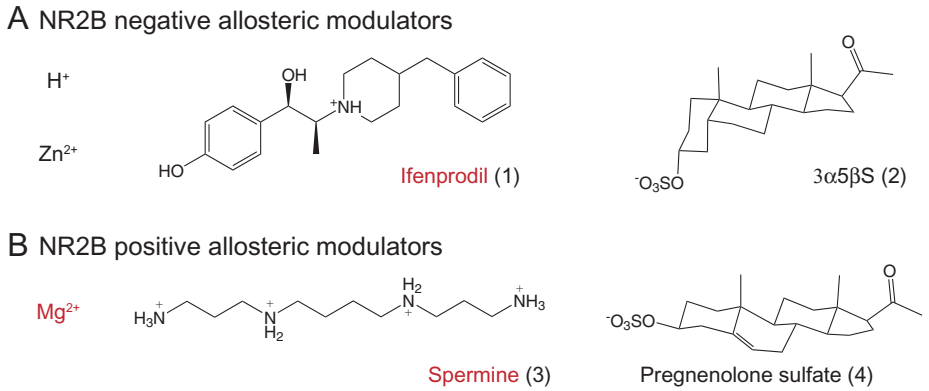

Figure 2 Positive and negative allosteric modulators of NR2B-containing NMDARs. (A) Allosteric modulators that inhibit NR2B-containing receptors. (B) Allosteric modulators that potentiate NR2B-containing receptors. The agents that selectively modulate NMDARs incorporating the NR2B subunit are named in red. NMDAR, N-methyl-D-aspartate receptors; 3α5βS, 3α-hydroxy-5β-pregnan-20-one sulphate.

spread with strong expression levels in the cortex, hippocampus, septum, striatum and thalamic nuclei. NR2B is also present in the developing cerebellum, though at lower levels. NR2B expression peaks around P7–P10, at a stage where NR2A expression rises sharply while NR2D drops. The most striking change in the pattern of NR2B gene expression takes place between the first and second week post-natal and results in its almost complete disappearance from the cerebellum (while NR2C, and to a lesser extent, NR2A increase) together with a confinement to forebrain structures. In the cerebellum, this switch in NR2 subunit expression from NR2B to NR2A and NR2C has been shown to occur in the same cell population, the granule cells (Farrant *et al.*, 1994). In the adult brain, expression of NR2B is the highest in the cortex (most particularly layer II/III), hippocampus, amygdala, ventral nuclei of the thalamus and olfactory bulb (Watanabe *et al.*, 1993). Interestingly, in the adult spinal chord, NR2B expression is restricted to lamina 2 of the dorsal horn, a region that receives primary sensory afferents from nociceptors and thermoreceptors (Watanabe *et al.*, 1994). The restricted localization of NR2B-containing receptors in this region could explain, in part, why NR2B-selective antagonists have analgesic effects (Chizh *et al.*, 2001 and see below).

At the subcellular level, NMDARs, including those containing NR2B, have been detected on synaptic, perisynaptic and extrasynaptic sites (Köhr, 2006). In most neurons, however, the density of NMDARs is higher in dendritic spines, within the postsynaptic density, than in the dendritic shaft and somatic membrane. At immature glutamatergic synapses, it is well established that NR2B-containing receptors predominate and mediate direct synaptic transmission. With maturation, the increase in NR2A expression usually results in faster decay kinetics and decreased sensitivity of synaptic currents to NR2B-selective antagonists. Moreover, several studies have shown that extrasynaptic NMDARs are enriched in NR2B subunits compared with synaptic receptors. These extrasynaptic NMDARs may be activated by spillover of glutamate released from multiple neighbouring synaptic sites (Scimemi *et al.*, 2004). However, in the adult brain, the segregation of NR2B and NR2A to extrasynaptic and synaptic sites is not absolute, and there are clear examples of adult synapses where NR2B subunits predominate (see for instance, Lopez de Armentia and Sah, 2003). The fact that at many adult synapses, NR2B subunits might incorporate preferentially into triheteromeric NR1/NR2A/NR2B receptors (Luo *et al.*, 1997), a receptor subtype shown only recently to have a low sensitivity to NR2B-selective antagonists (Hatton and Paoletti, 2005), is likely to explain why certain groups have proposed that NR2B is excluded from synaptic sites. Finally, there is accumulating evidence that NMDARs are also present at presynaptic sites where they can influence transmitter release. In particular, NR2B-containing autoreceptors have been described on primary sensory afferents of the spinal chord (Ma and Hargreaves, 2000), and at synapses on entorhinal cortical neurons (Woodhall *et al.*, 2001). Similarly to postsynaptic NMDARs, presynaptic NMDARs may also participate in certain forms of synaptic plasticity (Pinheiro and Mulle, 2008).

Allosteric inhibition by protons

Extracellular protons (H^+) are potent inhibitors of NMDARs. They inhibit NMDARs in a non-competitive and voltage-independent manner, indicating that they are not acting as channel blockers (Tang *et al.*, 1990; Traynelis and Cull-Candy, 1990; Vyklicky *et al.*, 1990). As a consequence of NMDAR inhibition by protons, mild extracellular acidosis accompanying ischaemia and seizures may minimize glutamate-induced neuronal damage (Giffard *et al.*, 1990; and see Dingledine *et al.*, 1999). Sensitivity of NMDARs to extracellular protons depends on subunit composition, with influence of both NR1 and NR2 subunits. Receptors containing the NR2C subunit are the least sensitive with an IC_{50} of ~pH 6.5 (Traynelis *et al.*, 1995; Low *et al.*, 2003). Receptors made of NR1a and NR2A have an intermediate pH sensitivity with an IC_{50} of ~pH 6.9 (when extracellular ambient zinc is chelated; Low *et al.*, 2000; 2003). NR1a/NR2B and NR1a/NR2D receptors display the highest pH sensitivity with an IC_{50} of ~pH 7.4 (Traynelis *et al.*, 1995). This implies that, under normal conditions, about half of NR1a/NR2B receptors are under tonic proton inhibition; it also suggests that small changes in extracellular pH can significantly alter the amount of current flowing through these receptors. Accordingly, endogenous alkaline transients fol-

lowing neuronal activity have recently been shown to boost postsynaptic NMDAR responses in hippocampal CA1 pyramidal neuron (Makani and Chesler, 2007). Proton sensitivity is also modulated by alternative splicing of the NR1 subunit. Inclusion of NR1 exon-5 (NR1b subunit) reduces proton sensitivity, an effect that has been attributed to the shielding of the proton sensor by positively charged residues present at the C-terminus end of exon-5 (Traynelis *et al.*, 1995; 1998). It is interesting to note that exon-5-containing NR1b/NR2A and NR1b/NR2B receptors have equal proton sensitivity, with IC_{50}s close to pH 6.7 (Traynelis *et al.*, 1995).

Although many residues, both on NR1 and NR2 subunits, have been shown to control proton inhibition (Traynelis *et al.*, 1998; Masuko *et al.*, 1999; Low *et al.*, 2000; 2003), the precise location of the proton 'sensor' (assuming that it forms a discrete site) remains unknown. Extensive mutagenesis has revealed that residues that influence proton sensitivity most strongly cluster in two neighbouring regions (Low *et al.*, 2003; but see Gielen *et al.*, 2008): (i) the lurcher region, composed of the 'SYTANLAAF' motif and located in the extracellular end of the second transmembrane segment (M2); and (ii) the linkers connecting the ABD S2 segment to the transmembrane segments (S2-M2 and S2-M3 linkers). These regions are closely associated with the activation gate of NMDARs (Chang and Kuo, 2008). The proton sensor is therefore likely to be tightly coupled to the movement of the NMDAR channel gate. Single-channel studies at NR1/NR2B receptors reveal that protons prolong one of the shut-time components, while it has almost no effect on channel open time (Banke *et al.*, 2005). These data strongly suggest that protons preferentially act by stabilizing a closed state rather than destabilizing the open state.

