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Pyridine bioisosteres of potent GluN2B subunit containing NMDA receptor antagonists with benzo[7]annulene scaffold

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Abstract

It has been reported that benzo[7]annulen-7-amines bearing electron withdrawing substituents such as **3d** with a 2-Cl or **3e** with a 2-NO_2 moiety show very high affinity towards the ifenprodil binding site of GluN2B subunit containing NMDA receptors. Therefore, bioisosteres of **3** with an electron deficient pyridine ring instead of the chloro- or nitrobenzene ring were envisaged. Starting from pyridine-2,3-dicarboxylic acid (**5**) a five-step synthesis of the key intermediate, the ketone **10**, was developed. Reductive amination with various primary amines and $NaBH(OAc)_3$ led to the homologous secondary amines **11a**-**c**. Subsequent methylation yielded the tertiary amines **12b** and **12c**. Receptor binding studies with [^{3}H]ifenprodil revealed K_i-values above 100 nM for the most active phenylpropyl- and phenylbutylamines **11b** and **11c**. The >100-fold reduced GluN2B affinity of pyridines **11b** and **11c** compared to the GluN2B affinity of the corresponding chloro- and nitrobenzene derivatives **3d** and **3e**

indicates that the pyridine ring is not tolerated as bioisosteric replacement of the chloro- or nitrobenzene ring in this type of compounds.

Keywords: NMDA receptor, GluN2B antagonists, ifenprodil binding site, affinity, selectivity, structure-affinity relationships, [7]annuleno[b]pyridin-7-amines; pyridine-benzene bioisosteres

1. Introduction

Glutamate receptors are transmembrane proteins in neurons binding specifically the excitatory neurotransmitter (*S*)-glutamate. They are classified in metabotropic and ionotropic glutamate receptors. Whereas metabotropic glutamate receptors belong to the class of G-protein coupled receptors inducing or inhibiting metabolic processes, ionotropic glutamate receptors are ligand-gated ion channels controlling the flow of cations (Na^+, K^+. Ca^{2+}) across the cell membrane. The group of ionotropic glutamate receptors consists of three receptor types: the 2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl)propanoic acid (AMPA), the kainate and the *N*-methyl-D-aspartate (NMDA) receptor. [1-4]

The NMDA receptor represents a ligand-gated and voltage dependent ion channel. In addition to the presence of the physiological agonist (*S*)-glutamate, activation of the NMDA receptor requires the presence of the coagonist glycine and depolarization of the surrounding membrane to remove the Mg^{2+} block. This is necessary, since at the resting potential the NMDA receptor associated ion channel is blocked by Mg^{2+}-ions.

Depolarization of the surrounding membrane can be achieved by glutamate-induced opening of AMPA and/or kainate receptors in the membrane. [1-4]

Upon activation of the NMDA receptor, Ca^{2+}- and Na^{+}-ions can penetrate into the neuron, whilst K^{+}-ions can leave the cell. The high Ca^{2+}-ion permeability represents a further characteristic feature of the NMDA receptor playing an important role in many physiological functions of the central nervous system like memory and learning. However, overactivation of the NMDA receptor leading to an excessive influx of Ca^{2+}-ions into the neuron is associated with the process of excitotoxicity resulting in apoptosis of the neuron.[3,4]

The NMDA receptor consists of four subunits, which form a heterotetramer. For humans, seven subunits are known, which are termed GluN1, GluN2A–D and GluN3A-B. The (*S*)-glutamate binding site is localized on GluN2 subunits and the glycine binding site on GluN1 and GluN3 subunits. Since GluN3 subunits are predominantly expressed in the embryonic brain, a functional NMDA receptor usually consists of two GluN1 and two GluN2 subunits. [5-7]

A single subunit of the NMDA receptor is subdivided into four domains. The C-terminal domain (CTD) is located on the intracellular site of the membrane and connects the receptor with the cytoskeleton. The transmembrane domain (TMD) is composed of four α-helices forming the channel pore and anchoring the receptor into the membrane. The TMD contains the binding site for open channel blockers, which is termed PCP binding site according to the prototypical ligand phencyclidine (1-(1-phjenylcyclohexyl)piperidine, PCP). In order to reach this binding site the ion channel has to be opened. Therefore, compounds interacting with this binding site (e.g.

ketamine, memantine, amantadine, MK-801) are termed open-channel blockers. [6] The ligand binding domain (LBD) for the agonists glutamate (GluN2 subunit) and glycine (GluN1 subunit) is located on the extracellular site. The extracellularly located N-terminal domain (NTD) far away from the ion channel pore contains binding sites for several non-competitive positive and negative allosteric modulators including polyamines, ifenprodil, Zn^{2+}-ions, NO and protons. [6,7] The ifenprodil binding site, named after the negative allosteric modulator ifenprodil, is found exclusively on the GluN2B subunit. Therefore, compounds interacting with the ifenprodil binding site can only modulate the opening state of GluN2B subunit containing NMDA receptors. As a consequence, high selectivity is achieved in the sense that only those NMDA receptors containing the GluN2B subunit can be modulated by ifenprodil-like compounds. [8]

Recently, X-ray crystal structure analyses have shown that the ifenprodil binding site is located at the interface between the GluN1 and GluN2B subunit. [9-12] Negative allosteric modulators interacting selectively with the ifenprodil binding site have a potential for the treatment of neurodegenerative and neurological diseases. In particular, negative allosteric modulators of GluN2B-NMDA receptors could be exploited for the treatment of depression, cerebral ischemia, stroke, Parkinson's, Alzheimer's and Huntington's disease. [7, 13-17]

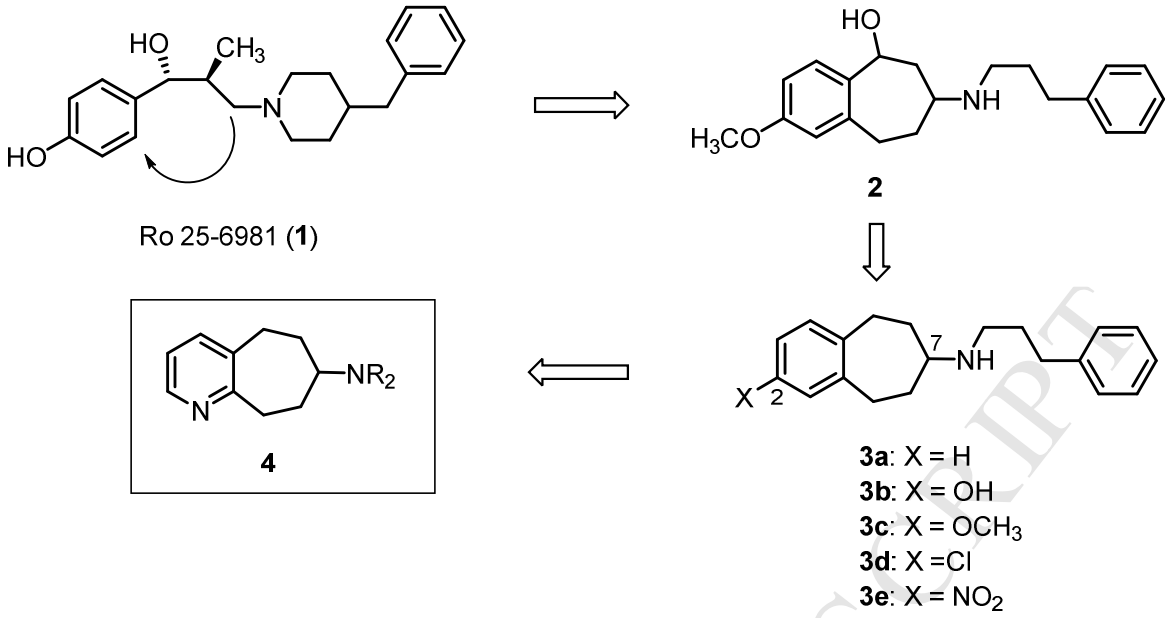


Figure 1: Development of pyridine based GluN2B antagonists.

