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Structure-based discovery of antagonists for GluN3-containing N-methyl-D-aspartate receptors

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Abstract

NMDA receptors are ligand-gated ion channels that assemble into tetrameric receptor complexes composed of glycine-binding GluN1 and GluN3 subunits (GluN3A-B) and glutamate-binding GluN2 subunits (GluN2A-D). NMDA receptors can assemble as GluN1/N2 receptors and as GluN3-containing NMDA receptors, which are either glutamate/glycine-activated triheteromeric GluN1/N2/N3 receptors or glycine-activated diheteromeric GluN1/N3 receptors. The glycine-binding GluN1 and GluN3 subunits display strikingly different pharmacological selectivity profiles. However, the pharmacological characterization of GluN3-containing receptors has been hampered by the lack of methods and pharmacological tools to study GluN3 subunit pharmacology in isolation. Here, we have developed a method to study the pharmacology of GluN3 subunits in recombinant diheteromeric GluN1/N3 receptors by mutating the orthosteric ligand-binding pocket in GluN1. This method is suitable for performing compound screening and characterization of structure-activity relationship studies on GluN3 ligands. We have performed a virtual screen of the orthosteric binding site of GluN3A in the search for antagonists with selectivity for GluN3 subunits. In the subsequent pharmacological evaluation of 99 selected compounds, we identified 6-hydroxy-[1,2,5]oxadiazolo[3,4-*b*]pyrazin-5(4*H*)-one (TK80) a novel competitive antagonist with preference for the GluN3B subunit. Serendipitously, we also identified [2-hydroxy-5-((4-(pyridin-3-yl)thiazol-2-yl)amino)benzoic acid (TK13) and 4-(2,4-dichlorobenzoyl)-1*H*-pyrrole-2-carboxylic acid (TK30), two novel non-competitive GluN3 antagonists. These findings demonstrate that structural differences between the orthosteric binding site of GluN3 and GluN1 can be exploited to generate selective ligands.

Keywords

NMDA receptor; GluN3 subunit; antagonist; selectivity; virtual screening; *Xenopus* oocyte electrophysiology

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1. Introduction

N-methyl-D-aspartate (NMDA) receptors are ligand-gated cation-selective channels that belong to the family of ionotropic glutamate receptors (iGluRs) (Traynelis et al., 2010). Most native NMDA receptors are composed of two GluN1 subunits and two GluN2 subunits and are activated by simultaneous binding of glycine and glutamate to GluN1 and GluN2, respectively (Laube et al., 1998; Ulbrich and Isacoff, 2007). GluN3-containing NMDA receptors are either glutamate/glycine-activated triheteromeric receptors composed of GluN1, GluN2, and GluN3 subunits or glycine-activated diheteromeric receptors composed of GluN1 and GluN3 subunits (Chatterton et al., 2002; Das et al., 1998; Pérez-Otaño et al., 2001; Sasaki et al., 2002; Smothers and Woodward, 2007; Smothers and Woodward, 2009; Ulbrich and Isacoff, 2007; Ulbrich and Isacoff, 2008).

Although the GluN3A subunit was cloned almost two decades ago (Ciabarra et al., 1995; Sucher et al., 1995) followed by cloning of the GluN3B subunit in the beginning of this century (Andersson et al., 2001; Chatterton et al., 2002; Matsuda et al., 2002; Nishi et al., 2001), many aspects of the relationship between structure and function for both GluN3A and GluN3B subunits remain elusive (Cavara and Hollmann, 2008; Henson et al., 2010; Low and Wee, 2010; Pachernegg et al., 2012). The GluN3 subunits appears to function as modulatory subunits that reduce the susceptibility of NMDA receptors to Mg^{2+} -blockage and reduce Ca^{2+} -permeability (Cavara et al., 2010; Chatterton et al., 2002; Pérez-Otaño et al., 2001; Sasaki et al., 2002). Furthermore, GluN3 subunits are involved in synapse maturation (Das et al., 1998; Henson et al., 2012; Pérez-Otaño et al., 2006; Roberts et al., 2009), synaptic plasticity (Larsen et al., 2011), and are neuroprotective in various cells (Kárádóttir et al., 2005; Martínez-Turrillas et al., 2012; Micu et al., 2006; Nakanishi et al., 2009; Salter and Fern, 2005). Consequently, the GluN3 subunits could be promising new targets for therapeutic intervention in neuropathological conditions that include excitotoxicity and cognitive impairment (Cavara and Hollmann, 2008; Henson et al., 2010; Stys and Lipton, 2007). In this regard, agonists and antagonists that are selective for the GluN3 subunits are of crucial importance in order to evaluate the physiological roles and therapeutic potential of GluN3-containing NMDA receptors. It appears feasible to develop GluN3-selective agonists and antagonists due to the observed structural and pharmacological differences between the glycine-binding NMDA receptor subunits GluN1 and GluN3. Studies using soluble ligand-binding domains (LBDs) of GluN1 and GluN3A have revealed that glycine binds with a 650-fold higher affinity at GluN3A over GluN1, indicating that the glycine binding site of GluN3 is different from that of GluN1 (Yao and Mayer, 2006). Furthermore, GluN3A is reported to have a selectivity profile strikingly different from that of GluN1 with respect to binding affinities of known glycine-site antagonist; 5,7-dichlorokynurenic acid (DCKA), L-689,560 and other high-affinity glycine-site antagonists exhibit strong selectivity for binding GluN1 over GluN3A (Yao and Mayer, 2006). Crystal structures of GluN1, GluN3A, and GluN3B LBDs in complex with glycine or D-serine have also revealed differences in the agonist binding pocket that could be exploited to achieve selectivity (Yao et al., 2008).

Advances in our understanding of the functional properties and pharmacology of GluN3-containing NMDA receptors have been hampered by the fundamental problem of establishing expression systems that allows GluN3 function to be studied in isolation. Recombinant co-expression of GluN1, GluN2, and GluN3 subunits generates multiple receptor populations (i.e. GluN1/N2 and GluN1/N2/N3), confounding the study of GluN3-containing receptors (Das et al., 1998; Pérez-Otaño et al., 2001; Sasaki et al., 2002). Expression of recombinant GluN1/N3 receptors in *Xenopus* oocytes provides a convenient solution to this problem (Awobuluyi et al., 2007; Chatterton et al., 2002; Madry et al., 2007). However, glycine has dual action at these GluN1/N3 receptors, in that glycine

appears to act agonistically at the GluN3 subunit and inhibitory through binding to the GluN1 subunit (Awobuluyi et al., 2007; Madry et al., 2007) resulting in bell-shaped concentration-response curves. In this study, we establish a method suitable for evaluation of compounds at recombinantly expressed GluN3-containing NMDA receptors by mutating the orthosteric ligand-binding pocket in GluN1. Based on virtual screening of the orthosteric binding site in the LBD of GluN3A subunit followed by pharmacological evaluation of 99 compounds, we identify antagonists with preference for GluN3-containing NMDA receptors. The novel antagonists have been characterized and the pharmacological mechanism of inhibition has been described. This discovery provides evidence that structural differences between GluN1 and GluN3 subunits can be exploited to generate GluN3-selective ligands that could be useful tools to study the physiological roles of GluN3 subunits.