Modification of proton sensitivity appears to be a common downstream mechanism of a number of NMDARs' allosteric modulators. Thus, ifenprodil, exon-5 insert and polyamines at NR2B-containing receptors, and extracellular zinc at NR2A-containing receptors, all alter NMDAR function by shifting the pKa of the proton sensor (Traynelis *et al.*, 1995; Mott *et al.*, 1998; Choi and Lipton, 1999; Low *et al.*, 2000 and see below). These data reinforce the idea that the proton sensor and the channel gate are structurally and functionally integrated.

Modulators binding the interlobe cleft of the NR2B NTD

One important recent development in NMDAR function and pharmacology has been the discovery that the NTDs of NR2A and NR2B subunits form discrete modulatory domains binding non-competitive antagonists with strong subunit selectivity (Herin and Aizenman, 2004). So far, only the zinc ion has been identified as being a ligand of the NR2A-NTD (Choi and Lipton, 1999; Fayyazuddin *et al.*, 2000; Low *et al.*, 2000; Paoletti *et al.*, 2000). Zinc binds the large interlobe crevice of the NTD and, by interacting with residues from both lobes 1 and 2, promotes its closure by a hinge bending mechanism, as seen in other clamshell-like domains (Paoletti *et al.*, 2000). Binding of zinc to the NR2A-NTD is responsible for the exquisite (nM) sensitivity of NR2A-containing receptors to extracellular zinc (IC_{50} ~ 15 nM for NR1/NR2A receptors; Chen *et al.*, 1997; Paoletti *et al.*, 1997; Choi and Lipton, 1999; Fayyazuddin *et al.*, 2000; Low *et al.*, 2000; Paoletti *et al.*, 2000; Hatton and Paoletti, 2005). The NTD of NR2B (but not that of NR2C and NR2D) also binds zinc, but with a much lower affinity. This modulatory site accounts for the low micromolar voltage-independent zinc inhibition of NR1/NR2B receptors (IC_{50} ~ 1 μM; Traynelis *et al.*, 1998; Rachline *et al.*, 2005). Thus, when applied at nanomolar concentrations (up to ~300 nM), zinc selectively inhibits receptors containing the NR2A subunit, a property than can be used to discriminate between NMDAR subtypes in physiological experiments (see Neyton and Paoletti, 2006). Moreover, because zinc is concentrated and released at many glutamatergic synapses in the CNS (Vogt *et al.*, 2000; Paoletti *et al.*, 2009), zinc is a potential candidate for an endogenous ligand of the NTDs of both NR2A and NR2B subunits. However, whether extracellular zinc acts as an *in vivo* regulator of NMDAR activity remains unknown.

Besides the zinc ion, the NR2B-NTD also binds ifenprodil [compound (1), Figure 2] and derivatives, a large family of synthetic organic compounds (Perin-Dureau *et al.*, 2002). Although first described as a cerebral vasodilator (Carron *et al.*, 1971), ifenprodil was later reported to have a neuroprotective action through non-competitive antagonism of NMDARs (Carter *et al.*, 1988). In 1993, Williams made the remarkable observation that ifenprodil displays strong (>100-fold) preference among the various NR1/NR2 receptor subtypes, by selectively inhibiting receptors containing the NR2B subunit (IC_{50} ~ 150 nM; Williams, 1993). Ever since this discovery, ifenprodil and derivatives have proven extremely useful as pharmacological tools to study the structure and function of NMDARs. They have also triggered intense pharmaceutical interest because of their therapeutic potential for a range of neurological and psychiatric disorders (see below).

Studies on chimeric NR2A/NR2B subunits and on isolated NTDs produced in bacteria mapped ifenprodil-binding site to NR2B-NTD (Gallagher *et al.*, 1996; Perin-Dureau *et al.*, 2002; Wong *et al.*, 2005; Ng *et al.*, 2007; 2008; Han *et al.*, 2008). An extensive mutagenesis scan, combined with molecular modelling, further supported the hypothesis of ifenprodil binding in the large interlobe cleft of the NR2B-NTD (Perin-Dureau *et al.*, 2002). Functional studies also revealed that zinc and ifenprodil act in a mutually exclusive manner at NR2B-NTD, competing for putative binding sites that partially overlap (Rachline *et al.*, 2005). No model of zinc binding has been proposed yet, but the zinc ion is likely to interact with histidine 127, glutamate 47 and aspartate 265, all located in the NR2B-NTD interlobe cleft (Rachline *et al.*, 2005), and bearing side chains commonly found in zinc-binding sites (Alberts *et al.*, 1998). The first experimentally validated 3D model of ifenprodil docked in its binding pocket has recently been described (Mony *et al.*, 2009). In this model, ifenprodil occupies the NTD cleft, almost perpendicular to the plane of the hinge. The NTD cleft is in a closed conformation and ifenprodil directly interacts with residues both from lobes 1 and 2, lending support to a model in which ifenprodil binding promotes closure of the NR2B-NTD. The ifenprodil molecule adopts a conformation in which its benzyl group contacts hydrophobic residues at or near the NTD hinge, while its

phenol moiety makes hydrogen bonds with polar residues at the entrance of the cleft. The central piperidine moiety makes both Van der Waals interactions with F176 and ionic interactions with D101. The fact that this latter residue may also be directly involved in zinc coordination would explain why ifenprodil and zinc cannot occupy the NR2B-NTD cleft simultaneously (Rachline *et al.*, 2005).

Opposing an ifenprodil-binding site entirely formed by NTD-NR2B residues, some data in the literature suggest that determinants of ifenprodil binding may reside on the NR1 subunit. Thus, Masuko *et al.* (1999) found mutations in NR1-NTD lobe 1 that affect ifenprodil inhibition, while Han *et al.* (2008) showed that isolated NR1-NTDs, similarly to isolated NR2B-NTDs (but not NR2A-NTDs), bind radiolabelled ifenprodil. The NR1 residues highlighted by Masuko *et al.* (1999) are located at positions homologous to residues participating in hydrophobic dimerization interfaces in other receptors containing LIVBP-like domains. Rather than directly binding ifenprodil, these residues may therefore be involved in the transduction of the ifenprodil-induced conformational changes of NR2B-NTD (see Perin-Dureau *et al.*, 2002). Moreover, it is possible that such hydrophobic residues fortuitously bind ifenprodil when NR1-NTD is isolated in a polar solvent. In fully assembled NR1/NR2 receptors, this potential binding site is likely to be masked following NR1 and NR2 NTD dimerization.

Ifenprodil is the prototypical member of a large, and growing, family of NR2B-selective antagonists that can be usefully grouped as 'prodils'. Among them are synthesized analogues offering improved potency (at NR1/NR2B receptors) and selectivity versus additional 'off' target activities such as adrenergic and sigma receptors (Kew and Kemp, 1998; Chenard and Menniti, 1999; Nikam and Meltzer, 2002; Borza and Domany, 2006). The best characterized compounds and, consequently, those most commonly used as pharmacological tools are traxoprodil or CP-101,606 [Chenard *et al.*, 1995, (5)], besonprodil [CI-1041, (6), Chizh *et al.*, 2001] and Ro 25-6981 [Fischer *et al.*, 1997, (7)], all of which are ~10-fold more potent than ifenprodil; the recently disclosed RGH-896 or radiprodil (8) can also be considered to form part of this chemical group (Figure 3A). These 'second generation' compounds, which share the same structural features as ifenprodil, are likely to bind in a similar mode to the NR2B-NTD (see Malherbe *et al.*, 2003 for Ro 25-6981). Large-scale screening approaches and medicinal chemistry efforts have also led to the identification of further novel NR2B-selective antagonists such as MK-0657 (9) (Figure 3B) and EVT-101 (structure not disclosed). These agents, like radiprodil, have good potency at NR2B-containing receptors and enhanced oral bioavailability compared with earlier agents, and have been progressed into the clinic by Merck, Evotec and Gedeon Richter/Forest Laboratories respectively (see Table 1). Recently, a more diverse array of structures have been described as NR2B antagonists including (Figure 3B) indole- and benzimidazole-2-carboxamides [Borza *et al et al.*, 2006; 2007, (10)], 2-(3,4-dihydro-1H-isoquinolin-2yl)-pyridines [Büttelmann *et al.*, 2003, (11)], benzamidines [Claiborne *et al.*, 2003, (12)], N-(phenylalkyl)cinnamides [Tamiz *et al.*, 1999, (13)], N1-(benzyl)cinnamamidines [Curtis *et al.*, 2003; Kiss *et al.*, 2005, (14)], 1-Benzyloxy-4,5-dihydro-1H-imidazol-2-yl-amines [Alanine *et al.*, 2003, (15)], Taisho's HON0001 [Suetake-Koga *et al.*, 2006, (16)] and di-imidazoles (17). Many of these structurally distinct compounds are highly potent, with low nanomolar affinities at NR1/NR2B. However, whether these latest generation NR2B-selective antagonists have a similar binding mode to ifenprodil remains to be determined. The recently proposed 3D model of ifenprodil-binding site into NR2B-NTD (Mony *et al.*, 2009) provides a useful approach to tackle this question.

