Negative allosteric modulators of NMDA receptors with GluN2B subunit: Alanine-derived benzoxazolone bioisosteres of 2-methyl-3-benzazepine-1,7-diols

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Abstract
Inspired by besonprodil, the phenol of potent negative allosteric modulators of GluN2B-N-methyl-D-aspartate (NMDA) receptors was replaced by a benzoxazolone system. To increase the similarity to the lead compounds, an additional methyl moiety was installed in the 8-position of tricyclic oxazolobenzazepines, resulting in compounds 6. The additional methyl moiety originates from alanine, which was introduced by a Mitsunobu reaction of benzoxazolylethanol 7 with N-triflyl-protected alanine methyl ester. A crucial feature of the synthesis was the protection of the oxazolone ring by an allyl moiety, which was cleaved off at the end of the synthesis by RhCl₃-catalyzed isomerization. Due to the additional methyl moiety, the intramolecular Friedel–Crafts acylation of acid 10 to afford ketone 11 required careful optimization to minimize the formation of the side product tetrahydroisoquinoline 16. Alkylation or reductive alkylation of secondary amine 13 led to diastereomeric oxazolobenzazepines cis-14 and trans-14, which were separated by flash chromatography. Phenylbutyl derivatives cis-6a and trans-6a revealed twofold higher GluN2B affinity than analog 5a without 8-CH₃ group. The methylated oxazolobenzazepines 6 and 14 did not interact with the phencyclidine binding site of NMDA receptors and σ₂ receptors. However, the σ₁ receptor preferred cis-configured oxazolobenzazepines. The highest σ₁ receptor affinities were obtained for cis-14a (Ki = 26 nM) and cis-6b (Ki = 30 nM).

KEYWORDS
β-elimination of sulfinate, cis/trans-isomers, GluN2B subunit, phenol-benzoxazolone bioisosteres, radioligand receptor binding studies

1 | INTRODUCTION

The group of ionotropic glutamate receptors comprises three subtypes named according to their prototypical agonists kainate, 2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl)propionate (AMPA), and N-methyl-D-aspartate (NMDA) receptor.[1,2] A typical heterotetrameric NMDA receptor consists of two GluN1 and two GluN2 subunits. The GluN1 subunit existing in eight splice variants (GluN1α–h) contains the binding site for glycine. The binding site for (S)-glutamate is located on the GluN2 subunit. Four different genes encode for the amino acid
sequence of four different GluN2 subunits termed GluN2A–D. GluN3A and GluN3B subunits are predominantly expressed during the pre- and perinatal period of life and are therefore not further discussed here.[3–6]

Whereas the different splice variants of the GluN1 subunit are expressed ubiquitously throughout the nervous system, GluN2 subunits show a distinct expression pattern and determine the gating kinetics of the NMDA receptor.[7,8] We are particularly interested in ligands negatively modulating GluN2B subunit-containing NMDA receptors (GluN2B-NMDA receptors). GluN2B-NMDA receptors are expressed predominantly in the cortex, hippocampus, and striatum. The expression in only some regions of the central nervous system reduces the side effect potential of GluN2B-selective NMDA receptor antagonists. Compared to NMDA receptors with the GluN2A subunit, GluN2B-NMDA receptors show slower kinetics, that is, the Ca²⁺ influx through the ion channel is slower for GluN2B-NMDA receptors.[3,5–8]

Activation of NMDA receptors plays a key role in synaptic plasticity and thus long-term potentiation correlating with processes such as learning and memory.[9–11] On the contrary, the NMDA receptor is also involved in the process of excitotoxicity following the massive release of the excitatory neurotransmitter (S)-glutamate and thus activation of glutamate receptors, in particular NMDA receptors.[12,13]

The therapeutic potential of ligands inhibiting GluN2B-NMDA receptors comprises acute neuronal damages (e.g., stroke, brain injury, epileptic seizure) and chronic neurodegenerative processes (e.g., Parkinson’s disease, Alzheimer’s disease,[14] multiple sclerosis).[3,6,11–13] The prototypical GluN2B selective NMDA receptor antagonist ifenprodil[15,16] (1; Figure 1) interacting with the ifenprodil binding site located within the amino-terminal domain of the NMDA receptor[17] is approved to improve the cerebral blood circulation. In some ongoing clinical trials, ifenprodil is tested for the treatment of drug addiction,[17–19] idiopathic pulmonary fibrosis, and Covid-19 infections.[20,21] In animal models ifenprodil was able to improve the symptoms of Alzheimer’s disease.[22] Traxoprodil (2; Figure 1) also interacts with high affinity with the ifenprodil binding site of GluN2B subunit-containing NMDA receptors and shows neuroprotective activity. In a clinical trial for the treatment of traumatic brain injury, traxoprodil did not lead to a significant benefit for the patients although only low side effects were observed.[23,24]