2. Materials and methods

2.1 Molecular biology

cDNAs encoding the GluN1-1a (GenBank: U11418; hereafter GluN1), GluN2A (GenBank: D13211), GluN2B (GenBank: M91562), GluN2C (GenBank: D13212), and GluN2D (GenBank: D13214) subunits were generously provided by Dr. S. Nakanishi (Osaka Bioscience Institute, Osaka, Japan). cDNAs encoding GluA1 (GenBank: X17184) and GluK2 (GenBank: Z11548) subunits were generously provided by Dr. S. Heinemann (Salk Institute for Biological Studies, San Diego, CA). cDNAs encoding the short variant GluN3A-1 (GenBank: U29873; hereafter GluN3A) and GluN3B (GenBank: NM_133308) subunits were generously provided by Dr. D. Zhang (Sanford-Burnham Medical Research Institute, La Jolla, CA). The GluN1 mutants were made by QuikChange Site-Directed mutagenesis (Stratagene, Agilent Technologies, Santa Clara, CA) and verified by DNA sequencing (SeqWright, Houston, TX). Amino acid residues are numbered based on the full-length polypeptide sequence, including the signal peptide (initiating methionine is 1).

2.2 Two-electrode voltage-clamp recordings

For expression in *Xenopus laevis* oocytes, cDNAs were linearized by restriction enzymes and used as templates to synthesize cRNA using the mMessage mMachine kit (Ambion, Life Technologies, Paisley, UK). Defolliculated stage V–VI oocytes ready to inject were obtained from EcoCyte Biosciences (Castrop-Rauxel, Germany) or prepared as previously described (Traynelis et al., 1998). Oocytes were coinjected with cRNAs encoding GluN1 and GluN2 or GluN3 at a 1:2 ratio, and maintained at 18°C in Barth's solution containing 88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO₃, 0.82 mM MgSO₄, 0.33 mM Ca(NO₃)₂, 0.91 mM CaCl₂, 10 mM HEPES (pH 7.5 with NaOH) supplemented with 100 IU/ml penicillin, 100 µg/ml streptomycin and 100 µg/ml gentamycin (Invitrogen, Life Technologies, Paisley, UK).

Two-electrode voltage-clamp (TEVC) recordings were performed at room temperature 3–6 days post-injection using an OC-725C TEVC amplifier (Warner Instruments, Hamden, CT). Glass electrodes had at tip resistance of 0.5–2.5 MΩ and were pulled from thin-walled glass capillary tubes (World Precision Instruments, Hertfordshire, UK) using a PC-10 puller (Narishige, East Meadow, NY). Voltage and current electrodes were filled with 0.3 and 3 M KCl, respectively. During recordings oocytes were placed in a recording chamber and perfused with the extracellular oocyte recording solution composed of 90 mM NaCl, 1 mM KCl, 10 mM HEPES, 0.5 mM BaCl₂ and 0.01 mM EDTA (pH 7.4 with NaOH). Current responses were recorded at a holding potential of –40 mV and –60 mV for GluN1/N2 and GluN1/N3 receptors, respectively (unless otherwise stated). Compound solution were made of extracellular oocyte recording solution and applied to the oocyte by gravity-driven

perfusion using a computer controlled 8-modular-valve positioner (Digital MVP, Hamilton, Reno, NV).

Current-voltage relationships were determined by performing current measurements at different holding potentials in the following order of solutions: 1) control (i.e. extracellular oocyte recording solution), 2) agonist, 3) agonist plus inhibitor, and finally 4) control. The average of the two current-voltage relationships in control was then subtracted from the relationships in agonist and agonist plus inhibitor.

2.3 Compound screening

Library compounds were obtained from Alinda Chemical (Moscow, Russia), ASDI (Newark, DE), ASINEX (Winston-Salem, NC), BioNet (Key Organics, Cornwall, UK), Chembridge (San Diego, CA), ChemDiv (San Diego, CA), Enamine (Kiev, Ukraine), InterBioScreen (Moscow, Russia), Life Chemicals (Orange, CT), Matrix Scientific (Columbia, SC), Maybridge (Cornwall, UK), Pharmeks (Moscow, Russia), Princeton BioMolecular Research (Monmouth, NJ), Sinova (Bethesda, MD), and Specs (Delft, The Netherlands). Stock solutions were made in DMSO or equimolar NaOH aqueous solution depending on compound solubility. DMSO concentration was constant in both control and test solution when DMSO-dissolved compounds were evaluated, and the DMSO concentration never exceeded 1%. The compound library was screened by TEVC recordings. Current responses were recorded at a holding potential of -60 mV. Compounds were evaluated at two oocytes for each receptor subtype.

2.4 Data analysis

Data were analyzed using GraphPad Prism 5 (GraphPad Software, La Jolla, CA). Agonist concentration-response data for individual oocytes were fitted to the Hill equation

$I = I_{\max} / (1 + 10^{(\log EC_{50} - \log [A]) \cdot n_H})$. $I_{\max}$ is the maximum current in response to agonist, EC_{50} is the concentration of agonist that produces half-maximum activation, $[A]$ is the concentration of agonist, and n_H is the Hill coefficient. Antagonist concentration-response data was also fitted to the Hill equation to produce IC_{50} values (i.e. the concentration of antagonist that produces half-maximum inhibition). $\log EC_{50}$ or $\log IC_{50}$ and n_H from the individual oocytes were used to calculate mean and S.E.M. For graphical presentation, the data for individual oocytes were normalized to the maximum current response in the same recording and averaged. The averaged data points were then fitted to the Hill equation and plotted together with the resulting curve.

For bell-shaped concentration-response curves (i.e. concentration-response data with two components, one that stimulates and one that inhibits), the data for individual oocytes were simultaneously fitted to the following three equations using global nonlinear regression (least-squares fitting method):

$$I = dip + I_s + I_i$$

$$I_s = -dip / (1 + 10^{(\log EC_{50} - \log [A]) \cdot n_H \{s\}})$$

$$I_i = -dip / (1 + 10^{(\log [A] - \log IC_{50}) \cdot n_H \{i\}})$$

The value for *dip* is the negative of the maximal response if no inhibition is present. The curves are assumed to begin at zero current at low agonist concentrations and end at zero current at high agonist concentrations. For example, if the maximal response when no inhibition is present is 100%, then the dip is -100% and I_s goes from 0% to 100% and I_i goes from 100% to 0%. Adding this gives 0% at the highest and lowest agonist concentrations. EC_{50} and IC_{50} are the concentrations of agonist that produces half-

maximum stimulatory and inhibitory effects, $[A]$ is the concentration of agonist, and $n_H\{s\}$ and $n_H\{I\}$ are the Hill coefficients. Due to the inherent ambiguity of the fitting algorithm, global non-linear regression (least-squares fitting method) was performed by simultaneously fitting data for all oocytes using shared values for EC_{50} , IC_{50} , and Hill coefficients and allowing only the value of dip to change among oocytes. This method will determine shared values for EC_{50} , IC_{50} , and Hill coefficients that best describe all the recorded data, thereby minimizing the contribution of ambiguous fitting results (Herman and Lee, 2012). For graphical representation, the individual oocytes were subsequently normalized to the fitted maximal response if no inhibition is present (i.e. the negative of the value of dip) and averaged. The averaged data points were then re-fitted to produce the bell-shaped concentration-response fit and plotted together with the resulting curve. Statistical analyses of the results were performed where appropriate. Unpaired Student's t test was conducted. Significance was set at $P < 0.05$. K_i -values were estimated for competitive antagonists from IC_{50} -values using the Cheng-Prusoff equation (Cheng and Prusoff, 1973).