The transduction cascade that couples binding of the modulatory NTD ligand to receptor inhibition (i.e. channel gate closure) has been recently dissected in the case of the high-affinity zinc inhibition of NR1/NR2A receptors (Gielen *et al.*, 2008, and see Figure 4B). First, zinc binds the interlobe cleft of the NR2A-NTD and promotes its closure. This closure then exerts tension on the linkers connecting the NTDs to the ABDs, an effect that triggers disruption of the ABD dimer interface; this in turn relieves the strain on the transmembrane segments, and together with proton binding, allows closure of the channel gate. This mechanism shows common features with that underlying fast desensitization of AMPA and kainate receptors, where conformational rearrangements at the ABD dimer interface also occur (Sun *et al.*, 2002). The inhibition of NR2B-containing receptors following zinc or ifenprodil binding to the NR2B-NTD may proceed through a similar mechanism to that described for zinc on NR1/NR2A receptors. The fact that ifenprodil inhibition of NR1/NR2B receptors reflects an enhancement of tonic proton inhibition (Pahk and Williams, 1997; Mott *et al.*, 1998), similarly to zinc inhibition of NR1/NR2A receptors (Choi and Lipton, 1999; Low *et al.*, 2000), argues in this direction. However, zinc inhibition of NR1/NR2B receptors appears not to depend on pH (Traynelis *et al.*, 1998; Low *et al.*, 2000), suggesting that zinc binding to NR2B-NTD may inhibit NR1/NR2B receptors through a different mechanism.

Polyamines: NR2B-selective positive allosteric modulators

Polyamines are polybasic aliphatic amines that are positively charged at physiological pH. The endogenous polyamines, spermine [(3), Figure 2], spermidine and putrescine, are synthesized from ornithine, a by-product of the urea cycle. Polyamines are widely distributed throughout the body and are found at high intracellular levels, where they interact with nucleic acids and proteins, including plasma membrane ion channels. In the CNS, there is also evidence that, under certain conditions, polyamines may be present in the extracellular medium, where they would act as modulators of neuronal excitability. First, polyamines are released in a Ca^{2+}-dependent manner following neuronal stimulation; second, high-affinity uptake systems for polyamines exist; and third, extracellular polyamines interact with various ion channels and receptors, including calcium channels and NMDARs (Rock and Macdonald, 1995).

Extracellular polyamines have multiple effects on NMDAR responses (Rock and Macdonald, 1995). Early patch-clamp

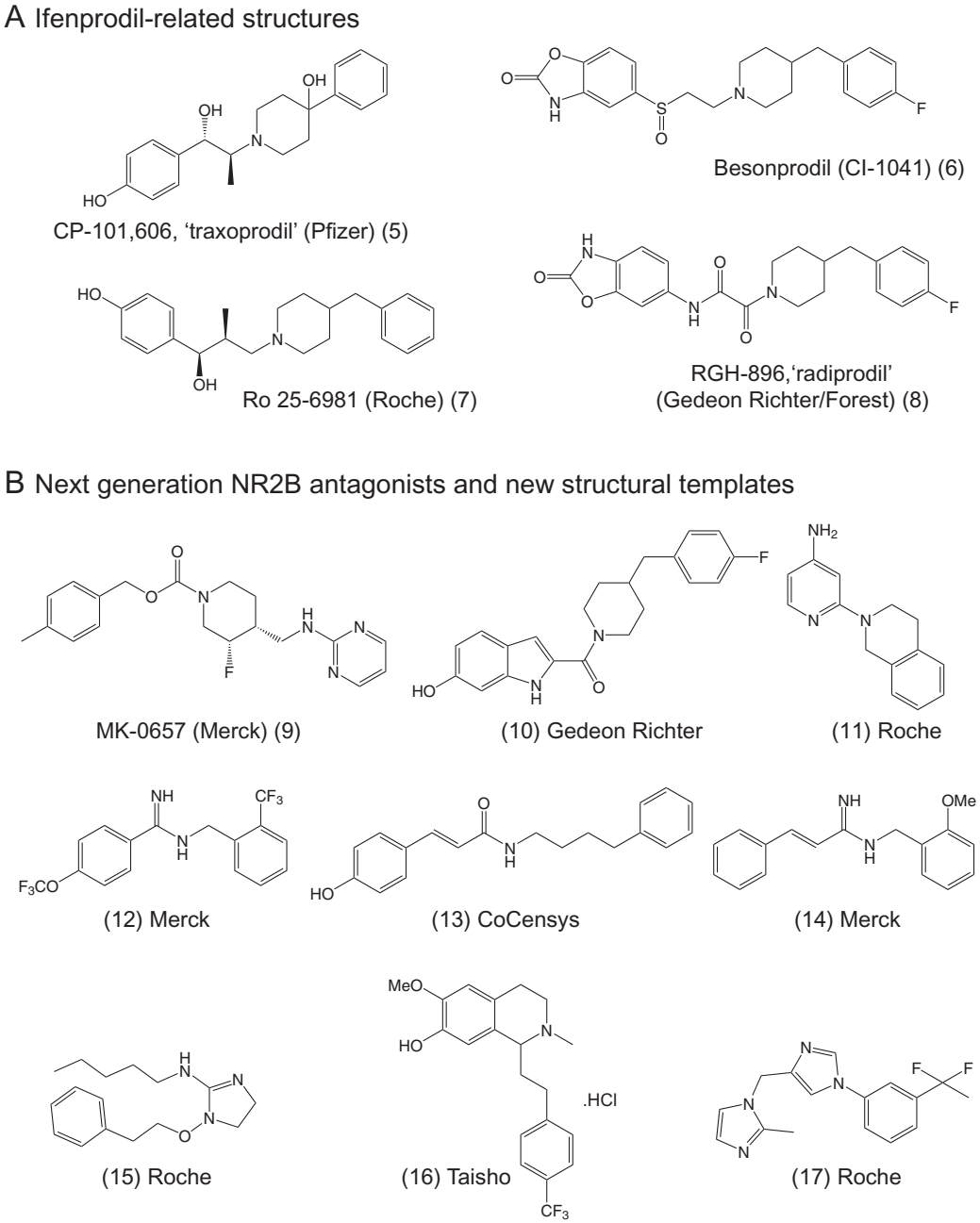

Figure 3 Structure of NR2B-selective NMDAR antagonists. (A) 'Second generation' compounds closely related in structure to the prototypical NR2B antagonist ifenprodil. (B) The latest generation of NR2B-selective antagonists and new structural templates. This represents a current perspective based on publications, patents, company press releases and analyst information; literature references, where available, are cited in the text. NMDAR, N-methyl-D-aspartate receptors.

studies on native NMDARs from cultured hippocampal neurons showed highly variable effects of polyamines, ranging from a strong enhancement to a marked inhibition depending upon the particular cell examined (Benveniste and Mayer, 1993). It is now well established that this variability can be accounted for different levels of expression of NMDAR subpopulations in individual cell, together with differential effects of polyamines depending on receptor subunit composition.