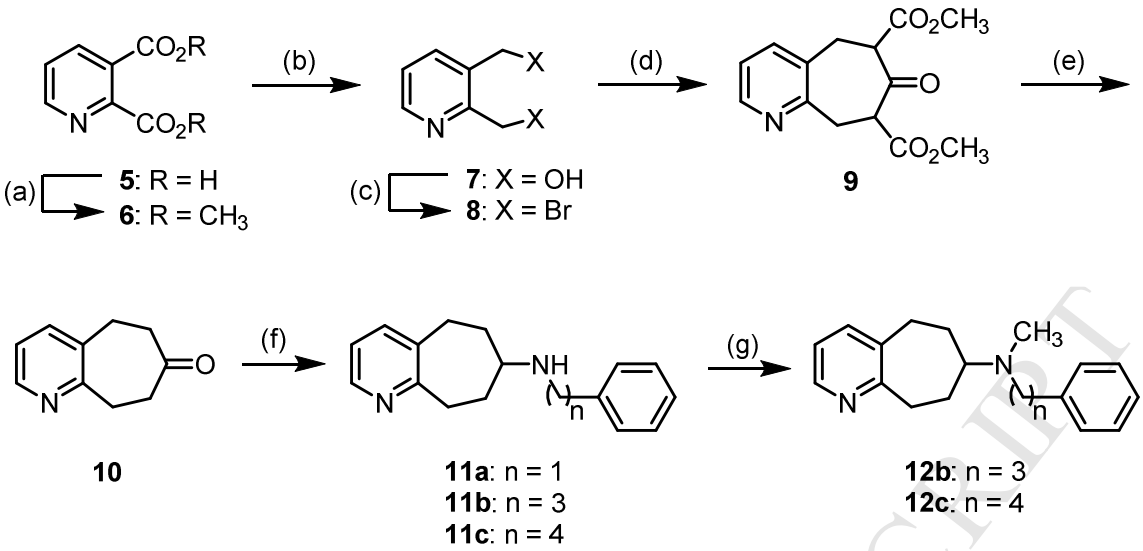
In order to develop novel high-affinity and selective negative allosteric modulators interacting with the ifenprodil binding site, the phenylpropylamine Ro 25-6981 (**1**) [18,19] served as lead compound in this project. (Figure 1) Formal connection of the methylene moiety adjacent to the N-atom of the piperidine ring with the benzene ring led to the benzo[7]annulen-7-amines **2** showing high GluN2B affinity (cis: K_i = 16 nM), high selectivity over related receptors, and cytoprotective activity (cis: IC_{50} = 12 nM). [20] Removal of the benzylic OH moiety and introduction of various substituents in 2-position resulted in high-affinity GluN2B ligands. Whereas electron releasing OH (**3b**) and OCH_3 (**3c**) moiety resulted in compounds with high GluN2B affinity ((**3a**: K_i = 16 nM; **3b**: K_i = 28 nM; **3c**: K_i = 10 nM), electron withdrawing substituents were able to further increase the GluN2B affinity in the low single-digit nanomolar range (**3d**: K_i = 2.1 nM; **3e**: K_i = 1.6 nM). [21,22] Since the substituents Cl in **3d** and NO_2 in **3e** have quite different properties, the idea came up that an electron deficient aromatic system is responsible for the high GluN2B affinity. In order to prove this hypothesis, the bioisosteric replacement of the benzene ring of **3** by an electron-deficient pyridine

ring was envisaged. Moreover, a pyridine ring would avoid the NO_2 moiety, which could lead to the formation of toxic metabolites *in vivo*. Herein, we report on the synthesis and pharmacological evaluation of pyridine bioisosteric GluN2B ligands of type **4**. (Figure 1)

2. Results and discussion

2.1. Synthesis

For the synthesis of pyridine derivatives of type **4**, ketone **10** should serve as key building block. The synthesis of **10** was accomplished in five steps starting with quinolinic acid **5**. Since initial attempts to reduce the diacid **5** with $LiAlH_4$ failed to yield the diol **7**, the diacid **5** was converted into the dimethyl ester **6** by a Fischer esterification with methanol and concentrated H_2SO_4. [23] (Scheme 1) Then, the diester **6** was reduced with $NaBH_4$ to give the diol **7** in 54 % yield. [23] The dibromide **8** was obtained by reaction of diol **7** with PBr_3. In order to avoid decomposition of the highly reactive and unstable dibromide **8**, fast work-up was performed and the product was directly used in the next reaction step. For the establishment of the [7]annulene ring in the oxodiester **9**, two subsequent S_N2 reactions of dimethyl 3-oxoglutarate and dibromide **8** were performed. [24] After purification, the exact spectroscopic analysis of oxodiester **9** turned out to be rather complex due to the existence of a mixture of four enantiomeric pairs of isomers (two pairs of keto-enol tautomers, two pairs of diastereomers). Therefore, the oxodiester **9** was hydrolyzed and decarboxylated with HCl to provide the ketone **10**. The ketone **10** was purified by reversed-phase column chromatography, since considerable decomposition was observed during normal phase silica gel chromatography.



Scheme 1: Synthesis of the ketone **10**. Reagents and reaction conditions: (a) MeOH, H_2SO_4, reflux, 16 h, 75 %. (b) $NaBH_4$, EtOH, -15 °C to reflux, 2 h, 54 %. (c) PBr_3, THF, 0 °C to rt, 1 h. (d) Dimethyl 3-oxoglutarate, K_2CO_3, KI, THF, reflux, 16 h. (e) 6 M HCl, 95 °C, 4 h, 11 % over 3 steps. (f) $Ph(CH_2)_nNH_2$, $NaBH(OAc)_3$, CH_2Cl_2, rt, 3 h, 22-52 %. (g) Formalin, $NaBH(OAc)_3$, CH_2Cl_2, rt, 3 h, 23-32 %.

Reductive amination of ketone **10** with homologous phenylalkylamines and $NaBH(OAc)_3$ [25] led to the secondary amines 1**1a**-**c**. (Scheme 1) The secondary amines **11b** and **11c** were converted into the tertiary amines **12b** and **12c** by reductive methylation with formaldehyde and $NaBH(OAc)_3$.

Some transformations gave only low yields of products. However, the low yields are due to purification problems of the rather polar pyridine derivatives and our high requirements regarding the purity of the compounds for the *in vitro* binding assays.

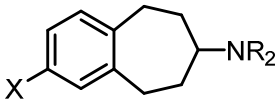
2.2. Pharmacological evaluation

2.2.1. GluN2B affinity

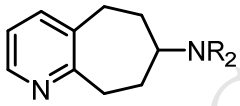
In order to determine the affinity of the synthesized compounds towards GluN2B subunit containing NMDA receptors, competitive binding assays with the radioligand

[^{3}H]ifenprodil were performed. [26] Cell membrane preparations from stably transfected mouse fibroblast cells (L(tk-) cells) were used as receptor source. The results are summarized in Table 1.