2.5 Computational modeling and virtual screening

Three hybrid models of the GluN3A LBD in a conformation induced by antagonist binding were built by adapting the agonist-bound GluN3A:D-serine complex (PDB: 2RC8) to the receptor conformations of GluN1:DCKA (PDB: 1PBQ), GluA2:6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (PDB: 3B7D) and GluA2:(S)-NS1209 (PDB: 2CMO). Restrained minimization was performed with MacroModel 9.6 (Schrödinger Inc., Portland, OR). Commercially available compounds were prepared with Ligprep 2.2 (Schrödinger Inc., Portland, OR) using Lipinski-type rules for drug-likeness and REOS-type filters for common reactive moieties and toxicophores (Lipinski et al., 2001; Walters and Murcko, 2002). The prepared compounds were then docked with Glide 5.0 in a standard Virtual Screening Workflow, in which the top 10% of each round of screening are taken into the next level of Glide's scoring functions (HTVS, SP and XP) (Schrödinger Inc., Portland, OR).

Default/recommended settings were used, with the exception of custom constraints: the key hydrogen bonds to the upper lobe (D1) of the LBD for amino acid recognition made by all known orthosteric iGluR ligands were required during docking (i.e. Arg638 sidechain as bidentate donor and backbone carbonyl of Ser631 as acceptor in GluN3A). The top-scoring 5,000 poses from the combined virtual screens were clustered into groups of close analogues. The top scoring members of each cluster were then inspected to check the quality of the binding poses. Chemotypes presenting pharmacophores similar to the known classes of iGluR antagonists, i.e. amino acids, kynurenate-like and quinoxalinedione-like heterocycles, were given extra weight. Potential agonists had been ruled out by molecular weight filters, as well as the implicit need to span the modeled gap between the domains in order for a compound to score well.

3 Results

3.1 Electrophysiological evaluation of the agonist binding site in GluN3

To enable evaluation of GluN3-selective antagonists, we aimed to establish a straightforward system suitable for screening of a compound library for GluN3 activity. Recombinant diheteromeric GluN1/N3 receptors can be functionally expressed in *Xenopus* oocytes. However, glycine binds to both GluN1 and GluN3, and previous studies have found that GluN1/N3 receptors have a bell-shaped glycine concentration-response relationship, where the receptor current is diminished at high glycine concentration (Chatterton et al., 2002). We observed that glycine concentrations above 10 μ M result in diminished receptor responses for GluN1/N3B receptors (Fig. 1C). By contrast, GluN1/N3A receptors displayed

negligible current responses (< 10 nA) upon glycine application (Fig. 1B,D). The absence of responses at GluN1/N3A and the bell-shaped concentration-response relationship at GluN1/N3B are likely caused by glycine having both stimulating and inhibitory activity at the GluN1/N3 receptors. Thus, glycine acts agonistically at the GluN3 subunit and inhibitory through binding to the GluN1 subunit (Awobuluyi et al., 2007; Madry et al., 2007). To overcome this problem caused by overlapping pharmacology at the GluN1 and the GluN3 agonist binding sites, we exploited the finding by Awobuluyi *et al.* (2007) that glycine binding to GluN3 subunit alone is enough to activate GluN1/N3 receptors in *Xenopus* oocytes (Awobuluyi et al., 2007). Awobuluyi *et al.* (2007) has shown that single mutations in the GluN1 orthosteric ligand-binding pocket (e.g. F484A) altered the concentration-response profiles for the GluN1/N3B receptor, with no glycine-induced inhibition of responses up to a glycine concentration of $1000\text{ }\mu\text{M}$ (Awobuluyi et al., 2007). However, at glycine concentrations $> 1000\text{ }\mu\text{M}$ the inhibitory component of glycine is still present (see the text below). We hypothesize that the change in concentration-response relationship observed by mutating the GluN1 LBD, thereby disrupting glycine binding to the GluN1 subunit, is caused by a change in the inhibitory component of glycine at the GluN1/N3 receptors. GluN1/N3 receptors without the inhibitory component will obtain a plateau of maximal response upon glycine activation, allowing determination of glycine EC_{50} -values at GluN3. We subsequently tested this idea by introducing mutations into GluN1 that will result in activation of GluN1/N3 receptors by glycine binding to the GluN3 subunit only.

We mutated several GluN1 residues previously demonstrated to be important for glycine sensitivity (Kuryatov et al., 1994) and studied the properties of the GluN1 mutations in GluN1/N2A receptors expressed in *Xenopus* oocytes using TEVC recordings. The F484A mutation drastically reduced glycine sensitivity, resulting in a 3000-fold increase in glycine EC_{50} at GluN1/N2A receptors (Fig. 1A, Table 1). F484 in GluN1 forms a 'lid' in the orthosteric ligand-binding pocket that has been proposed to sterically prevent bound agonist from leaving the closed cleft conformation (Furukawa and Gouaux, 2003) (Fig. 2A). Glycine EC_{50} was increased 6000-fold at GluN1/N2A by the T518L mutation of GluN1 (Fig. 1A, Table 1) and a similar increase in glycine EC_{50} at GluN1/N2A was observed by introducing the R523A mutation (Fig. 1A, Table 1). These two mutations are directly disrupting hydrogen bonds that coordinate glycine in the agonist-binding pocket of GluN1 (Furukawa and Gouaux, 2003) (Fig. 2A). Two other mutations A714L and V689L that are reported to stabilize the open cleft conformation of the GluN1 LBD (Furukawa and Gouaux, 2003) resulted in minor reductions in glycine sensitivity; 25- and 60-fold increases in glycine EC_{50} at GluN1/N2A (Fig. 1A, Table 1). Based on the results from the evaluation of the GluN1 LBD single mutations at the GluN1/N2A receptor, two mutations (i.e. F484A and T518L) with pronounced effects on glycine potency were selected for characterization at the GluN3-containing receptors.

The mutated GluN1 subunit (i.e. GluN1(F484A) and GluN1(T518L)) expressed together with either GluN3A or GluN3B subunits produced functional receptors. Similar to wildtype GluN1/N3 receptors, the glycine concentration-response curves for these receptors are bell-shaped (Fig. 1B–C, parameters from the bell-shaped fit are listed in Table 2). Although the concentration-response relationship remains bell-shaped, the potency of the inhibitory component of the receptor response was reduced. Thus, the onset of the glycine-induced reduction of responses occurred at higher glycine concentrations: $> 100\text{ }\mu\text{M}$ for GluN1(F484A)/N3A and GluN1(T518L)/N3A and $> 1000\text{ }\mu\text{M}$ for GluN1(F484A)/N3B and GluN1(T518L)/N3B receptors (Fig. 1B–D). The double mutant GluN1(F484A/T518L)/N2A receptor is non-functional for glycine concentration up to 30 mM (Fig. 1A). At GluN3-containing receptors, a marked change in the glycine concentration-response curves was observed by introducing the GluN1(F484A/T518L) subunit. The GluN1(F484A/T518L)/N3A and GluN1(F484A/T518L)/N3B receptors reach a plateau of maximal response at

glycine concentration > 1000 μ M. No reduction in current responses is observed for glycine concentration up to 30 mM (Fig. 1B–D). The EC₅₀-values were 57 μ M and 95 μ M for GluN1(F484A/T518L)/N3A and GluN1(F484A/T518L)/N3B receptors, respectively (Table 2).