Spermine (and spermidine) produces three different effects on NMDARs: a voltage-dependent block, a glycine-dependent potentiation and a voltage-independent and glycine-independent potentiation. The voltage-dependent block is due to spermine entering the pore and occluding ion fluxes, much like the block produced by extracellular Mg^{2+} (Rock and MacDonald, 1992). It is highly voltage-dependent with IC_{50} values of 350 μM at −60 mV and 27 mM at 0 mV (NMDAR responses from cultures hippocampal neurons; Benveniste and Mayer, 1993). In contrast to Mg^{2+}, at very hyperpolarized potentials, polyamines may escape their blocking site by permeating the NMDAR channel (Araneda *et al.*, 1999). The polyamine block has the same subunit dependence as the

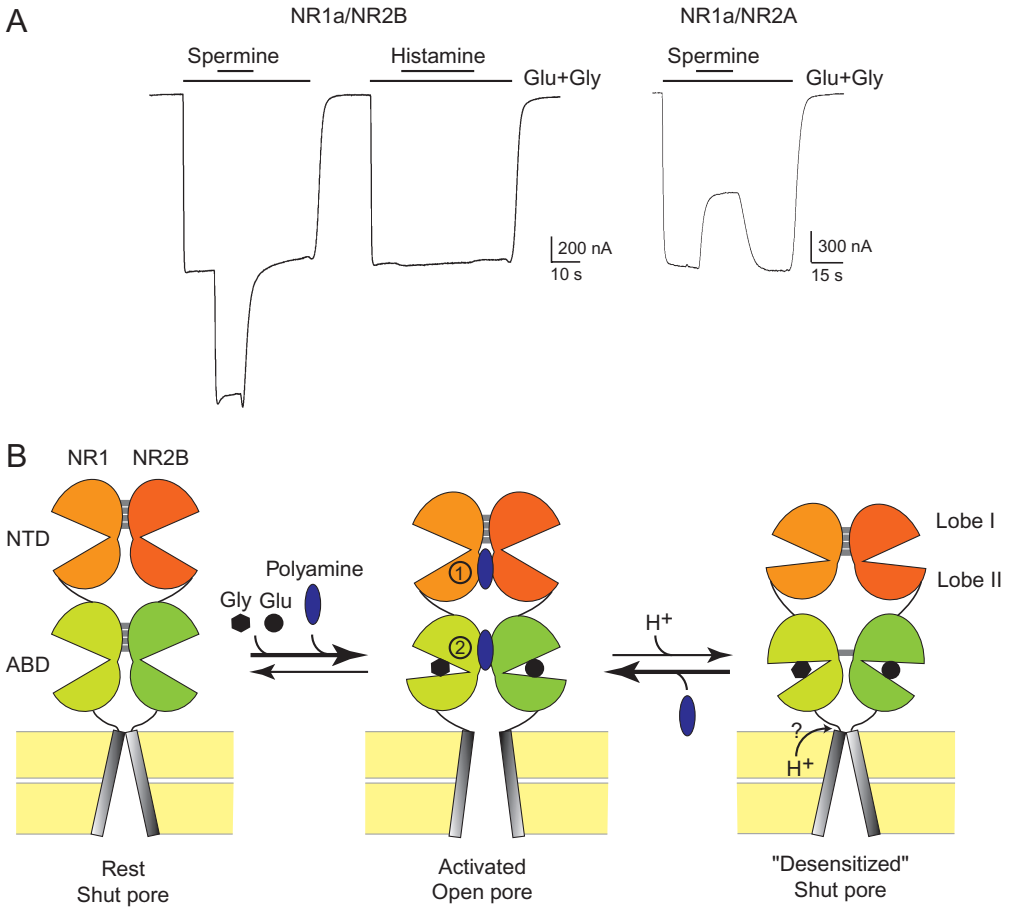

Figure 4 Spermine, but not histamine, potentiates NR1a/NR2B receptors. (A) Typical current traces obtained from oocytes expressing the NR1-1a subunit (NR1a) with the NR2B (left panel) or the NR2A (right panel) subunit. Spermine was applied at a concentration of 200 μM and histamine at 100 μM, each during an application of agonists (100 μM glutamate and glycine, saturating concentrations). Holding potential −40 mV (left panel) and −30 mV (right panel). The bars above the current traces indicate the duration of agonist, spermine and histamine applications. Note that application of 100 μM histamine has no effect on NR1a/NR2B receptors while spermine does (same cell). Note also that spermine potentiation is absent on NR1a/NR2A receptors. (B) Two hypothetical mechanisms of how a polyamine could potentiate NR2B-containing receptors. These models are based on the mechanism proposed for allosteric inhibition of NR1/NR2A receptors (Gielen *et al.*, 2008). While the full receptor is a tetramer, only a NR1/NR2B heterodimer is shown. It is hypothesized that NTDs dimerize, and that closures of the NTDs can inactivate the receptors (i.e. induce channel gate closure) by pulling apart the ABD dimer interface ('desensitized' state). Mechanism (1): the polyamine molecule binds between the NR1 and NR2 NTD lobes II, making NTD closure, and ABD dimer interface disruption, more difficult. Mechanism (2): the polyamine molecule directly binds and stabilizes the ABD dimer interface. Entry into the 'desensitized' state is thus disfavoured. This mechanism resembles that described for cyclothiazide-induced suppression of desensitization at AMPA receptors (Sun *et al.*, 2002; Mayer, 2006). In both models, proton binding, which stabilizes a closed state of the channel (Banke *et al.*, 2005), is proposed to be closely associated with ABD dimer interface breaking (Gielen *et al.*, 2008). ABD, agonist-binding domain; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate; Glu, glutamate; Gly, glycine; NTD, N-terminal domain.

Mg^{2+} block, being less pronounced for NR2C-containing and NR2D-containing receptors than for NR2A-containing or NR2B-containing receptors (these latter two having the same degree of blockade; Williams *et al.*, 1994). Finally, mutations in the pore at the critical asparagines residues (Q/R/N site) that suppress Mg^{2+} block also suppress spermine block, indicating a shared binding site deep in the pore (Kashiwagi *et al.*, 1997; Traynelis *et al.*, 1998).

Polyamines can enhance NMDAR responses by increasing the apparent sensitivity for glycine (glycine-dependent potentiation). This is reflected by the fact that at low glycine concentrations, polyamines stimulate NMDAR responses to a greater extent than at saturating glycine concentrations (McGurk *et al.*, 1990). In the presence of 1 mM spermine, the apparent affinity for glycine increases by ~3-fold, an effect largely due to a decrease in the rate of glycine dissociation from its binding site on NR1 (Benveniste and Mayer, 1993). Glycine-dependent potentiation occurs both at NR2A-containing and NR2B-containing receptors but not at NR2C-containing and NR2D-containing receptors (Zhang *et al.*, 1994; Williams *et al.*, 1995). Moreover, in contrast to the voltage-independent and glycine-independent potentiation (see below), the glycine-dependent stimulation is not influenced by the type of NR1 subunit splice variants. The polyamine-binding site mediating the glycine-dependent potentiation is unknown, but may reside on the NR1 subunit that harbours the glycine-binding site. In physiological conditions, it is likely that this site is partially occupied by endogenous Ca^{2+} and/or Mg^{2+} because both cations also increase glycine sensitivity when present at