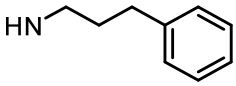
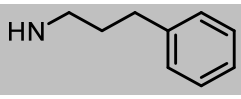
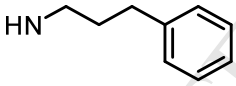
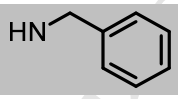
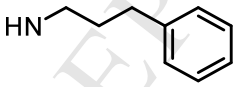
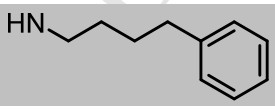
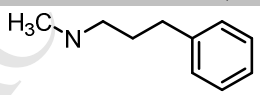
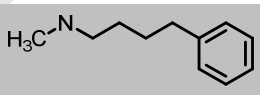
Table 1. Receptor affinity of the pyridine derivatives **11** and **12** compared with the affinity of lead and reference compounds.



3



11,12

Compound	NR_2	K_i ± SEM [nM]			
		GluN2B	PCP	σ_1	σ_2
3b[22]		28 ± 5.0	0 %	123	32 ± 13
3d[22]		2.1 ± 1.2	0 %	18 ± 7.0	7.4 ± 1.6
3e[22]		1.6 ± 0.9	3 %	18 ± 5	11 ± 4
11a		>1000	0 %	942	490
11b		302 ± 69	10 %	992	276
11c		149 ± 30	8 %	214	68 ± 13
12b		445 ± 123	0 %	261	68 ± 15
12c		477 ± 64	5 %	263	115 ± 14
Ifenprodil		10 ± 0.7	-	125 ± 24	98 ± 34

Data for compounds with K_i values higher than 100 nM in the first test were not repeated. For very low affinity compounds only inhibition (in %) of radioligand binding at a test compound concentration of 1 µM (i.e. IC_{50} > 1 µM) is given.

The phenylbutylamine **11c** reveals the highest affinity towards the ifenprodil binding site with a K_i value of 149 nM. The GluN2B affinity of **11b** with a shorter phenylpropylamine side chain is slightly reduced (K_i = 302 nM), whereas the benzylamine **11a** with only one methylene moiety between the phenyl ring and the amino moiety shows considerably lower GluN2B affinity (K_i > 1000 nM). These results are in good accordance to the structure-affinity relationships obtained for the benzene analogous lead compounds of type **3** in which a phenylpropyl or phenylbutyl side chain represented the optimum chain length for high GluN2B affinity. Conversion of the secondary amines **11b** and **11c** into tertiary methylamines **12b** and **12c** resulted in a slightly reduced GluN2B affinity.

The loss of GluN2B affinity of the pyridine derivatives **11b** and **11c** compared to the GluN2B affinity of the corresponding substituted benzene derivatives **3d** (2-Cl) and **3e** (2-NO_2) clearly indicates that despite the reduced electron density the pyridine ring is not an appropriate bioisosteric replacement of the chlorobenzene (**3d**) and nitrobenzene moiety (**3e**). This result was surprising as the pyridine ring can also serve as an H-bond acceptor. In the hydrogen bond basicity scale the pyridine ring is given with a pK_{BHX} value of 1.86. [27] It is speculated that either the wrong position of the N-atom in the pyridine ring or the rather polar properties of the pyridine ring itself might be responsible for the reduced GluN2B affinity.

2.2.2. Receptor selectivity

In order to test, whether the pyridine derivatives **11** and **12** also interact with the PCP binding site within the channel pore of the NMDA receptor, the affinity towards this binding site was evaluated in radioligand receptor binding studies as well. [28,29] Table 1 clearly shows that the pyridine derivatives **11** and **12** did not interact with the

PCP binding site even at the rather high test compound concentration of 1 µM. This result correlates nicely with the negligible PCP affinity of the corresponding benzene derivatives **3**.

It has been reported that ifenprodil and similar GluN2B ligands also interact with σ_1 and σ_2 receptors. Therefore, the σ_1 and σ_2 receptor affinities of **11** and **12** were recorded in radioligand receptor binding studies as well. [30-32] According to the data given in Table 1, the σ_1 affinity of the pyridine derivatives **11** and **12** is in the same range as their GluN2B affinity. However, the σ_2 affinity often exceeds the GluN2B affinity indicating a slight preference for binding at σ_2 receptors over GluN2B receptors. The substituted benzo[7]annulene derivatives **3** show also rather high σ_2 affinity, but the GluN2B affinity is always higher than the σ_2 affinity. Replacement of the nitrobenzene or chlorobenzene moiety of potent GluN2B ligands **3d** and **3e** by a pyridine ring shifts the receptor profile towards preferred σ_2 affinity. The changed ratio of GluN2B : σ_2 receptor interaction of the pyridine derivatives **11** and **12** can be attributed to the considerably reduced GluN2B affinity.

3. Conclusion

In order to prove the hypothesis of beneficial effects of electron deficient aromatic systems on GluN2B affinity, a set of pyridine annulated [7]annulenes was prepared. Bioisosteric replacement of the nitrobenzene moiety of **3e** by the pyridine ring in **11b** led to 200-fold reduced GluN2B affinity. Therefore, it was concluded that an electron deficient aromatic system is not alone responsible for the high GluN2B affinity of the nitrobenzene derivative **3e**. Polar interactions of the NO_2 moiety with the receptor protein, in particular the establishment of an H-bond network with the conserved

water molecule in the binding pocket, is responsible for the very high GluN2B affinity of **3e**.[20] Obviously, a similar H-bond network cannot be formed by the pyridine analogs **11** and **12**.

4. Experimental Part

4.1. Chemistry, General methods

Oxygen and moisture sensitive reactions were carried out under nitrogen, dried with silica gel with moisture indicator (orange gel, VWR, Darmstadt, Germany) and in glassware (Schlenk flask or Schlenk tube). Temperatures were controlled with dry ice and NaCl (-15 °C), ice/water (0 °C) and magnetic stirrer MR 3001 K (Heidolph, Schwalbach, Germany) or RCT CL (IKA, Staufen, Germany), together with temperature controller EKT HeiCon (Heidolph) or VT-5 (VWR) and PEG or silicone bath. All solvents were of analytical or technical grade quality. Demineralized water was used. CH_2Cl_2 was distilled from CaH_2; THF was distilled from sodium/benzophenone; MeOH was distilled from magnesium methanolate. Thin layer chromatography (tlc): tlc silica gel 60 F_{254} on aluminum sheets (VWR). Flash chromatography (fc): Silica gel 60, 40–63 µm (VWR), silica 60 RP – 18 (EM Industries Inc.); parentheses include: diameter of the column (d), length of the stationary phase (l), fraction size (V) and eluent. Automated flash chromatography: Isolera™ Spektra One (Biotage®); parentheses include: cartridge size, flow rate, eluent, fractions size was always 20 mL. Melting point: Melting point system MP50 (Mettler Toledo, Gießen, Germany), open capillary, uncorrected. MS: MicroTOFQII mass spectrometer (Bruker Daltonics, Bremen, Germany); deviations of the found exact masses from the calculated exact masses were 5 mDa or less; the data were analyzed with DataAnalysis® (Bruker Daltonics). NMR: NMR spectra were recorded in deuterated solvents on Agilent DD2 400 MHz and 600 MHz spectrometers

(Agilent, Santa Clara CA, USA); chemical shifts (δ) are reported in parts per million (ppm) against the reference substance tetramethylsilane and calculated using the solvent residual peak of the undeuterated solvent; coupling constants are given with 0.5 Hz resolution; assignment of ^{1}H and ^{13}C NMR signals was supported by 2-D NMR techniques where necessary.IR: FT/IR IR Affinity®-1 spectrometer (Shimadzu, Düsseldorf, Germany) using ATR technique.