To provide evidence that mutated GluN1(F484A/T518L) subunits form functional receptors with GluN3 subunits we evaluated surface expression of functional glycine-activated receptors in *Xenopus* oocytes injected with either the individual subunits alone or co-injected with GluN1(F484A/T518L) and GluN3A or GluN3B (Fig. 2). *Xenopus* oocytes individually injected with cRNA encoding GluN1, GluN1(F484A/T518L), GluN3A, and GluN3B subunits did not show any current responses to application of 10 μ M, 100 μ M, and 1000 μ M glycine (Fig. 2C–F). By contrast, robust glycine-activated current responses were obtained when GluN1(F484A/T518L) were co-injected with GluN3A or GluN3B (Fig. 2A–B). These results demonstrate that both GluN1(F484A/T518L) and GluN3 subunits are required to form functional receptors and strongly indicates surface expression of functional diheteromeric GluN1(F484A/T518L)/N3A and GluN1(F484A/T518L)/N3B receptors in accordance with previous reports using single-molecule fluorescence in *Xenopus* oocytes (Ulbrich and Isacoff, 2007; Ulbrich and Isacoff, 2008).

Thus, the diheteromeric GluN3-containing NMDA receptors containing GluN1 subunits that are presumably incapable of binding glycine (i.e. GluN1(F484A/T518L)/N3A and GluN1(F484A/T518L)/N3B receptors) provide a straightforward and robust system that reach a plateau of maximal response facilitating determination of EC₅₀-values and pharmacological characterization of GluN3 ligands.

3.2 Virtual screening of the GluN3A LBD

Preliminary investigation included a close comparison of the sequence and structure in the vicinity of the glycine-binding sites of GluN1, GluN3A, and GluN3B as represented by X-ray structures of the respective LBDs in complexes with D-serine, (PDB: 1PB8, PDB: 2RC8, and PDB: 2RCB, respectively) (Furukawa and Gouaux, 2003; Yao et al., 2008). Between GluN3A and GluN3B, first shell binding site sequence conservation is 100%, and only a few differences are noted in the second and third shells of residues around the ligand, such as A847/S747, A824/T724, R798/W698, and D804/A704 (amino acids residues according to GluN3A/GluN3B), with the upper lobe of the LBD (D1) being even more highly conserved than the lower lobe (D2). Due to the high similarity of the GluN3A and GluN3B X-ray structures and because slightly higher quality structural data was available for GluN3A, this subtype was chosen to represent both GluN3A and GluN3B LBDs. The GluN1 binding site displays major differences from those of GluN3 subunits in both subdomains of the LBD (i.e. D1 and D2). (Fig. 3A,B). The distal end of the pocket is tightly closed by W731 in GluN1, but capped by the smaller, more flexible M844 in GluN3A (Fig. 3A,B). GluN1 has no inter-domain hydrogen bond (“lock”) at Q405—A714, whereas GluN3A does, at E522—T825. In each of these respects, the GluN3 binding sites are more similar to α -amino-3-hydroxy-4-methylisoxazole-5-propionic acid (AMPA) and kainate receptor subunits than other NMDA receptor subunits, with particular similarity to the kainate receptor subunit GluK1. The binding site residues distinguishing GluN3A as a glycine-sensitive subunit are firstly D845 that complements the α -amino group (Asp in all GluN subunits; Glu in GluA and GluK subunits), and secondly the lack of an H-bond donor at A802 to recognize a distal carboxylic acid group (V689 in GluN1, Thr in GluN2A as well as all GluA and GluK subunits) (Fig. 3A,B). In summary, the GluN3 binding site is quite distinct from GluN1 and contains a unique combination of residues, with various features in common with members of each different class of iGluR. Therefore, with high resolution

structures in hand, the prospects were good that virtual screening could uncover selective ligands.

Ideally, virtual screening for new and potentially selective classes of chemical matter that engage the target (i.e. novel chemotypes) would be performed on a suitable experimental X-ray structure or structures of the GluN3 LBD. However, iGluRs undergo a large conformational change (domain closure) upon agonist binding, and no *apo* or antagonist-bound structures of GluN3 are published. Furthermore, variation is seen in the degree of domain closure/opening induced by different antagonists at various iGluRs, and it was unknown how the GluN3 LBD conformation should be to maximize the chances of finding new antagonists. Therefore, an ensemble of three models of the states that GluN3 might adopt when an antagonist is bound were built, covering a range of conformations. To a first approximation, the two subdomains of the LBD act as rigid bodies, connected by a hinge consisting of two strands in a β -sheet, a few residues long, that allow relative ligand-induced bending and some twisting motion (Bjerrum and Biggin, 2008). The starting point was a high quality domain-closed structure of GluN3A in complex with D-serine (PDB: 2RC8) (Yao et al., 2008). The two domains were separated by cutting the middle of the hinge, at S650-T651 and I870-E871. Each domain was superimposed on the corresponding domain of an antagonized structure of a homologous iGluR LBD, and then the hinge rebuilt and the complex relaxed with molecular mechanics. The first template chosen was GluN1:DCKA (PDB: 1PBQ) (Furukawa and Gouaux, 2003), a somewhat closed structure, and the only example of an antagonized conformation of a NMDA receptor subunit LBD (Fig. 3C). The second was GluA2:CNQX (PDB: 3B7D) (Menuz et al., 2007), since CNQX is known to possess GluN3 activity and induce little domain closure compared to *apo* in GluA2. The third model was based on the hyperextended GluA2:(S)-NS1209 complex (PDB: 2CMO) (Kasper et al., 2006) as an upper bound to the known degree of domain opening.

A collection of approximately 4 million structures of commercially available compounds were virtually screened at each of the three models (see Materials and methods section 2.5). With the goal of obtaining a broad distribution of chemotypes, the top-scoring 5,000 poses from the combined screens were clustered into groups of close analogues. Two hundred of these compounds were shortlisted for purchase, of which 99 were ultimately obtained from commercial vendors and evaluated at GluN3-containing receptors expressed in *Xenopus* oocytes using TEVC recordings.

3.3 Identification of antagonists for GluN3-containing NMDA receptors

The purchased compounds were functionally screened at GluN1(F484A/T518L)/N3A and GluN1(F484A/T518L)/N3B receptors expressed in *Xenopus* oocytes using TEVC recordings. Compounds were evaluated as agonist at a single concentration of 100 μ M and as antagonist at a single concentration of 100 μ M in the presence of 100 μ M glycine. Current responses to application of the compounds were compared to current responses in the same recording to 1 mM glycine (agonist screen) or 100 μ M glycine (antagonist screen). 8 of the 99 library compounds were identified as hits in the antagonist screen (Fig. 4), whereas none of the 99 compounds displayed activity in the agonist screen (data not shown).

A compound was classified as a hit in the antagonist screen if it displayed > 25% inhibition of the current response to 100 μ M glycine. The identified hits were subsequently evaluated for potency at GluN3-containing NMDA receptors and for selectivity for the GluN3 subunit over the GluN1 subunit.

First, the 8 identified hits were evaluated for potency at GluN3-containing NMDA receptors. Concentration-response data were generated using TEVC recordings of responses from GluN1(F484A/T518L)/N3A and GluN1(F484A/T518L)/N3B receptors expressed in

Xenopus oocytes. The identified hits were generally more potent at the GluN1(F484A/T518L)/N3B receptor compared to the GluN1(F484A/T518L)/N3A based on the determined IC₅₀-values (Table 3). Only one hit (i.e. TK67) had a different profile being a more potent antagonist at the GluN1(F484A/T518L)/N3A receptor (Table 3, Fig. 4).