Table 1 NR2B-selective compounds in clinical development

NR2B antagonist (route of administration)	*Most advanced phase of development (indication)*	Pharmacological activity *(in vitro)*	*Activity in preclinical species (in vivo relevant model)*	*Recent clinical data*	*References*
CP-101,606 Traxoprodil (i.v.)	Phase 2 (experimental medicine studies in pain, Parkinson's and major depression recently completed. Previously discontinued for stroke/ traumatic brain injury)	Potent and selective NR2B antagonist: NR2B IC_{50} = 8–13† to 60* nM NR2A IC_{50} = 10 μM† (selectivity > 1000-fold)	Efficacious in rat models of acute inflammatory pain, for example, carageenan (ED_{50} = 37 mg·kg^{-1}) neuropathic pain (ED_{50} = 4.1 mg·kg^{-1}) and visceral pain (mustard oil, MED 10 mg·kg^{-1}). Also neuroprotective in models of stroke (middle cerebral artery occlusion) and efficacious in rodent models of Parkinson's disease.	Phase 2 experimental medicine study in 19 patients with chronic pain (spinal cord injury, radiculopathy) indicated significant reduction in pain score following a controlled i.v. infusion of CP-101,606 to 100 ng·mL^{-1}. CP-101,606 was reasonably well tolerated, although some CNS AEs such as dizziness, depression and hypoaesthesia were reported. A phase 2 trial in Parkinson's disease (up to 200 ng·mL^{-1}) has also been completed. Some improvement in levodopa-induced dyskinesias but no significant effect on Parkinsonism. AEs included abnormal thinking, dissociation and amnesia. Recent data demonstrate efficacy of CP-101,606 in treatment of refractory major depressive disorder.	http://www.pfizer.com Trube *et al.* (1996) Boyce *et al.* (1999; 2002) Chenard and Menniti (1999) Sang *et al.* (2003) Nutt *et al.* (2008) Preskorn *et al.* (2008)
RGH-896 Radiprodil (p.o.)	Phase 2. (neuropathic pain and CNS indications)	Potent and selective NR2B antagonist: NR2B IC_{50} = 3–10 nM†* NR2A IC_{50} > 10 μM† (selectivity > 1000-fold)	Efficacious in rat models of acute inflammatory pain, for example, FCA (anti-allodynic effects at 0.25 and 4 mg·kg^{-1}) and neuropathic pain (MED = 5 mg·kg^{-1}).	Phase 1 completed successfully. Phase 2 studies in neuropathic pain stated as planned as early as 2006 (co. press release), however, no further public details are available on the progression of this molecule.	http://www.richter.hu http://www.frx.com/ Farkas *et al.* (2003) Horvath (2004)
MK-0657 (compound 33) (p.o.)	Phase 2 (neuropathic pain, Parkinson's disease, major depression)	Potent and selective NR2B antagonist: NR2B IC_{50} = 3 nM‡ (Selectivity data not disclosed.)	Efficacious in rat carageenan model of acute inflammatory pain, ED_{50} = 3 mg·kg^{-1} No further details have been disclosed.	Stated as approved for clinical development and ready to enter phase 1 clinical trials (co. press release). Subsequently, a phase 1 trial in 18 Parkinson's disease patients with 7 mg MK-0657 + levodopa was completed in 2008, and a study within major depression is underway, 4–8 mg·day^{-1} (see www.clinicaltrials.gov). No further details have been disclosed.	http://www.merck.com McCauley (2007) McCauley *et al.* (2008)
EVT-101 (p.o.)	Phase 1 (pain and Alzheimer's disease)	Potent and selective NR2B antagonist: NR2B IC_{50} = 2 nM†* NR2A/C/D IC_{50} > 10 μM† (Selectivity > 1000-fold)	Protection against NMDA-induced seizures ED_{50} = 4.6 mg·kg^{-1} No further details have been disclosed.	Phase 1 single (up to 15 mg) and multiple (up to 8 mg) dose studies complete in a total of 90 patients. No AEs reported. T1/2 defined as 11 h. Further phase 1b studies incorporating fMRI studies demonstrated CNS effects of EVT-101. Phase 2 efficacy studies in third molar extraction (dental pain) and spinal cord injury (neuropathic pain) are planned (co. press release).	http://www.evotec.com/

The table summarizes the currently available public data on NR2B-selective compounds that are being evaluated in the clinic assembled from company press releases, analyst presentations, clinical trials register (http://www.clinicaltrials.gov/) and the publications cited. Chemical structures of the NR2B antagonists listed, where available, are given in Figure 3; note that there are no known positive modulator compounds currently in clinical development.

*Determination by electrophysiology (whole-cell patch clamp or oocyte recording).

†[3H]-Ro256981 or [3H]-MK801 binding assay.

‡FLIPR-Ca2+ assay.

AE, adverse event; CNS, central nervous system; ED_{50}, effective dose exhibiting 50% reversal; FCA, Freund's complete adjuvant; FLIPR, fluorescent imaging plate reader; fMRI, functional magnetic resonance imaging; IC_{50}, effective concentration exhibiting 50% inhibition; MED, minimum effective dose; NMDA, N-methyD-aspartate.

mM concentrations (Wang and MacDonald, 1995; see Paoletti *et al*., 1995).

The third effect of polyamines on NMDARs is a potentiation, which is both voltage-independent and glycine-independent because it can be observed both at negative and positive membrane potentials and with saturating glycine concentrations (McGurk *et al*., 1990; Lerma, 1992; Benveniste and Mayer, 1993; Lu *et al*., 1998; Figure 4A). With spermine, the EC_{50} for this effect is ~150 μM and results in a maximal potentiation around threefold (at pH 7.3; Benveniste and Mayer, 1993). Remarkably, the voltage-independent and glycine-independent polyamine potentiation is observed exclusively at receptors that incorporate the NR2B subunit (Williams, 1994a; Williams *et al*., 1994; Zhang *et al*., 1994; Traynelis *et al*., 1995; Figure 4A). Studies on recombinant NR1/NR2B receptors have revealed that the NR2B-specific polyamine potentiation arises from the relief of tonic proton inhibition. Thus, at physiological pH, polyamines enhance NR1/NR2B responses by shifting the pKa value of the proton sensor towards more acidic values (Traynelis *et al*., 1995). Accordingly, at NR1/NR2B receptors, there is a strong correlation between the proton sensitivity and the degree of polyamine potentiation (Traynelis *et al*., 1995; 1998).

Many mutations that affect the NR2B-specific polyamine potentiation have been described. These mutations, usually of acidic residues, are found both on NR1 and NR2 subunits and are scattered throughout the extracellular region. Most of these mutations (if not all) also modify proton sensitivity (Traynelis *et al*., 1995; Williams *et al*., 1995; Kashiwagi *et al*., 1996; Masuko *et al*., 1999). In consequence, it is still unclear whether these mutations alter polyamine potentiation directly, by disrupting the polyamine-binding pocket, or indirectly, by changing the proton sensitivity. Based on our recent finding that proton inhibition of NMDARs involves disruption of the ABD dimer interface (Gielen *et al*., 2008), we propose two mechanisms by which polyamines could relieve tonic proton inhibition and thus enhance activity of NR2B-containing receptors (Figure 4B). In a first model, the polyamine molecule would act similarly than cyclothiazide on AMPA receptors (Sun *et al*., 2002): it would bind at the ABD dimer interface and stabilize the dimer assembly. By doing so, it would decrease pH sensitivity by rendering ABD dimer disruption more difficult (an effect that would also account for the spermine-induced acceleration of NMDAR current deactivation; Rumbaugh *et al*. 2000). In a second model, the polyamine would bind at the level of the NTDs, between the two 'bottom' lobes of a NTD pair. By 'gluing' together these lobes, the polyamine would render NTD closure less favourable, an effect that in turn would tend to stabilize the ABD dimer interface (and thus decrease proton sensitivity; Gielen *et al*., 2008). In agreement with this mechanistic scheme, 3D homology modelling of a NR1/NR2B NTD pair indicates that many of the acidic residues of NR1 and NR2B known to control glycine-independent spermine potentiation locate on the side of the NTD 'bottom' lobes, with residues of NR2B facing those of NR1. This suggests a polyamine-binding site at the boundary between the two NTD 'bottom' lobes (Huggins and Grant, 2005). The absence of some of these acidic residues in the NTDs of the other NR2 subunits could explain why the glycine-independent potentiation by polyamines requires the NR2B subunit. A model in which the polyamine molecule binds the NR1/NR2B NTD interface and holds the NTDs open, while ifenprodil binds in the NR2B-NTD cleft and promotes its closure, is fully consistent with the proposal of Kew and Kemp (1998) that spermine and ifenprodil bind distinct sites that interact in a negative allosteric manner (with binding of spermine promoting ifenprodil dissociation and vice versa; see also Han *et al*., 2008).