4.2. HPLC method for the determination of the purity

Pump: LPG-3400SD, degasser: DG-1210, autosampler: ACC-3000T, UV-detector: VWD-3400RS, interface: DIONEX UltiMate 3000, data acquisition: Chromeleon 7 (equipment and software from Thermo Fisher Scientific, Lauenstadt, Germany); column: LiChrospher® 60 RP-select B (5 µm), LiChroCART® 250-4 mm cartridge; flow rate: 1.0 mL/min; injection volume: 5.0 µL; detection at λ = 210 nm; solvents: A: demineralized water with 0.05 % (V/V) trifluoroacetic acid, B: CH_3CN with 0.05 % (V/V) trifluoroacetic acid; gradient elution (% A): 0 - 4 min: 90 %; 4 - 29 min: gradient from 90 % to 0 %; 29 - 31 min: 0 %; 31 - 31.5 min: gradient from 0 % to 90 %; 31.5 - 40 min: 90 %.

4.3. Synthetic procedures

4.3.1. Dimethyl pyridine-2,3-dicarboxylate (6)

Quinolinic acid (**5**, 15.6 g, 94.0 mmol, 1.00 eq.) was dissolved in MeOH (100 ml) and concentrated H_2SO_4 (10 ml) was added dropwise. The reaction mixture was heated to reflux for 16 h. H_2O (80 ml) was added and the mixture was adjusted to pH 8 by addition of NaOH platelets and extracted with CH_2Cl_2 (3x100ml). The combined organic layers were dried (anhydr. Na_2SO_4) and the solvent was removed in vacuo. Yellow solid, yield 13.8 g (75 %). $C_9H_9NO_4$ (195.2 g/mol). Mp = 55 °C. ^{1}H NMR (600

MHz, CD_3OD): δ = 3.96 (*s*, 3H, C*H*$_3$), 3.99 (*s*, 3H, C*H*$_3$), 7.70 (*dd*, *J* = 8.0, 4.9 Hz, 1H, 5-*H*), 8.35 (*dd*, *J* = 8.0, 1.6 Hz, 1H, 4-*H*), 8.78 (*dd*, *J* = 4.9, 1.6 Hz, 1H, 6-H). ^{13}C NMR (151 MHz, CD_3OD): δ = 55.1 (1C, CO_2CH_3), 55.5 (1C, CO_2CH_3), 126.7 (1C, 5-*C*), 127.5 (1C, 4-*C*), 139.4 (1C, 3-*C*), 152.0 (1C, 6-*C*), 152.9 (1C, 2-C), 168.2 (1C, *C*=O), 166.9 (1C, *C*=O). Exact MS (APCI): m/z = 196.0609 (calcd. 196.0604 for $C_9H_{10}NO_4^+$ [M+H$^+$]).

4.3.2. Pyridine-2,3-dimethanol (7)

Diester **6** (250 mg, 1.3 mmol, 1.0 eq.) was dissolved in EtOH (10 ml) and $NaBH_4$ (240 mg, 6.40 mmol, 5.00 eq.) was added portion wise at -10 °C. The resulting slurry was heated to reflux for 2 h and then MeOH (10 ml) and H_2O (1 ml) were added. The mixture was filtered over Celite® and the filtrate was dried (Na_2SO_4). The solvent was removed in vacuo and the residue was purified via fc (ø = 2 cm, l = 20 cm, V = 10 ml, R_f = 0.75, CH_2Cl_2:MeOH:triethylamine = 15:5:0.1). Colorless oil, yield 96 mg (54 %). $C_7H_9NO_2$ (139.2 g/mol). R_f = 0.75 (CH_2Cl_2:MeOH:triethylamine = 15:5:0.1). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 4.64 (*s*, 2H, 8-C*H*$_2$-OH), 4.73 (*s*, 2H, 7-C*H*$_2$-OH), 7.22 (*dd*, *J* = 7.6, 4.9 Hz, 1H, 5-*H*), 7.72 (*dd*, *J* = 7.6, 4.9 Hz, 1H, 4-*H*), 8.40 (*d*, *J* = 4.9, 1H, 6-*H*). ^{13}C NMR (151 MHz, $CDCl_3$): δ = 62.7 (1C, 8-*C*H$_2$-OH), 65.9 (1C, 7-*C*H$_2$-OH), 121.7 (1C, 5-*C*), 135.7 (1C, 4-*C*), 137.7 (1C, 3-*C*), 145.5(1C, 6-*C*), 161.5 (1C, 2-*C*). Exact MS (APCI): m/z = 140.0700 (calcd. 140.0706 for $C_7H_{10}NO_2^+$ [M+H$^+$]).

4.3.3. 5,6,8,9-Tetrahydro-7*H*-[7]annuleno[*b*]pyridin-7-one (10)

Diol **7** (140 mg, 1.0 mmol, 1.0 eq.) was dissolved in THF (20 ml) and PBr_3 (0.28 ml, 3.0 mmol, 3.0 eq.) was added slowly under ice bath cooling. The reaction mixture was stirred for 1 h at rt and then saturated $NaHCO_3$ solution was

added until pH 8 was adjusted. The layers were separated, and the aqueous layer was extracted with EtOAc (3x20 ml). The combined organic layers were dried over anhydrous Na_2SO_4 and the solvent was removed in vacuo. The residue was dissolved in THF (5 ml) and filtrated over Celite®. The solvent was removed in vacuo. The resulting dibromide **8** (131 mg, 0.5 mmol, 1.0 eq.) was dissolved in THF (10 ml) and K_2CO_3 (205 mg, 1.5 mmol, 3.0 eq.) and KI (4 mg, 0.03 mmol, 0.05 eq.) were added. Dimethyl 3-oxoglutarate (0.86 ml, 0.8 mmol, 1.2 eq.) was added dropwise and the reaction mixture was heated to reflux for 16 h. The slurry was filtered over Celite®. The solvent was removed in vacuo and the residue was purified via fc (ø = 2 cm, l = 27 cm, V = 10 ml, R_f = 0.75, ethyl acetate:triethylamine = 97:3). Oxodiester **9** (75 mg, 0.3 mmol, 1.0 eq.) was dissolved in 6 M HCl (10 ml) and heated up to 95 °C for 4 h. 6 M NaOH (15 ml) was added to the mixture until pH 8 was adjusted and the mixture was extracted with EtOAc (3x20 ml). The solvent was removed in vacuo and the residue was purified via flash column chromatography (ø = 2.5 cm, l = 6 cm, V = 12 ml, H_2O : acetonitrile : trifluoroacetic acid = 80:20:0.1). Colorless oil, yield 18 mg (16 %). $C_{10}H_{11}NO$ (161.2 g/mol). FTIR (neat): $\tilde{\nu}$ (cm^{-1}): 752 ($\delta_{Ar\text{-}H\ (out\ of\ plane)}$), 1697 ($\nu_{C=N}$), 1770 ($\nu_{C=O}$), 2954 ($\nu_{C\text{-}H}$). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 2.63 – 2.66 (*m*, 2H, 6-C*H*$_2$), 2.67 – 2.70 (*m*, 2H, 8-C*H*$_2$), 2.92 - 2.95 (*m*, 2H, 5-C*H*$_2$), 3.15 – 3.19 (*m*, 2H, 9-C*H*$_2$), 7.17 (*dd*, *J* = 7.6, 4.9 Hz, 1H, 3-*H*), 7.51 (*dd*, *J* = 7.6, 1.6 Hz, 1H, 4-*H*), 8.43 (*dd*, *J* = 4.9, 1.6 Hz, 1H, 2-*H*). ^{13}C NMR (151 MHz, $CDCl_3$): δ = 32.6 (9-*C*), 33.3 (1C, 5-*C*), 42.8 (1C, 6-*C*), 45.1 (1C, 8-*C*), 122.5 (1C, 3-*C*), 135.5 (1C, 4a-*C*), 137.2 (1C, 4-*C*), 147.6 (1C, 2-*C*), 160.1 (1C, 9a-*C*), 210.5 (1C, *C*O). Exact MS (APCI): m/z = 162.0918 (calcd. 162.0913 for $C_{10}H_{12}NO^+$ [M+H$^+$]). Purity (HPLC - Method I): 45.7 % (t_R = 8.11 min)