We subsequently explored whether the hits were selective for GluN3 over GluN1 by evaluating the activity of the compounds at GluN1/N2A receptors. Four compounds (i.e. TK13¹, TK30¹, TK67, and TK80¹) displayed minor inhibition at GluN1/N2A receptors compared to the GluN3-containing receptors, and thus displayed preference for GluN3-containing receptors (Table 3–4, Fig. 4B). Three compounds (i.e. TK40, TK51, and TK69) showed preference for the GluN1 subunit, and were consequently excluded from further characterization (Table 3–4, Fig. 4B). Compound TK31 displayed high IC₅₀-values (> 100 μM) at all evaluated receptors, and was therefore excluded from further characterization (Table 3–4, Fig. 4B).

3.4 Mechanism of action for novel antagonists with preference for GluN3-containing NMDA receptors

TK13 and TK30 displayed 100% inhibition and similar IC₅₀-values at the single mutant GluN1(F484A)/N3A and GluN1(T518L)/N3A receptors compared to the double mutated GluN1(F484A/T518L)/N3A receptor and were expected to interact with the GluN3 subunit as predicted from the virtual screen (Table 3). TK80 displayed selectivity for GluN3B over GluN3A-containing receptors, indicating interactions with the GluN3 subunit. In contrast a 35% decrease in maximal inhibition by TK67 was observed at the single mutant GluN1(F484A)/N3A receptor together with a more than 20-fold increase in IC₅₀-value of TK67 observed at the single mutated GluN1(T518L)/N3A receptor compared to the double mutated GluN1(F484A/T518L)/N3A receptor (Table 3). The sensitivity of TK67 to mutations in the GluN1 agonist binding pocket (i.e. more than 20-fold increase in IC₅₀ and decreased maximal inhibition) indicates that TK67 could be interacting with the mutated GluN1(F484A/T518L) subunit.

TK30 (Fig. 4B) was the most potent of the novel antagonists with IC₅₀-values of 14 μM and 7.4 μM at GluN1(F484A/T518L)/N3A and GluN1(F484A/T518L)/N3B receptors, respectively, and no comparable inhibitory activity at the four conventional GluN1/N2 receptors, GluA1, and GluK2 (IC₅₀-values > 100 μM) (Table 3–4, Fig. 5C). TK13 (Fig. 4B) displays IC₅₀-values of 67 μM and 49 μM at GluN1(F484A/T518L)/N3A and GluN1(F484A/T518L)/N3B receptors, respectively, and IC₅₀-values > 300 μM at GluN1/N2 receptors, GluA1, and GluK2 (Table 3–4, Fig. 5B), whereas TK80 (Fig. 4B) displayed selectivity for GluN1(F484A/T518L)/N3B over GluN1(F484A/T518L)/N3A receptors with an IC₅₀-value of 79 μM at GluN1(F484A/T518L)/N3B and no comparable inhibitory activity at GluN1(F484A/T518L)/N3A, the four conventional GluN1/N2 receptors, GluA1, and GluK2 (IC₅₀-values > 300 μM) (Table 3–4, Fig. 5A,D).

To evaluate the mechanism of action for inhibition of GluN3-containing NMDA receptors by TK13, TK30, and TK80, we first assessed whether increasing the concentration of glycine from 100 μM to 3000 μM could surmount inhibition by TK13, TK30, and TK80. The high glycine concentration did not reduce the extent of inhibition of half-maximal effective concentration of TK13 at GluN1(F484A/T518L)/N3A receptors, indicating that the inhibition was non-competitive (Fig. 6A,C). The extent of inhibition of GluN1(F484A/

¹Abbreviations: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; DCKA, dichlorokynurenic acid; iGluR, ionotropic glutamate receptor; LBD, ligand-binding domain; NMDA, N-methyl-D-aspartate; TEVC, two-electrode voltage-clamp; TK13, [2-hydroxy-5-((4-(pyridin-3-yl)thiazol-2-yl)amino)benzoic acid; TK30, 4-(2,4-dichlorobenzoyl)-1H-pyrrole-2-carboxylic acid; TK80, 6-hydroxy-[1,2,5]oxadiazolo[3,4-b]pyrazin-5(4H)-one.

T518L)/N3A receptors by a half-maximal effective concentration of TK30 was reduced in a high glycine concentration, but the inhibition was insurmountable and TK30 inhibition appear non-competitive (Fig. 6C). However, the inhibition by a half-maximal effective concentration of TK80 was abolished in a high glycine concentration, indicating that TK80 inhibition of GluN1(F484A/T518L)/N3B receptors was surmountable and thus competitive (Fig. 6B,C). To strengthen the conclusion regarding mechanism, glycine concentration-response curves in the absence and presence of increasing concentrations of TK13, TK30, and TK80 were generated at the GluN1(F484A/T518L)/N3A or GluN1(F484A/T518L)/N3B receptors (Fig. 6D–F). For TK13 and TK30, similar glycine EC₅₀-values were obtained in the presence of increasing concentrations of TK13 and TK30 compared to the glycine EC₅₀-value at GluN1(F484A/T518L)/N3A obtained in the absence of inhibitor, however a decrease in the maximal response were observed in the presence of either TK13 or TK30 (Fig. 6D–E, Table 5). These findings confirm a non-competitive mechanism of inhibition by TK13 and TK30. By contrast, TK80 displays right-shifting of the glycine concentration-response curves in the presence of increasing concentration of TK80 with increasing glycine EC₅₀-values compared to the glycine EC₅₀-value at GluN1(F484A/T518L)/N3B obtained in the absence of inhibitor. The glycine curves remain parallel with the same maximal activation consistent with a competitive mechanism of inhibition by TK80 (Fig. 6F, Table 5). For the competitive antagonist, the K_i -value was estimated using the Cheng-Prusoff equation. TK80 has estimated K_i -values of 39 μ M at GluN1(F484A/T518L)/N3B, > 290 μ M at GluN1/N2A, > 260 μ M at GluN1/N2B and GluN1/N2C, and > 210 μ M at GluN1/N2D.

The current-voltage relationship of TK13, TK30, and TK80 inhibition of GluN1(F484A/T518L)/N3A and GluN1(F484A/T518L)/N3B receptors, respectively, demonstrated that inhibition is mediated by a voltage-independent mechanism as the degree of inhibition at –60 mV and +30 mV was similar for all three compounds (4–5 oocytes; $P > 0.05$, Student's t test). (Fig. 7A–C).

The effects of TK13, TK30, and TK80 were also evaluated at wild-type GluN1/N3B receptors expressed in *Xenopus* oocytes. TK13 (100 μ M), TK30 (30 μ M), and TK80 (100 μ M) resulted in 55 ± 3.1 %, 70 ± 4.0 %, and 65 ± 4.3 % inhibition, respectively, of current responses to 10 μ M glycine (6–8 oocytes) (Fig. 8A–D).

4 Discussion

In the present study, we have identified a GluN1 LBD mutant (GluN1(F484A/T518L)) that completely eliminates the inhibitory action of glycine at GluN1/N3 receptors. Using this GluN1 LBD mutant, we have established a method to evaluate compounds for activity at the GluN3 subunit in GluN3-containing receptors. We performed a virtual screen of the GluN3A LBD in the search for antagonists with selectivity for GluN3-containing receptors. By exploiting this structure-based approach, we have succeeded in identifying one novel competitive antagonist (TK80) with preference for the GluN3B subunit. In addition, we serendipitously identified two novel antagonists (TK13 and TK30) that appear to be non-competitive GluN3 antagonists.