The neurotransmitter histamine has been proposed to act as an endogenous activator of the NR2B-specific polyamine modulatory site (Williams, 1994b). However, results from our lab (Figure 4A) and that of Steven Traynelis (pers. comm.) show a lack of effect of histamine on responses mediated by NR1a/NR2B receptors. The reason for this discrepancy remains unclear. Finally, Mg^{2+}, but not Ca^{2+}, mimics the glycine-independent potentiating effect of polyamine at NR2B-containing receptors (Paoletti *et al*., 1995; Kew and Kemp, 1998). With an Mg^{2+} EC_{50} of ~2.0 mM close to physiological concentration of extracellular Mg^{2+}, the NR2B-specific polyamine potentiating site is likely to be partially occupied by the magnesium ion under physiological conditions.

Negative and positive allosteric modulation by neurosteroids

Most steroids act like hormones being synthesized by glandular tissues and released into the general circulation system. Steroids diffuse through membranes and bind intracellular receptors, which in turn interact with transcription factors to enhance or suppress gene expression. Because of the necessity for activation of the transcriptional and translational machineries, the physiological responses induced by steroids usually take hours to days. Steroids synthesized in the periphery can cross the blood–brain barrier and produce changes in mood and behaviour (Belelli and Lambert, 2005). The brain is also capable of synthesizing steroids *de novo* (Robel and Baulieu, 1994). Neurosteroids are normally present at nanomolar concentrations in the CNS, but their levels can increase significantly following stress, for instance. In contrast to the classical genomic effects of steroids, endogenous neurosteroids act locally and produce acute effects on neuronal excitability, with time delays ranging from seconds to minutes, suggesting direct modulatory effects on membrane proteins. It is now well established that gamma-amino butyric acid-A (GABA-A) receptors, which mediate most of the inhibitory transmission in the CNS, are major targets of neurosteroids (Belelli and Lambert, 2005). Neurosteroids also regulate NMDARs. At these receptors, neurosteroids exert either positive or negative effects depending both on the neurosteroid chemistry and the receptor subunit composition.

Pregnenolone sulfate [PS (4), Figure 2], one of the most abundant neurosteroids and a negative modulator of GABA-A receptors, was initially shown to potentiate native NMDARs but not AMPA and kainate receptors (Wu *et al*., 1991; Bowlby, 1993). PS is a derivative of pregnenolone, which is formed by cleavage of cholesterol side chain in glial cells. Potentiation of NMDAR responses by PS is voltage independent, does not

affect single-channel conductance and occurs only at relatively high concentrations of PS (>1 μM). Interestingly, when applied in the extracellular medium, PS increases NMDAR activity recorded not only from excised outside-out patches but also from cell-attached patches, indicating that the lipophilic PS may penetrate the cell membrane and reach NMDAR channels by diffusion (Bowlby, 1993). PS differentially modulates activity of recombinant NR1/NR2 NMDARs. It potentiates NR1/NR2A and NR1/NR2B receptors (with EC_{50} in the 10–30 μM range) while it inhibits, in a non-competitive manner, NR1/NR2C and NR1/NR2D receptors (with IC_{50} in the 100–200 μM range; Malayev *et al.*, 2002; Horak *et al.*, 2006). The mechanism of action of PS at NR1/NR2B receptors has been studied using fast perfusion techniques on transfected human embryonic kidney (HEK)-293 cells. Strikingly, the degree of PS potentiation is an order-of-magnitude larger when PS is applied just before than during NMDAR activation, indicating that the PS affinity is strongly decreased after glutamate binding (Horak *et al.*, 2004).

PS enhances NMDAR responses by acting at a site that likely differs from the site(s) responsible for spermine potentation (Park-Chung *et al.*, 1997). By constructing chimeric NR2B/NR2D (Jang *et al.*, 2004) or NR2A/NR2C (Horak *et al.*, 2006) subunits, the PS potentiating site has been recently mapped to a region encompassing the M2–M3 extracellular loop and the M3 transmembrane segment. In the NR2 M2–M3 loop, helices J and K appear to be important for PS sensitivity (Jang *et al.*, 2004). Because helix J is a key constituent of the dimer interface between neighbouring NR1-NR2 ABDs, Jang *et al.* (2004) have proposed that PS binds and stabilizes this interface very much like the positive allosteric modulator cyclothiazide on AMPA receptors. There are, however, experimental data that are difficult to reconcile with a PS-binding site at the ABD dimer interface. First, PS potentiation appears to be functionally independent from proton inhibition (Jang *et al.*, 2004), whereas stability of the ABD dimer is closely correlated to proton sensitivity (Gielen *et al.*, 2008). Second, the transmembrane segment M3 appears critical for the potentiating effect of PS, suggesting that this region may contain residues directly binding PS (Jang *et al.*, 2004). Thus, the potentiating effect of PS on NMDARs may be mediated by a site in the membrane region rather than at an extracellular location. The lipophilic nature of PS and the very slow kinetics of its action at NR1/N2B receptors (Horak *et al.*, 2004) are compatible with this hypothesis. It is interesting to note that at GABA-A receptors, transmembrane sites are responsible for the regulatory effects of neurosteroids on these receptors (Hosie *et al.*, 2006).

The PS analogue 3α-hydroxy-5β-pregnan-20-one sulphate [3α5βS (2), Figure 2] is another endogenous neurosteroid that modulates NMDAR activity. 3α5βS inhibits native and recombinant NMDARs, with little selectivity among the different NR1/NR2 receptor subtypes. It acts in a voltage-independent and non-competitive manner, strongly arguing for a binding site outside the pore and the agonist-binding pockets (Park-chung *et al.*, 1994; Park-Chung *et al.*, 1997; Malayev *et al.*, 2002; Petrovic *et al.*, 2005). Thus, despite strong chemical similarity, PS and 3α5βS produce opposite effects at NR1/NR2A and NR1/NR2B receptors, the former acting as a positive allosteric modulator and the latter as a negative allosteric modulator. Structure-activity studies on the steroid molecules have led to the identification of important structural features that determine the action of neurosteroids at NMDARs. The presence of a negative charge (bulky or not) at the C3 position seems mandatory for both the potentiating and inhibitory effects of neurosteroids on NMDARs (Park-Chung *et al.*, 1997; Weaver *et al.*, 2000). Moreover, the geometry of the neurosteroid strongly influences the sign of the modulatory effect. Neurosteroids having a bent geometry (3α5βS and 3β5βS) inhibit native NMDAR responses (mostly from NR2A-containing and/or NR2B-containing receptors), whereas those having a more planar configuration (PS and 3β5αS) potentiate such responses (Weaver *et al.*, 2000). Thus, whether a neurosteroid enhances or decreases NMDAR activity critically depends on the geometry of its A–B ring junction, a parameter that is determined by the stereochemistry of the C5 position.

NR2B-containing receptor function and the therapeutic potential of subtype-selective NMDAR modulation

There is currently great interest in determining whether different NMDAR subtypes make specific contributions to physiological and pathological neuronal processes. Dissection of the roles of NMDAR subtypes can be performed using genetic (genetically modified mice, RNAi-mediated gene interference) or pharmacological tools. All of these approaches have recognized limitations (e.g. see Neyton and Paoletti, 2006 for the pharmacological approach) and, hence, despite intense investigation, there are many controversial issues in the field. Several parameters may strongly influence the role that a NMDAR subtype may play: (i) its particular receptor subunit composition, which determines the level of receptor activity and kinetic behaviour; (ii) its subcellular location (synaptic or extrasynaptic); (iii) its coupling to downstream signalling cascades (these latter two parameters being likely dependent on the subunit composition); and (iv) potential changes in its expression or activity linked to disease.

Based on our current understanding of the roles of the different NMDAR subtypes in normal physiology and in pathophysiological conditions, it appears that subtype-selective modulation of receptor function offers emerging therapeutic potential for the treatment of a range of CNS disorders. Thus, NR2B-selective antagonists may offer utility for the treatment of disorders including chronic pain, Parkinson's disease, Huntington's disease, Alzheimer's disease, cerebral ischaemia and major depression.