4.3.4. *N*-Benzyl-6,7,8,9-tetrahydro-5*H*-[7]annuleno[b]pyridin-7-amine (11a)

Ketone **10** (34 mg, 0.2 mmol, 1.0 eq.) was dissolved in CH_2Cl_2 (5 ml) and Na_2SO_4 (50 mg) was added. Benzylamine (23 µl, 0.2 mmol, 1.0 eq.) was added and the mixture was stirred for 5 min. $NaBH(OAc)_3$ (58 mg, 0.3 mmol, 1.3 eq.) was added and the mixture was stirred for 3 h. H_2O (0.5 ml) was added and the slurry was filtered. The solvent was removed in vacuo and the residue was purified via fc (ø = 2.5 cm, l = 6 cm, V = 12 ml, H_2O : acetonitrile : trifluoroacetic acid = 80:20:0.1). Yellow oil, 12 mg (22 %). $C_{17}H_{20}N_2$ (252.4 g/mol). FTIR (DMSO): $\tilde{\nu}$ (cm^{-1}): 705, 675 ($\delta_{Ph\text{-}H\ (out\ of\ plane)}$), 1658 ($\nu_{C=N}$), 3473 ($\nu_{N\text{-}H}$). ^1H NMR (400 MHz, $CDCl_3$): δ = 1.42 – 1.49 (*m*, 2H, 6,8-*H*), 2.12 - 2.17 (*m*, 2H, 6,8-*H*), 2.65 – 2.70 (*m*, 1H, 9-*H*), 2.72 – 2.77 (*m*, 2H, Ph-CH_2), 2.85 – 2.91 (*m*, 3H, 5,9-C*H*, C*H*-NH), 3.17 – 3.21 (*m*, 1H, 5-*H*), 7.03 (*dd*, *J* = 7.5, 4.9 Hz, 1H, 3-*H*), 7.25 – 7.27 (*m*, 3H, 3',4'-*H*), 7.33 - 7.34 (*m,* 2H, 2'-*H*), 7.38 (*dd*, *J* = 7.5, 1.7 Hz, 1H, 4-*H*), 8.30 (*dd*, *J* = 4.9, 1.7 Hz, 1H, 2-*H*). ^{13}C NMR (151 MHz, CDCl3): δ = 29.6 (1C, 9-C), 30.7 (1C, 8-C), 32.2 (1C, 6-C), 32.6 (1C, 5-C), 50.6 (1C, Ph-C), 62.7 (1C, CH-N), 121.4 (1C, 3-C), 127.0 (1C, 4'-C), 127.2 (2C, 3'-C), 128.4 (2C, 2'-C), 131.9 (1C, 4-C), 136.3 (1C, 4a-C), 137.1 (1C, 1'-C), 146.4 (1C, 2-C), 162.2 (1C, 9a-C). Exact MS (APCI): m/z = 253.1707 (calcd. 253.1705 for $C_{17}H_{21}N_2^+$ [M+H^+]). Purity (HPLC): 78.8 % (t_R = 6.11 min).

4.3.5. *N*-(3-Phenylpropyl)-6,7,8,9-tetrahydro-5*H*-[7]annuleno[b]pyridin-7-amine (11b)

Ketone **10** (51 mg, 0.3 mmol, 1.0 eq.) was dissolved in CH_2Cl_2 (5 ml) and Na_2SO_4 (50 mg) was added. 4-Phenylbutylamine (47 µl, 0.3 mmol, 1.1 eq.) was added and the mixture was stirred for 5 min. $NaBH(OAc)_3$ (69 mg, 0.3 mmol, 1.1 eq.) was added and the mixture was stirred for 4 h. H_2O (0.5 ml) was added and the slurry was filtered. The solvent was removed in vacuo and the residue was purified via flash

column chromatography (ø = 2.5 cm, l = 6 cm, V = 12 ml, H_2O : acetonitrile : trifluoroacetic acid = 80:20:0.1). Green oil, 47 mg (52 %). $C_{19}H_{24}N_2$ (280.4 g/mol). FTIR (neat): $\tilde{\nu}$ (cm^{-1}): 744, 698 ($\delta_{Ph\text{-}H\ (out\ of\ plane)}$), 799 ($\delta_{Ar\text{-}H\ (out\ of\ plane)}$), 1685 ($\nu_{C=N}$), 2924 ($\nu_{C\text{-}H}$), 3280 ($\nu_{N\text{-}H}$). ^{1}H NMR (600 MHz, $CDCl_3$): δ = 1.28 – 1.34 (*m*, 1H, 6-*H*), 1.35 – 1.40 (*m*, 1H, 8-*H*), 1.80 – 1.86 (*m*, 2H, Ph-CH_2-CH_2), 2.10 – 2.13 (*m*, 2H, 6,8-*H*), 2.65 – 2.71 (*m*, 5H, N-CH_2, Ph-CH_2, 9-*H*), 2.78 – 2.83 (*m*, 2H, 9-*H*, C*H*-N), 2.86 – 2.90 (*m*, 1H, 5-*H*), 3.12 – 3.16 (*m*, 1H, 5-*H*), 7.01 (*dd*, *J* = 7.5, 4.9 Hz, 1H, 3-*H*), 7.16 – 7.19 (*m*, 3H, 3',4'-*H*), 7.26 – 7.29 (*m*, 2H, 2-*H*), 7.35 (*dd*, *J* = 7.5, 1.6 Hz, 1H, 3-*H*), 8.28 (*dd*, *J* = 4.9, 1.6 Hz, 1H, 2-*H*). ^{13}C NMR (151 MHz, $CDCl_3$, 300K): δ = 31.4 (1C, 9-*C*), 32.2 (1C, 8-*C*), 32.8 (1C, Ph-CH_2-*C*H_2), 34.1 (1C, Ph-*C*), 34.3 (1C, 6-*C*), 35.3 (1C, 5-*C*), 46.8 (1C, N-*C*H_2), 61.2 (1C, *C*H-N), 121.6 (1C, 3-*C*), 126.1 (1C, 4'-*C*), 128.7 (2C, 3'-*C*), 128.7 (2C, 2'-*C*), 136.6 (1C, 4-*C*), 137.5 (1C, 4a-C), 142.3 (1C, 1'-*C*), 146.7 (1C, 2-*C*), 162.39 (1C, 9a-*C*). Exact MS (APCI): m/z = 281.2006 (calcd. 281.2012 for $C_{19}H_{25}N_2^+$ [M+H^+]). Purity (HPLC): 95.6 % (t_R = 10.80 min).