4.1 Diheteromeric GluN1/N3 receptors with mutated GluN1 as a method for evaluation of GluN3 pharmacology

We have utilized the fact that only activation of the GluN3 subunit is required to activate GluN1/N3 receptors (Awobuluyi et al., 2007; Madry et al., 2007) to identify a GluN1 LBD mutant (GluN1(F484A/T518L)) that eliminates the inhibitory contribution of the GluN1 subunit in GluN1/N3 receptors. Co-expression of this GluN1 LBD mutant with GluN3 subunits produces concentration-response data that can be fitted to the Hill equation and allows determination of glycine EC₅₀-values. The glycine EC₅₀-values for GluN3 subunits

co-expressed with GluN1(F484A/T518L) are about 50–100-fold less potent compared to the glycine EC₅₀ at GluN1/N2A receptor (EC₅₀ = 1.1 μM, (Hansen et al., 2012)).

Robust determination of EC₅₀-values relies on the ability to determine the maximal response of the receptor. The double mutant GluN1(F484A/T518L)/N3 receptors that we report here, completely abolish GluN1-mediated inhibition and the receptors reach a plateau of maximal response that is unchanged, even at supersaturating glycine concentrations. Single mutant GluN1(F484A)/N3 receptors do not allow unambiguous determination of glycine EC₅₀-values due to the bell-shaped glycine concentration-response curve and the unreliable estimation of the maximal response to glycine. Nonetheless, the estimated glycine EC₅₀-values of the bell-shaped curves from GluN1(F484A)/N3 and GluN1(T518L)/N3 receptors are in the same range (< 2-fold difference) as the glycine EC₅₀-values determined for the double mutant GluN1(F484A/T518L)/N3 receptors. Furthermore, the estimated glycine EC₅₀-values are similar to estimated EC₅₀-values reported from the previously published study on GluN1(F484A)/N3 receptors (Madry et al., 2007).

4.2 Structure-based discovery and mechanism of inhibition of the novel GluN3-antagonists

We performed a virtual screen of the GluN3A LBD that exploited the structural differences of agonist binding sites in GluN1 and GluN3 subunits to achieve selectivity for the GluN3 subunit. The competitive mechanism of the novel antagonist (TK80) was expected as the compound was found in a virtual screen of the orthosteric ligand-binding pocket of a GluN3 subunit. TK80 is remarkably selective for GluN3B over GluN3A. This selectivity profile provides strong evidence for the possibility of developing compounds selective for either GluN3A or GluN3B. There was no expectation that the virtual screening should discover ligands discriminating between GluN3A and GluN3B subunits as the backbone conformation and binding pocket shape and environment are almost identical in the GluN3:D-serine complexes. Our evaluation of compound activity does not rule out the possibility that part of the inhibition of GluN1/N2 receptors observed for some compounds can be attributed to binding to the GluN2 subunit rather than the GluN1 subunit. However, since a saturating concentration of glutamate (100 μM) was used, which is 30- to 200-fold higher than glutamate EC₅₀ at the respective GluN1/N2 receptor subtypes (Erreger et al., 2007), it is unlikely that the compounds act as competitive antagonist at the glutamate binding site of the GluN2 subunit.

The identification of two novel non-competitive antagonists (TK13 and TK30) was surprising considering the method by which they were identified. A non-competitive mechanism of inhibition indicates that these antagonists may not bind in the orthosteric ligand-binding pocket, and were therefore not expected to be identified from a virtual screen of the orthosteric ligand-binding pocket. A potential caveat could be that TK13 and TK30 interact with the mutated GluN1 subunit. However, the two non-competitive compounds have similar IC₅₀-values at the single mutant GluN1(F484A)/N3A and GluN1(T518L)/N3A receptors compared to the double mutant GluN1(F484A/T518L)/N3A receptor, and it is therefore highly unlikely that TK13 and TK30 inhibits through binding to the mutated agonist binding site of the GluN1 subunit, furthermore both TK13 and TK30 display inhibition of wild-type GluN1/N3B receptors. By contrast, the TK67 compound showed profound sensitivity for mutations in the GluN1 LBD indicating that TK67 potentially interacts with the mutated GluN1 subunit and affects receptor function through binding to the mutated GluN1 subunit.

The novel GluN3-antagonists identified in this study display inhibition of wild-type GluN1/N3B receptors comparable to the inhibition observed at the double mutant GluN1(F484A/T518L)/N3 receptors. Effects at wild-type GluN1/N3B receptors, independent of mutated

GluN1 subunit, provide evidence for the potential use of GluN3-antagonists as pharmacological tools to study neuronal GluN3-containing NMDA receptors.

5 Conclusion

To date, the physiological role of GluN3-containing receptors remains largely unknown. The lack of useful pharmacological tools, such as GluN3-selective agonists or antagonists, has slowed down our progress to understand the physiological roles of GluN3-containing receptors. Generation of selective GluN3 ligands together with studies on GluN3 pharmacology and functional properties have largely been hampered by the fundamental problem of investigating GluN3 function in isolation. By the discovery of a double mutant GluN1 LBD, that completely eliminates the GluN1-mediated glycine inhibition of GluN1/N3 receptors, this study has proposed a method to study the activity of the GluN3 subunit in isolation. In addition, the present discovery of the competitive antagonist TK80 with preference for the GluN3B subunit supports the idea that the structural differences between GluN1 and GluN3 subunits can be exploited to generate GluN3-selective ligands. Consequently, the findings presented in this study provide a clear rationale for future efforts to develop GluN3-selective ligands that can be used as pharmacological tools or potentially for therapeutic gain.

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Research Highlights

- A method to study pharmacology of GluN3 subunit in GluN1/N3 receptors was developed
- A virtual screen of the orthosteric binding site of GluN3A
- 99 selected compounds were evaluated for GluN3 activity and selectivity
- We identified a novel competitive antagonist with preference for the GluN3B subunit
- Two non-competitive antagonists with preference for GluN3 subunits were identified

Fig. 1. Concentration-response data for glycine at NMDA receptors with wild type or mutated GluN1 subunits. A–C, concentration-response data for glycine at GluN1/N2A (A), GluN1/N3A (B), and GluN1/N3B (C) receptors. Receptors were expressed in *Xenopus* oocytes and current responses recorded from two-electrode voltage-clamp electrophysiology. Data are mean $\pm$ S.E.M. from 6–28 oocytes. EC₅₀-values and parameters for bell-shaped fitting are listed in Table 1 and Table 2. D, representative two-electrode voltage-clamp recording of responses from GluN1/N3A (top), GluN1(F484A)/N3A (upper middle), GluN1(T518L)/N3A (lower middle), and GluN1(F484A/T518L)/N3A (bottom) receptors to increasing concentration of glycine.

Fig. 2.

Co-expression of GluN1(F484A/T518L) and GluN3A or GluN3B subunits results in surface expression of functional receptors in *Xenopus* oocytes. A–F, representative two-electrode voltage-clamp recordings of current responses to application of 10 μ M, 100 μ M, and 1000 μ M glycine to *Xenopus* oocytes co-injected with GluN1(F484A/T518L) and GluN3A (A) or GluN3B (B), and to *Xenopus* oocytes injected with the individual subunits alone: GluN1 (C), GluN1(F484A/T518L) (D), GluN3A (E), or GluN3B (F). Data are from 10–13 oocytes from 3 different batches of oocytes.