Notably, while non-selective NMDAR antagonists commonly exhibit a range of side effects including behavioural, cardiovascular and potentially cytotoxic activities, which have limited their therapeutic development, NR2B-selective antagonists are relatively well tolerated (Kemp *et al.*, 1999; Chazot, 2004; Gogas, 2006). In addition to the inherent sparing of non-NR2B-containing receptor function as a consequence of subtype selectivity, including a relative lack of effect in brain regions with little or no NR2B expression, such as the cerebellum, ifenprodil and related compounds also display an activity-dependent mode of action; they bind with a higher affinity to the activated and desensitized states of the

receptor than to the unliganded resting state (Kew *et al*., 1996; Fischer *et al*., 1997; Gill *et al*., 2002). Thus, these compounds preferentially block NMDARs continuously or repetitively activated, as may be the case in pathological conditions, while leaving those that are physiologically activated relatively unaffected. In addition, as ifenprodil inhibition of the NR2B-containing receptors is mediated through an enhancement of proton inhibition (Pahk and Williams, 1997; Mott *et al*., 1998), antagonist efficacy would be further increased under pathological conditions where pH levels also fall, such as in ischaemia (Silver *et al*., 1992). Thus, both the mechanism of action and subunit selectivity contribute to the relative lack of adverse events/good tolerability profile associated with this compound class (Kemp *et al*., 1999; Gogas, 2006).

Although most studies point towards an improved therapeutic index with the NR2B-subtype-selective approach, recent studies have raised concerns regarding the role of NR2B in mediating phencyclidine (PCP)-like behavioural effects and the potential for abuse liability as previously associated with pan-NMDAR antagonists, such as ketamine. Preclinical studies with Ro 25-6981 (Chaperon *et al*., 2003) and CP-101,606 (Nicholson *et al*., 2007) suggest a role of NR2B in producing the subjective and reinforcing effects associated with PCP in rodents and primates. Although noted to be generally well tolerated in the clinic, some indications of such activity have emerged in recent clinical studies with CP-101,606 (Table 1). Further work is, therefore, required to explore these aspects in more detail, especially for the latest NR2B-selective antagonists progressed into the clinic, to determine if an acceptable balance between efficacy and side effects exists to support their further development for the treatment of the range of disorders outlined below.

Therapeutic potential of NR2B-selective antagonists

Pain

Clinically, broad-spectrum NMDAR antagonists (e.g. ketamine, dextromethorphan) are reportedly used off label for the treatment of neuropathic pain (Chizh *et al*., 2001; 2007); they have good efficacy, but suffer from a generally unacceptable side-effect profile due to a very narrow therapeutic index. The rationale for developing subtype-selective NMDAR antagonists therefore stems from a desire to maintain NMDAR- mediated efficacy while moving away from the side effects resulting from indiscriminate broad NMDAR blockade. Initial focus on NR2B derived from the more localized expression of NR2B in the dorsal horn of the spinal cord as well as in higher brain centres thought to be important for the relay and conscious perception of pain, for example, the thalamus and anterior cingulate cortex (Figure 1). The availability of the 'prodil' class of NR2B-selective antagonists (Figure 3) has led to rapid progress in validating NR2B-containing NMDARs as a target for the treatment of pain, with many studies reporting efficacy of such agents in models of acute (Taniguchi *et al*., 1997; Boyce *et al*., 1999) and chronic inflammatory (Wilson *et al*., 2006), neuropathic (Boyce *et al*., 1999) and even visceral pain (Boyce *et al*., 2002) at doses that are devoid of overt behavioural side effects. More recent research has also defined a key involvement of NR2B-containing receptors in mechanisms, such as 'wind up' and 'central sensitization', that operate spinally and supraspinally, and which may explain the apparent broad-spectrum activity of NR2B antagonists across the various disease models. These pharmacological studies have been complemented by additional insight into the role of NR2B-containing receptors in pain gained from studies with transgenic mice overexpressing NR2B in the forebrain which display a selective enhancement of persistent pain and allodynia (Wei *et al*., 2001). Similarly, intrathecal administration of siRNAs against the NR2B subunit reduces the pain responses induced by peripheral inflammation (Tan *et al*., 2005).

The potential of NR2B antagonists for the treatment of pain has now gained clinical precedence based on the observed efficacy of CP-101,606 in a placebo-controlled crossover phase 2a study in spinal cord injury and monoradiculopathy patients, where a significant reduction in pain score was seen following i.v. dosing of CP-101,606 (Sang *et al*., 2003). CP-101,606 was reasonably well tolerated in this study but did yield some CNS side effects (Table 1). Despite these encouraging data, subsequent progress in this field has been hampered by the lack of compounds with good orally bioavailability, appropriate selectivity versus human ether-a-gogo related gene (hERG), and concerns regarding CNS side effects and abuse potential as discussed above. Despite these challenges, Gedeon Richter and Forest Laboratories, Merck and Evotec have now identified orally bioavailable agents that have progressed into clinical development (Table 1). To date, only limited information concerning the safety profile of these agents in phase 1 trials is available, and this provides some basis for optimism (Table 1). However, it will only be with efficacy data from appropriate phase 2 studies that these initial findings can be put into context of therapeutic index and the prospects for further development fully evaluated.

Parkinson's disease

The degeneration of nigral dopaminergic neurons and the depletion of dopamine from the nigro-striatal pathway results in overactivation of glutamatergic projections to the striatum and basal ganglia output nuclei. In addition, NMDARs have been proposed to play a role in the development of levodopa-induced dyskinesias. Accordingly, the therapeutic potential of NMDAR antagonists has been investigated, and broad-spectrum antagonists have been shown to exhibit antiparkinsonian or antidyskinetic activity in rodent and monkey models, and the low affinity NMDAR antagonist amantadine has been shown to exhibit antidyskinetic activity in humans (Del Dotto *et al*., 2001). Subsequently, NR2B-selective antagonists have been shown to exhibit efficacy in preclinical models of Parkinsonism in both rodents and non-human primates (e.g. Steece-Collier *et al*., 2000; Loschmann *et al*., 2004; Wessell *et al*., 2004), with an apparently improved tolerability profile relative to broad-spectrum antagonists. While ifenprodil failed to show a significant benefit in a small clinical trial in Parkinson's disease patients as adjunct therapy to L-DOPA (Montastruc *et al*., 1992), in a recent study, CP-101,606 exhibited antidyskinetic but not antiparkinso-

nian effects, although at doses associated with dissociation and amnesia (Nutt *et al*., 2008). Further studies are required to determine whether therapeutic efficacy can be achieved at lower doses, in the absence of adverse cognitive effects. A phase 1 study with MK-0657 as adjunct therapy to levodopa has also been reported as completed, although data have not yet been disclosed (Table 1).

Huntington's disease

Huntington's disease is an autosomal dominant inherited disease characterized by selective neuronal degeneration, most prominently of striatal GABAergic medium-sized spiny neurons. The disorder results from expression of mutant forms of the *huntingtin* gene, which contain an expanded trinucleotide CAG repeat sequence encoding an extended polyglutamate tract in the translated protein. Studies of animal and cellular models expressing mutant forms of the huntingtin protein have implicated NMDAR dysregulation and subsequent excitotoxic damage in the pathophysiology of the disease. For example, in a mouse model of Huntington's disease, striatal GABAergic medium-sized neurons show a selective enhancement of NMDAR currents mediated by NR2B-containing receptors (Zeron *et al*., 2002) and this increase may underlie the selective neuronal loss in Huntington's disease (Li *et al*., 2003). Thus, early intervention with selective NR2B antagonists at the onset of pathology, or presymptomatically, may offer therapeutic potential in this disorder.