4.3.6. *N*-(4-Phenylbutyl)-6,7,8,9-tetrahydro-5*H*-[7]annuleno[b]pyridin-7-amine (11c)

Ketone **10** (40 mg, 0.2 mmol, 1.0 eq.) was dissolved in CH_2Cl_2 (5 ml) and Na_2SO_4 (50 mg) was added. 4-Phenylbutylamine (47 µl, 0.2 mmol, 1.0 eq.) was added and the mixture was stirred for 5 min. $NaBH(OAc)_3$ (67 mg, 0.3 mmol, 1.3 eq.) was added and the mixture was stirred for 3 h. H_2O (0.5 ml) was added and the slurry was filtered. The solvent was removed in vacuo and the residue was purified via fc (ø = 2.5 cm, l = 6 cm, V = 12 ml, H_2O : acetonitrile : trifluoroacetic acid = 80:20:0.1). Green oil, 26 mg (36%). $C_{20}H_{26}N_2$ (294.2 g/mol). FTIR (neat): $\tilde{\nu}$ (cm^{-1}): 744, 698 ($\delta_{Ph\text{-}H\ (out\ of\ plane)}$), 799 ($\delta_{Ar\text{-}H\ (out\ of\ plane)}$), 1685 ($\nu_{C=N}$), 2924 ($\nu_{C\text{-}H}$), 3270 ($\nu_{N\text{-}H}$). ^{1}H NMR (600 MHz, $CDCl_3$, 300K): δ = 1.28 – 1.34 (*m*, 1H, 6-*H*), 1.36 – 1.40 (*m*, 1H, 8-*H*), 1.50 –

1.55 (*m*, 2H, N-CH_2-CH_2), 1.63 – 1.68 (*m*, 2H, Ph-CH_2-CH_2), 2.04 – 2.11 (*m*, 2H, 6,8-*H*), 2.60 – 2.63 (*m*, 2H, Ph-CH_2), 2.65 – 2.70 (*m*, 3H, 5-*H*, N-CH_2), 2.77 – 2.83 (*m*, 2H, 9-*H*, C*H*-NH), 2.86 – 2.90 (*m*, 1H, 9-*H*), 3.11 – 3.15 (*m*, 1H, 5-*H*), 7.01 (*dd*, *J* = 7.5, 4.6 Hz, 1H, 3-*H*), 7.16 – 7.17 (*m*, 3H, 3',4'-*H*), 7.25 – 7.27 (*m*, 2H, 2'-*H*), 7.35 (*dd*, *J* = 7.5, 1.6 Hz, 1H, 4-*H*), 8.28 (*dd*, *J* = 4.9, 1.6 Hz, 1H, 2-*H*). ^{13}C NMR (151 MHz, $CDCl_3$, 300K): *δ* = 29.6 (1C, Ph-CH_2-*C*H_2), 30.3 (1C, N-CH_2-*C*H_2), 31.5 (1C, 9-*C*), 32.9 (1C, 6-*C*), 34.4 (1C, 8-*C*), 35.3 (1C, 5-*C*), 36.2 (1C, *C*-Ph), 47.2 (1C, *C*H_2-NH), 61.3 (1C, *C*-NH), 123.8 (1C, 3-*C*), 126.0 (2C, 2'-*C*), 128.6 (2C, 3'-*C*), 128.7 (1C, 4'-*C*), 136.7 (1C, 4-*C*), 137.5 (1C, 4a-*C*), 142.7 (1C, 2-C), 146.7 (1C, 1'-*C*), 162.6 (1C, 9a-C). Exact MS (APCI): m/z = 295.2170 (calcd. 295.2169 for $C_{20}H_{27}N_2^+$ [M+H^+]). Purity (HPLC): 98.6 % (t_R = 11.67 min).

4.3.7. *N*-Methyl-*N*-(3-phenylpropyl)-6,7,8,9-tetrahydro-5*H*-[7]annuleno[b]pyridin-7-amine (12b)

3-Phenylpropylamine **11b** (40 mg, 0.1 mmol, 1.0) and formalin (37 %, 100 µl, 6.5 eq.) were dissolved in CH_2Cl_2 (5 ml) and Na_2SO_4 (50 mg) was added. $NaBH(OAc)_3$ (75 mg, 0.4 mmol, 2.5 eq.) was added and the mixture was stirred for 4.5 h. The slurry was filtered, and the solvent was removed in vacuo. The residue was purified via fc (*ø* = 2.5 cm, l = 6 cm, V = 12 ml, H_2O : acetonitrile : trifluoroacetic acid = 80:20:0.1). Green oil, 13 mg (32 %). $C_{20}H_{26}N_2$ (294.4 g/mol). FTIR (neat): $\tilde{\nu}$ (cm^{-1}): 748, 698 ($\delta_{Ph\text{-}H\ (out\ of\ plane)}$), 802 ($\delta_{Ar\text{-}H\ (out\ of\ plane)}$), 1678 ($\nu_{C=N}$), 2789 ($\nu_{N\text{-}C}$), 2927 ($\nu_{C\text{-}H}$). ^{1}H NMR (600 MHz, $CDCl_3$, 300K): *δ* = 1.33 – 1.48 (*m*, 2H, 6,8-*H*), 1.77 – 1.85 (*m*, 2H, N-CH_2-CH_2), 2.08 – 2.12 (*m*, 2H, 6,8-*H*), 2.26 (*s*, 3H, CH_3), 2.47 – 2.51 (*m*, 2H, N-CH_2), 2.61 – 2.65 (*m*, 2H, PH-CH_2), 2.69 – 2.88 (*m*, 4H, C*H*-N, 5,9-*H*), 3.11 – 3.16 (*m*, 1H, 5-*H*), 7.04 (*dd*, *J* = 7.5, 4.9 Hz, 1H, 3-*H*), 7.18 – 7.20 (*m*, 3H, 3',4'-*H*), 7.26 – 7.29 (*m*, 2H, 2'-*H*), 7.38 (*dd*, *J* = 7.5, 1.5 Hz, 1H, 4-*H*),

8.31 (*dd*, *J* = 4.9, 1.5 Hz, 1H, 2-*H*). ^{13}C NMR (151 MHz, $CDCl_3$, 300K): *δ* = 27.7 (1C, 8-*C*), 29.9 (1C, 6-*C*), 30.0 (1C, N-CH_2-CH_2), 32.5 (1C, 9-*C*), 33.7 (1C, Ph-*C*), 36.3 (1C, 5-*C*), 37.5 (1C, CH_3), 53.2 (1C, N-CH_2), 67.5 (1C, *C*H-N), 121.7 (1C, 3-*C*), 125.9 (1C, 4'-*C*), 128.5 (2C, 2'-*C*), 128.52 (2C, 3'-*C*), 136.5 (1C, 4-*C*), 137.3 (1C, 4a-*C*), 142.1 (1C, 1'-*C*), 146.7 (1C, 2-*C*), 162.3 (1C, 9a-*C*). Exact MS (APCI): m/z = 295.2157 (calcd. 295.2174 for $C_{20}H_{27}N_2^+$ [M+H$^+$]). Purity (HPLC): 92.2 % (t_R = 10.96 min).