Fig. 3.

The LBD of GluN1 and GluN3A subunits – crystal structures and homology model. A and B, crystal structures of the agonist binding pockets of the GluN1 and GluN3A LBDs in complex with D-serine (PDB: 1PB8 and PDB: 2RCB, respectively) (Furukawa and Gouaux, 2003; Yao et al., 2008). Carbon atoms of D-serine are shown in yellow and residues that interact with D-serine in the binding pocket are shown as sticks. Black dashes indicate polar interactions between D-serine and residues in the binding pockets. C, crystal structure of the GluN1 LBD in a closed conformation with the agonist D-serine bound (PDB: 1PB8) (Furukawa and Gouaux, 2003) (top-left), crystal structure of the GluN1 LBD in an open conformation with the antagonist DCKA bound (PDB: 1PBQ) (Furukawa and Gouaux, 2003) (top-right), crystal structure of the GluN3A LBD in a closed conformation with the

agonist D-serine bound (PDB: 2RCB) (Yao et al., 2008) (bottom-left), model of the GluN3A LBD in an open conformation (bottom-right).

Fig. 4.

Hit identification in the antagonist screen of library compounds. A, 99 compounds from the virtual screen were examined for antagonistic activity at GluN1(F484A/T518L)/N3A and GluN1(F484A/T518L)/N3B receptors expressed in *Xenopus* oocytes. Responses to co-application of 100 μ M test compounds and 100 μ M glycine were recorded using two-electrode voltage-clamp recordings. Data are given as percentage of control response (100 μ M glycine) in the same recording and are averaged from two oocytes. Compounds displaying > 25% inhibition of the maximal response were classified as hits and are highlighted in red. B, structure of the 8 identified hits.

Fig. 5. Inhibition data for TK13, TK30, and TK80. **A**, representative two-electrode voltage-clamp recording of responses from GluN1(F484A/T518L)/N3A (left) and GluN1(F484A/T518L)/N3B (right) receptors expressed in *Xenopus* oocytes showing inhibition by increasing concentration of TK80 in the continuous presence of 100 μM glycine at the GluN1(F484A/T518L)/N3B receptor. No inhibition was observed at the GluN1(F484A/T518L)/N3A receptor. **B–D**, concentration-response data for TK13 (**B**), TK30 (**C**), and TK80 (**D**) at receptors determined by two-electrode voltage-clamp recordings. Data at GluA1 and GluK2 receptors are presented as percent of 30 μM glutamate response in 300 μM of the compound. Data are mean ± S.E.M. from 3–9 oocytes. IC_{50} -values are listed in Table 3 and Table 4. The compounds were co-applied with 100 μM glycine at GluN1(F484A/T518L)/N3 receptors, with 100 μM glutamate and 0.5 μM glycine at GluN1/N2 receptors, and with 30

μ M glutamate at GluA1 and GluK2 receptors. Oocytes expressing GluK2 were incubated for 5 min. in 10 μ M concanavalin A.

Fig. 6.

Mechanism of inhibition for TK13, TK30, and TK80 at GluN3-containing NMDA receptors. A, representative two-electrode voltage-clamp recording of response from GluN1(F484A/T518L)/N3A receptors expressed in *Xenopus* oocytes showing that increasing the concentration of glycine from 100 μ M to 3000 μ M did not reduce the extent of inhibition by 100 μ M TK13. B, representative two-electrode voltage-clamp recording of response from GluN1(F484A/T518L)/N3B receptors expressed in *Xenopus* oocytes showing that increasing the concentration of glycine from 100 μ M to 3000 μ M surmounted inhibition by 100 μ M TK80. C, bar graph showing inhibition of responses to 100 μ M glycine or 3000 μ M glycine at GluN1(F484A/T518L)/N3A receptors by 100 μ M TK13 and 30 μ M TK30, and at GluN1(F484A/T518L)/N3B receptors by 100 μ M TK80. Data are shown as percentage of the response to 100 μ M or 3000 μ M glycine, respectively, and are mean $\pm$ S.E.M. from 4–7 oocytes. *, $P < 0.05$ (Student's t test). D–F, glycine concentration-response curves in the absence (0 μ M) and presence of increasing concentrations of TK13 (D), TK30 (E), and TK80 (F) at GluN1(F484A/T518L)/N3A or GluN1(F484A/T518L)/N3B receptors. EC₅₀-values and maximal responses are listed in Table 5. Data are from 4–6 oocytes.

Fig. 7.

Mechanism of inhibition for TK13, TK30, and TK80 at GluN3-containing NMDA receptors. A–C, bar graph and current-voltage relationship curves showing inhibition of GluN1(F484A/T518L)/N3A responses at membrane potentials of -60 mV to $+30$ mV by 100 μM TK13 (A) and 30 μM TK30 (B), and inhibition of GluN1(F484A/T518L)/N3B responses at membrane potentials of -60 mV and $+30$ mV by 100 μM TK80 (C). Data are shown as percentage of the response to 100 μM glycine (control) and are mean $\pm$ S.E.M. from 4–5 oocytes. The current-voltage relationship curves are normalized to the response to 100 μM glycine at -60 mV. $P > 0.05$ (Student's t test).

Fig. 8.

Effects of TK13, TK30, and TK80 at wild-type GluN1/N3B receptors. A–C, representative two-electrode voltage-clamp recordings of responses from GluN1/N3B receptors expressed in *Xenopus* oocytes showing inhibition of 10 μM glycine-activated current response by 100 μM TK13 (A), 30 μM TK30 (B), and 100 μM TK80 (C). D, bar graph showing inhibition of responses to 10 μM glycine at GluN1/N3B receptors by 100 μM TK13, 30 μM TK30, and by 100 μM TK80. Data are mean ± S.E.M. from 6–8 oocytes.

Table 1

Concentration-response data for glycine at GluN1/N2 receptors with wild type or mutated GluN1 subunits. EC₅₀ (pEC₅₀ ± S.E.M.) and Hill coefficient (*n_H*) are determined by two-electrode voltage-clamp recordings from *Xenopus* oocytes expressing the indicated recombinant receptors. *N* is the number of oocytes. Glycine was co-applied with 100 μM glutamate. N.E., no significant effect at 30 mM glycine.

	EC ₅₀ (pEC ₅₀ ± S.E.M.) [μM]	<i>n_H</i>	<i>N</i>
GluN1/N2A	1.2 (5.9 ± 0.02)	1.48	16
GluN1(A714L)/N2A	27 (4.6 ± 0.05)	1.28	6
GluN1(V689L)/N2A	59 (4.2 ± 0.04)	1.24	5
GluN1(R523A)/N2A	5400 (2.3 ± 0.01)	1.56	5
GluN1(F484A)/N2A	3100 (2.5 ± 0.02)	1.80	6
GluN1(T518L)/N2A	6400 (2.2 ± 0.03)	1.68	6
GluN1(F484A/T518L)/N2A	N.E.		6

Concentration-response data for glycine at GluN1/N3 receptors with one or two mutations in the agonist binding pocket of the GluN1 subunit. Concentration-response data are determined by two-electrode voltage-clamp recordings from *Xenopus* oocytes expressing the indicated recombinant receptor. See Materials and methods section 2.4 for details on data analysis of bell-shaped concentration-response data. *N* is the number of oocytes, *n_H* is the Hill coefficient. IC₅₀ is a measure of the GluN1-dependent inhibitory component. N.E. indicates no effect.