Alzheimer's disease

The low affinity, broad-spectrum NMDAR channel blocker memantine has been approved in both the USA and Europe for the treatment of moderate to severe Alzheimer's disease. As NMDAR activity is well recognized to play a critical role in learning and memory, the efficacy of memantine as a symptomatic, cognitive-enhancing therapy in Alzheimer's disease appears, at first sight, to be counter-intuitive. Memantine is a low-affinity, voltage-dependent uncompetitive antagonist with fast dissociation kinetics, and it has been proposed that it mediates efficacy through normalizing aberrant, disease-associated low level NMDAR activation without impairing physiological synaptic receptor activation, in a manner somewhat analogous to the endogenous NMDAR channel blocker, magnesium (Parsons *et al*., 2007). As such, it is thought to improve the 'signal to noise' characteristics of NMDAR signalling in the diseased brain.

NR2B-containing NMDARs, which are enriched in the extrasynaptic receptor population, represent plausible candidates for mediating such disease-associated 'background' NMDAR activation. Preclinical studies in healthy adult rats that notably do not exhibit the proposed disease-associated NMDAR dysfunction provide some support for the rationale of selectively targeting NR2B-containing receptors in terms of absence of cognitive impairment and evidence for cognitive enhancement. Thus, CP-101,606 at a dose which fully occupied hippocampal NR2B-containing receptors did not impair spatial learning or memory in the Morris water maze task in healthly adult rats (in contrast to the broad-spectrum channel blocker MK-801; Guscott *et al*., 2003). In addition, while CP-101,606 promoted impulsive-type responding, it improved task performance in a rat delayed match to position task (Higgins *et al*., 2005). Thus, NR2B-selective antagonists may have therapeutic potential as cognitive enhancers in Alzheimer's disease, and Evotec is currently developing EVT-101 for this indication (Table 1).

Cerebral ischaemia and traumatic brain injury

The role of CNS glutamate receptors and, particularly, the highly calcium permeable NMDARs in mediating the excitotoxic neuronal cell damage observed in both cerebral ischaemia and traumatic brain injury is well recognized. Accordingly, broad-spectrum NMDAR antagonists have been shown to be neuroprotective when administered before or shortly after traumatic brain injury or ischaemic insult in animal models (Kemp *et al*., 1999). However, the promise of preclinical data has not been realized in patients with several agents, failing to show efficacy in clinical studies (e.g. Morris *et al*., 1999; Lees *et al*., 2000; Albers *et al*., 2001). It has been speculated that the dose-limiting side effects associated with such broad-spectrum NMDAR antagonists may have contributed to the failure of these studies. NR2B-selective antagonists may offer potential as efficacious neuroprotective agents with an acceptable tolerability profile. In support, data from several studies suggest that activation of extrasynaptic (mostly NR2B-containing) receptors triggers pro-death signalling events, while activation of synaptic (mostly NR2A-containing) receptors favours neuronal survival (Hardingham and Bading, 2003; Zhou and Baudry, 2006; von Engelhardt *et al*., 2007; Liu *et al*., 2007; Chen *et al*., 2008; Martel *et al*., 2009). However, while NR2B-selective antagonists are neuroprotective in animal models with an improved therapeutic index relative to broad-spectrum antagonists (Kemp *et al*., 1999; Tahirovic *et al*., 2008), clinical studies have yielded disappointing outcomes, with CP-101,606 failing to demonstrate efficacy, despite an apparently acceptable tolerability profile (Merchant *et al*., 1999; Saltarelli *et al*., 2004).

Major depression

Following the demonstration of antidepressant-like activity of broad-spectrum NMDAR antagonists in animal models, two small crossover clinical studies have reported a positive antidepressant effect of the NMDAR channel blocker ketamine (Berman *et al*., 2000; Zarate *et al*., 2006). Notably, the antidepressant effect exhibited a rapid onset and persisted for several days after single dose administration (Zarate *et al*., 2006), presenting a potentially attractive therapeutic profile. However, the clinical applicability of ketamine is limited by its psychomimetic activity, which was observed in both studies. Preclinically, NR2B-selective antagonists have also been reported to exhibit antidepressant activity (Maeng and Zarate, 2007), and recently a randomized, double-blind study in patients with refractory major depressive disorder treated with CP-101,606 was reported (Preskorn *et al*., 2008). In this parallel group study, subjects with major depression and a history of treatment refractoriness to selective serotonin reuptake inhibitors (SSRI) first received 6 weeks open-label

treatment with the SSRI paroxetine followed by a single-blind placebo infusion. Paroxetine non-responders (n = 30) were then randomized to a single infusion of CP-101,606 or placebo plus continued treatment with paroxetine for up to a further 4 weeks. CP-101,606 was generally well tolerated and its administration produced a greater antidepressant effect than placebo, thus, illustrating antidepressant potential for NR2B subtype-selective antagonists in otherwise treatment refractory patients. A further clinical study in refractory depression has recently been initiated with MK-0657 (Table 1).

Positive modulation of NR2B-containing NMDA receptors

As discussed above, a number of NMDAR positive modulators have been identified, including polyamines, neurosteroids and Mg^{2+}, some of which exhibit subunit selectivity. Based on the increasing understanding of receptor structure and the molecular mechanisms underlying both positive modulation by such ligands and subunit-selective antagonism, it seems reasonable to expect that appropriately configured screening campaigns might identify novel, subunit-selective small molecule positive modulators. Subunit-selective positive modulation might represent a strategy to enhance receptor function, for example, under pathological conditions of receptor hypofunction, without triggering excitotoxicity through receptor overactivation. NMDAR hypofunction has been implicated in the pathophysiology of schizophrenia, initially based on the psychomimetic activity of the broad-spectrum ion channel blockers, ketamine and PCP, which have been reported to elicit a schizophrenia-like phenotype in healthy volunteers, encompassing the characteristic positive, negative and cognitive symptoms of the disease (see, e.g. Krystal *et al.*, 2005). Subsequent neurophysiological, neuroanatomical and biochemical studies have provided support for disease-associated NMDAR hypofunction (e.g. Umbricht and Krljes, 2005; Hahn *et al.*, 2006; Woo *et al.*, 2008). Efforts to enhance disease-associated receptor hypofunction to date have centred on strategies to enhance glycine site occupancy and, hence, receptor tone, either through the administration of agonists (glycine, D-serine) or via inhibition of the GLYT1 glycine transporter, and these studies have yielded encouraging preliminary data (Javitt, 2008). The preferred target NMDAR subunit for a novel small molecule positive modulator approach is unclear. In support of NR2B, its transgenic overexpression resulted in mice with improved learning and memory (Tang *et al.*, 1999), and gene knockout/knockdown resulted in learning and memory impairments (von Engelhardt *et al.*, 2008). Thus, selective positive modulation of NR2B-containing NMDARs might represent a novel therapeutic strategy for the treatment of schizophrenia and, potentially, other cognitive disorders.

Conclusion

By combining biochemical, structural and functional studies, much progress has been made in recent years in the comprehension of the mechanisms by which extracellular agents can affect NMDAR activity. In particular, it has become increasingly clear that the large NTD of NR2 subunits, which precedes the glutamate-binding domain, is a major site for subunit-specific allosteric modulation. In that respect, receptors incorporating the NR2B subunit appear particularly interesting because the NR2B-NTD not only harbours sites for negative allosteric modulators (such as zinc and ifenprodil) but might also confer a unique sensitivity to the positive allosteric modulators polyamines and Mg^{2+} by participating in an inter-subunit regulatory site. Finally, our increasing understanding of NMDAR structure, function and pharmacology is now translating into promising therapeutic strategies to target NMDAR dysregulation. The therapeutic utility of broad-spectrum NMDAR antagonists is typically limited by their associated side effects. Newer, subtype-selective negative modulators of receptor function, primarily targeting NR2B-containing receptors, have entered clinical development and offer improved potential for the treatment of patients suffering from a range of debilitating psychiatric and neurological disorders. Opportunities for selective positive modulation of NMDARs and to selectively target other NMDAR subtypes or their downstream regulatory cascades are currently more limited but are likely to be a focus of future research. The advent of biological therapeutic agents, such as antibodies (or fragments thereof), and RNA inhibition may also enable therapeutic interventions considered intractable using existing small molecule approaches.

Conflicts of interest

LM and PP declare no conflict of interest. MJG and JK are employees of GlaxoSmithKline PLC.

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