4.3.8. *N*-Methyl-*N*-(4-phenylbutyl)-6,7,8,9-tetrahydro-5*H*-[7]annuleno[b]pyridin-7-amine (12c)

4-Phenylbutylamine **11c** (53 mg, 0.2 mmol, 1.0) and formalin (37 % aq., 128 µl, 6.5 eq.) were dissolved in CH_2Cl_2 (5 ml) and Na_2SO_4 (50 mg) was added. $NaBH(OAc)_3$ (116 mg, 0.5 mmol, 3.0 eq.) was added and the mixture was stirred for 4 h. The slurry was filtered, and the solvent was removed in vacuo. The residue was purified via fc (ø = 2.5 cm, l = 6 cm, V = 12 ml, H_2O : acetonitrile : trifluoroacetic acid = 80:20:0.1). Green oil, 13 mg (23 %). $C_{21}H_{28}N_2$ (308.5 g/mol). FTIR (neat): $\tilde{\nu}$ (cm^{-1}): 744, 698 ($\delta_{Ph\text{-}H\ (out\ of\ plane)}$), 802 ($\delta_{Ar\text{-}H\ (out\ of\ plane)}$), 1678 ($\nu_{C=N}$), 2789 ($\nu_{N\text{-}C}$), 2927 ($\nu_{C\text{-}H}$). ^{1}H NMR (600 MHz, $CDCl_3$, 300K): *δ* = 1.35 – 1.40 (*m*, 1H, 6-*H*), 1.41 – 1.46 (*m*, 1H, 8-*H*), 1.50 – 1.55 (*m*, 2H, N-CH_2-C*H*$_2$), 1.61 – 1.66 (*m*, 2H, Ph-CH_2-C*H*$_2$), 2.09 – 2.12 (*m*, 2H, 6,8-*H*), 2.25 (*s*, 3H, C*H*$_3$), 2.46 – 2.48 (*m*, 2H, N-C*H*$_2$), 2.61 – 2.64 (*m*, 2H, Ph-C*H*$_2$), 2.67 – 2.71 (*m*, 1H, 9-*H),* 2.74 – 2.81 (*m*, 3H, 5,9-*H*, C*H*-N), 3.11 – 3.15 (*m*, 1H, 5-*H*), 7.05 (*dd*, *J* = 7.5, 4.9 Hz, 1H, 3-*H*), 7.16 – 7.18 (*m*, 3H, 3',4'-*H*), 7.25 – 7.28 (*m*, 2H, 2'-*H*), 7.38 (*dd*, *J* = 7.5, 1.5 Hz, 1H, 4-*H*), 8.31 (*dd*, *J* = 4.9, 1.5 Hz, 1H, 2-*H*). ^{13}C NMR (151 MHz, $CDCl_3$, 300K): *δ* = 27.5 (1C, 8-*C*), 27.7 (1C, N-CH_2-*C*H$_2$), 29.3 (1C, Ph-CH_2-*C*H$_2$), 29.9 (1C, 6-*C*), 32.4 (1C, 9-*C*), 35.1 (1C, Ph-*C*), 36.2 (1C, 5-*C*), 37.6 (1C, N-*C*H$_3$), 53.5 (N-*C*H$_2$), 67.5 (N-*C*H), 121.7 (1C, 3-*C*), 125.8 (1C, 4'-*C*), 128.4 (2C, 3'-*C*),

128.5 (2C, *C*), 136.5 (1C, 4-*C*), 137.2 (1C, 5a-*C*), 142.6 (1C, 1'-*C*), 146.7 (1C, 2-*C*), 162.3 (1C, 9a-*C*). Exact MS (HRMS): m/z = calc. for $C_{21}H_{29}N_2^+$ [M+H$^+$] 309.2325, found 309.2314. Purity (HPLC): 91.4 % (t_R = 12.76 min).

4.4. Receptor binding studies

4.4.1. Materials

Guinea pig brains and rat livers were commercially available (Harlan-Winkelmann, Borchen, Germany). Pig brains were a donation of the local slaughterhouse (Coesfeld, Germany). The recombinant L(tk-) cells stably expressing the GluN2B receptor were obtained from Prof. Dr. Dieter Steinhilber (Frankfurt, Germany). Homogenizers: Elvehjem Potter (B. Braun Biotech International, Melsungen, Germany) and Soniprep® 150, MSE, London, UK). Centrifuges: Cooling centrifuge model Rotina® 35R (Hettich, Tuttlingen, Germany) and High-speed cooling centrifuge model Sorvall® RC-5C plus (Thermo Fisher Scientific, Langenselbold, Germany). Multiplates: standard 96 well multiplates (Diagonal, Muenster, Germany). Shaker: self-made device with adjustable temperature and tumbling speed (scientific workshop of the institute). Harvester: MicroBeta® FilterMate 96 Harvester. Filter: Printed Filtermat Typ A and B. Scintillator: Meltilex® (Typ A or B) solid state scintillator. Scintillation analyzer: MicroBeta® Trilux (all Perkin Elmer LAS, Rodgau-Jügesheim, Germany).

4.4.2. Cell culture and preparation of membrane homogenates from GluN2B cells

Mouse L(tk-) cells stably transfected with the dexamethasone-inducible eukaryotic expression vectors pMSG GluN1a, pMSG GluN2B (1:5 ratio) were grown in Modified Earl's Medium (MEM) containing 10 % of standardized FCS (Biochrom AG, Berlin,

Germany). The expression of the NMDA receptor at the cell surface was induced after the cell density of the adherent growing cells had reached approximately 90 % of confluency. For the induction, the original growth medium was replaced by growth medium containing 4 µM dexamethasone and 4 µM ketamine (final concentration). After 24 h, the cells were rinsed with phosphate buffered saline solution (PBS, Biochrom AG, Berlin, Germany), harvested by mechanical detachment and pelleted (10 min, 5,000 x g).

For the binding assay, the cell pellet was resuspended in PBS solution and the number of cells was determined using a Scepter® cell counter (MERCK Millipore, Darmstadt, Germany). Subsequently, the cells were lysed by sonication (4 °C, 6 x 10 s cycles with breaks of 10 s). The resulting cell fragments were centrifuged with a high performance cool centrifuge (23,500 x g, 4 °C). The supernatant was discarded and the pellet was resuspended in a defined volume of PBS yielding cell fragments of approximately 500,000 cells/mL. The suspension of membrane homogenates was sonicated again (4 °C, 2 x 10 s cycles with a break of 10 s) and stored at -80 °C.

4.4.3. Preparation of membrane homogenates from pig brain cortex

Fresh pig brain cortex was homogenized with the potter (500-800 rpm, 10 up and down strokes) in 6 volumes of cold 0.32 M sucrose. The suspension was centrifuged at 1,200 x g for 10 min at 4 °C. The supernatant was separated and centrifuged at 31,000 x g for 20 min at 4 °C. The pellet was resuspended in 5-6 volumes of TRIS/EDTA buffer (5 mM TRIS/1 mM EDTA, pH 7.5) and centrifuged again at 31,000 x g (20 min, 4 °C). The final pellet was resuspended in 5-6 volumes of buffer and frozen (-80 °C) in 1.5 mL portions containing about 0.8 mg protein/mL.