Table 2

	EC ₅₀ (pEC ₅₀ ± S.E.M.) [μM]	<i>n_H</i>	IC ₅₀ (pIC ₅₀) [μM]	<i>n_H</i>	<i>N</i>
GluN1(F484A/T518L)/N3A	57 (4.2 ± 0.01)	1.40	N.E.		28
GluN1(F484A/T518L)/N3B	95 (4.0 ± 0.02)	1.57	N.E.		15
GluN1(F484A)/N3A	100 (4.0) ^a	1.58	800 (3.1) ^a	1.36	12
GluN1(F484A)/N3B	160 (3.8) ^a	1.62	26000 (1.6) ^a	1.31	12
GluN1(T518L)/N3A	32 (4.5) ^a	1.90	370 (3.4) ^a	1.45	13
GluN1(T518L)/N3B	58 (4.2) ^a	1.52	24000 (1.6) ^a	1.00	9

^a Bell-shaped concentration-response data are fitted by global non-linear regression determining shared values for EC₅₀, IC₅₀, and *n_H* that best describes all recorded data, thus no S.E.M. are given.

Table 3

Inhibition data for hit compounds at GluN3-containing receptors.

IC₅₀ (pIC₅₀ ± S.E.M.) and Hill coefficient (*n*_H) are determined by two-electrode voltage-clamp recordings from *Xenopus* oocytes expressing the indicated recombinant receptor. *N* is the number of oocytes. The compounds were co-applied with 100 μM glycine at GluN1(F484A)/T518L/N3 and GluN1(F484A)/N3A receptors and with 50 μM glycine at GluN1(T518L)/N3A receptors. IC₅₀ > 100 or IC₅₀ > 300 indicates that the compound showed some inhibition but less than 50% inhibition at 100 μM and 300 μM, respectively. Maximal inhibition is 100% unless otherwise stated. N.E., no significant effect at 300 μM. N.D., not determined.

	GluN1(F484A)/T518L)/N3A			GluN1(F484A)/T518L)/N3B			GluN1(F484A)/N3A			GluN1(T518L)/N3A		
	IC ₅₀ (pIC ₅₀ ± S.E.M.) [μM]	<i>n</i> _H	<i>N</i>	IC ₅₀ (pIC ₅₀ ± S.E.M.) [μM]	<i>n</i> _H	<i>N</i>	IC ₅₀ (pIC ₅₀ ± S.E.M.) [μM]	<i>n</i> _H	<i>N</i>	IC ₅₀ (pIC ₅₀ ± S.E.M.) [μM]	<i>n</i> _H	<i>N</i>
TK13	67 (4.2 ± 0.06)	-1.04	4	49 (4.3 ± 0.03)	-0.77	4	93 (4.0 ± 0.03)	-1.04	6	100 (4.0 ± 0.05)	-1.02	6
TK30	14 (4.8 ± 0.03)	-1.44	4	7.4 (5.1 ± 0.05)	-1.37	4	34 (4.5 ± 0.08)	-1.16	6	9.8 (5.0 ± 0.02)	-1.50	9
TK31	> 100		4	> 100		5	N.D.			N.D.		
TK40	41 (4.4 ± 0.03)	-1.26	4	10 (5.0 ± 0.04)	-1.39	4	N.D.			N.D.		
TK51	> 300		4	69 (4.2 ± 0.07)	-1.05	5	N.D.			N.D.		
TK67	14 (4.9 ± 0.04)	-1.18	6	67 (4.2 ± 0.11)	-1.07	4	25 (4.6 ± 0.02) ^a	-1.84	6	> 300		9
TK69	> 300		3	85 (4.1 ± 0.10)	-1.33	3	N.D.			N.D.		
TK80	N.E.		5	79 (4.1 ± 0.05)	-1.79	4	N.D.			N.D.		

^aMaximal inhibition is 65%.

Table 4

Inhibition data for hit compounds at GluN1/N2 receptors. IC₅₀ (pIC₅₀ ± S.E.M.) and Hill coefficient (*n_H*) are determined by two-electrode voltage-clamp recordings from *Xenopus* oocytes expressing the indicated recombinant receptor. *N* is the number of oocytes. The compounds were co-applied with 100 μM glutamate and 0.5 μM glycine. IC₅₀ > 300 indicates that the compound showed some inhibition but less than 50% inhibition at 300 μM. Maximal inhibition is 100%. N.D., not determined.

	GluN1/N2A				GluN1/N2B				GluN1/N2C				GluN1/N2D			
	IC ₅₀ (pIC ₅₀ ± S.E.M.) [μM]	n _H	N	IC ₅₀ (pIC ₅₀ ± S.E.M.) [μM]	n _H	N	IC ₅₀ (pIC ₅₀ ± S.E.M.) [μM]	n _H	N	IC ₅₀ (pIC ₅₀ ± S.E.M.) [μM]	n _H	N	IC ₅₀ (pIC ₅₀ ± S.E.M.) [μM]	n _H	N	
TK13	> 300		5	> 300		4	> 300		4	> 300		3	> 300		3	
TK30	270 (3.6 ± 0.06)	-1.39	9	> 300		3	120 (3.9 ± 0.07)	-1.77	5	90 (4.1 ± 0.03)	-1.83	6				
TK31	> 100		4	N.D.			N.D.			N.D.			N.D.			
TK40	0.051 (7.3 ± 0.02)	-1.48	8	N.D.			N.D.			N.D.			N.D.			
TK51	3.3 (5.5 ± 0.02)	-1.20	6	N.D.			N.D.			N.D.			N.D.			
TK67	> 300		5	N.D.			N.D.			N.D.			N.D.			
TK69	41 (4.4 ± 0.02)	-1.09	5	N.D.			N.D.			N.D.			N.D.			
TK80	> 300		5	> 300		6	> 300		4	> 300		4	> 300		4	

Effects of TK13, TK30, and TK80 on the EC₅₀-value and maximal response to glycine at GluN1(F484A/T518L)/N3 receptors. EC₅₀ (pEC₅₀ ± S.E.M.) and maximal response (R_{max}) are determined by two-electrode voltage-clamp recordings from *Xenopus* oocytes expressing the indicated recombinant receptors. *N* is the number of oocytes.

Table 5

Glycine + inhibitor at GluN1(F484A/T518L)/N3A				
	EC ₅₀ (pEC ₅₀ ± S.E.M.) [μM]	R _{max} ± S.E.M. ^a [%]		<i>N</i>
30 μM TK13	65 (4.2 ± 0.03)	85 ± 2.1		5
100 μM TK13	67 (4.2 ± 0.02)	54 ± 2.2		6
30 μM TK30	58 (4.2 ± 0.02)	55 ± 5.6		5
100 μM TK30	62 (4.2 ± 0.04)	28 ± 7.8		4
Glycine + inhibitor at GluN1(F484A/T518L)/N3B				
	EC ₅₀ (pEC ₅₀ ± S.E.M.) [μM]	R _{max} ± S.E.M. ^a [%]		<i>N</i>
100 μM TK80	190 (3.7 ± 0.01)	99 ± 2.3		5
300 μM TK80	420 (3.4 ± 0.05)	100 ± 1.5		6

^aR_{max} for the glycine concentration response curve in the absence of inhibitor is set at 100%.