4.4.4. Preparation of membrane homogenates from guinea pig brain

5 guinea pig brains were homogenized with the potter (500-800 rpm, 10 up and down strokes) in 6 volumes of cold 0.32 M sucrose. The suspension was centrifuged at 1,200 x g for 10 min at 4 °C. The supernatant was separated and centrifuged at 23,500 x g for 20 min at 4 °C. The pellet was resuspended in 5-6 volumes of buffer (50 mM TRIS, pH 7.4) and centrifuged again at 23,500 x g (20 min, 4 °C). This procedure was repeated twice. The final pellet was resuspended in 5-6 volumes of buffer and frozen (-80 °C) in 1.5 mL portions containing about 1.5 mg protein/mL.

4.4.5. Preparation of membrane homogenates from rat liver

Two rat livers were cut into small pieces and homogenized with the potter (500-800 rpm, 10 up and down strokes) in 6 volumes of cold 0.32 M sucrose. The suspension was centrifuged at 1,200 x g for 10 min at 4 °C. The supernatant was separated and centrifuged at 31,000 x g for 20 min at 4 °C. The pellet was resuspended in 5-6 volumes of buffer (50 mM TRIS, pH 8.0) and incubated at rt for 30 min. After the incubation, the suspension was centrifuged again at 31,000 x g for 20 min at 4 °C. The final pellet was resuspended in 5-6 volumes of buffer and stored at -80 °C in 1.5 mL portions containing about 2 mg protein/mL.

4.4.6. General procedures for the binding assays

The test compound solutions were prepared by dissolving approximately 10 µmol (usually 2-4 mg) of test compound in DMSO so that a 10 mM stock solution was obtained. To obtain the required test solutions for the assay, the DMSO stock solution was diluted with the respective assay buffer. The filtermats were presoaked in 0.5 % aqueous polyethylenimine solution for 2 h at rt before use. All binding

experiments were carried out in duplicates in the 96 well multiplates. The concentrations given are the final concentration in the assay. Generally, the assays were performed by addition of 50 µL of the respective assay buffer, 50 µL of test compound solution in various concentrations (10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9} and 10^{-10} mol/L), 50 µL of the corresponding radioligand solution and 50 µL of the respective receptor preparation into each well of the multiplate (total volume 200 µL). The receptor preparation was always added last. During the incubation, the multiplates were shaken at a speed of 500-600 rpm at the specified temperature. Unless otherwise noted, the assays were terminated after 120 min by rapid filtration using the harvester. During the filtration, each well was washed five times with 300 µL of water. Subsequently, the filtermats were dried at 95 °C. The solid scintillator was melted on the dried filtermats at a temperature of 95 °C for 5 min. After solidifying of the scintillator at rt, the trapped radioactivity in the filtermats was measured with the scintillation analyzer. Each position on the filtermat corresponding to one well of the multiplate was measured for 5 min with the [^{3}H]-counting protocol. The overall counting efficiency was 20 %. The IC_{50} values were calculated with the program GraphPad Prism® 3.0 (GraphPad Software, San Diego, CA, USA) by non-linear regression analysis. Subsequently, the IC_{50} values were transformed into K_i values using the equation of Cheng and Prusoff.[33] The K_i values are given as mean value ± SEM from three independent experiments.

4.4.7. Affinity for the GluN2B binding site of the NMDA receptor

The competitive binding assay was performed with the radioligand [^{3}H]ifenprodil (60 Ci/mmol; BIOTREND, Cologne, Germany). The thawed cell membrane preparation from the transfected L(tk-) cells (about 20 µg protein) was incubated with various concentrations of test compounds, 5 nM [^{3}H]-ifenprodil, and TRIS/EDTA-

buffer (5 mM TRIS/1 mM EDTA, pH 7.5) at 37 °C. The non-specific binding was determined with 10 µM unlabeled ifenprodil. The K_d value of ifenprodil is 7.6 nM.[26]

4.4.8. Affinity for the PCP binding site of the NMDA receptor

The assay was performed with the radioligand [^{3}H]-(+)-MK-801 (22.0 Ci/mmol; Perkin Elmer). [17,18] The thawed membrane preparation of pig brain cortex (about 100 µg of the protein) was incubated with various concentrations of test compounds, 2 nM [^{3}H]-(+)-MK-801, and TRIS/EDTA buffer (5 mM TRIS/1 mM EDTA, pH 7.5) at rt. The non-specific binding was determined with 10 µM unlabeled (+)-MK-801. The K_d value of (+)-MK-801 is 2.26 nM. [34]

4.4.9. Affinity for the σ_1 receptor

The assay [28-30] was performed with the radioligand [^{3}H]-(+)-pentazocine (22.0 Ci/mmol; Perkin Elmer). The thawed membrane preparation of guinea pig brain cortex (about 100 µg of the protein) was incubated with various concentrations of test compounds, 2 nM [^{3}H]-(+)-pentazocine, and TRIS buffer (50 mM, pH 7.4) at 37 °C. The non-specific binding was determined with 10 µM unlabeled (+)-pentazocine. The K_d value of (+)-pentazocine is 2.9 nM.[35]

4.4.10. Affinity for the σ_2 receptor

The assay [28-30] was performed with the radioligand [^{3}H]di-*o*-tolylguanidine (specific activity 50 Ci/mmol; ARC, St. Louis, MO, USA). The thawed rat liver membrane preparation (about 100 µg protein) was incubated with various concentrations of the test compound, 3 nM [^{3}H]di-*o*-tolylguanidine and buffer containing (+)-pentazocine (500 nM (+)-pentazocine in TRIS buffer (50 mM TRIS, pH 8.0)) at rt. The non-specific

binding was determined with 10 µM non-labeled di-*o*-tolylguanidine. The K_d value of di-*o*-tolylguanidine is 17.9 nM.[36]

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Supporting Information

The Supporting Information contains purity data of prepared compounds, protein determination of biological material and NMR spectra.

Conflict of interest

The authors declare no conflict of interest.

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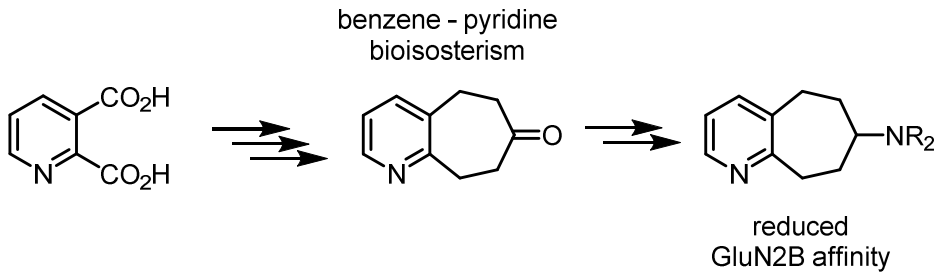
Graphical Abstract



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Research Highlights

> A series of [7]annulene[b]pyridin-7-amines was prepared.

> Bioisosteric replacement of nitrobenzene by pyridine.

> Pyridine bioisosteres show reduced GluN2B affinity.

> Interaction with the ifenprodil binding site.