In addition to high affinity towards GluN2B-NMDA receptors, ifenprodil interacts with related σ₁, σ₂, α₅, 5-HT₁A, and 5-HT₂ receptors.[25–28] It was shown that the selectivity of ifenprodil depends considerably on the configuration of the two centers of chirality.[29] Moreover, the bioavailability of ifenprodil is rather low due to fast biotransformation, in particular rapid glucuronidation of the phenol.[30]

To improve selectivity and bioavailability, the 2-amino-1-phenylpropanol substructure of ifenprodil (1) and traxoprodil (2) was rearranged in the class of 3-benzazepines 3 (Figure 1). Depending on the configuration, the 3-benzazepines 3 showed high

![Figure 1](image_url)

**FIGURE 1** Evolution of methylated oxazolo-benzazepines 6: Conformational restriction of lead compounds 1 and 2 and bioisosteric replacement of the phenol led to tetrahydro-3-benzazepines 3 and benzoxazolone 4, respectively. Merging of benzazepines 3 and benzoxazolone 4 resulted in oxazolo-benzazepines 5 and finally 6 with an additional methyl moiety in the 8-position.
affinity towards the ifenprodil binding site and high cytoprotective activity on mouse fibroblast cells expressing the GluN2B-NMDA receptor.\[^{31-34}\] To avoid phenol glucuronidation, the phenol of 1 and 2 was replaced by the benzoazolone system in besonprodil (4).\[^{35}\]

Inspired by besonprodil (4), the phenol of 3-benzazepinediol 3a was also replaced by the benzoazolone system in 5.\[^{36,37}\] However, in the first series of compounds a methyl moiety in 8-position was missing. Herein, we wish to report the synthesis and pharmacological evaluation of oxazolo-benzazepines of type 6 bearing an additional methyl moiety at 8-position to mimic the corresponding methyl moiety of ifenprodil (1), traxoprodil (2) and 3-benzazepine 3b.

## 2. RESULTS AND DISCUSSION

### 2.1. Synthesis

The synthesis of methylated oxazolo-benzazepines 6 (Scheme 1) followed the same strategy as the synthesis of nonmethylated analogs 5,\[^{37}\] but instead of glycine, alanine was employed as starting material. In the first step, Mitsunobu reaction\[^{38}\] of benzoxazolylethanol 7 with triflyl-protected alanine methyl ester\[^{39}\] provided ester 8 in 77% yield. The introduction of the allyl moiety and the subsequent saponification of the ester 9 were performed in a consecutive one-pot synthesis leading to the acid 10 in 89% yield (over two steps).

The intramolecular Friedel–Crafts acylation demonstrated the reduced reactivity of the methylated acid 10. Applying similar reaction conditions as for the glycine analog (P4O10, 10 equiv., CH2Cl2, reflux, 4 h)\[^{36,37}\] resulted in a low yield (19%) of the desired oxazolobenzazepinedione 11. In addition to nonconverted starting material 10, the ring contracted tetrahydroisoquinoline 16 (see Scheme 2) was isolated. The formation of the tetrahydroisoquinoline 16 is explained by decarbonylation of intermediate acylim ion forming the iminium ion 15, which attacks the phenyl ring.\[^{38}\] This competing side reaction could be avoided for substrates without methyl moiety by the very strong electron-withdrawing trifluoromethylsulfonyl moiety. In the case of acid 10, the destabilization of the resulting iminium ion 15 by the triflyl group is compensated partly by the stabilizing $+$I effect of the additional methyl moiety. To avoid the competing formation of the tetrahydroisoquinoline 16 and increase the yield of tricyclic ketone 11, reaction conditions were carefully optimized. Portion-wise addition of P4O10 (3 equiv.) to a diluted solution of acid 10 in CH2Cl2, stirring at room temperature for 60 h, and finally heating to reflux for 24 h provided the tricyclic ketone 11 in 43% yield. Nevertheless, the formation of tetrahydroisoquinoline 16 could not be avoided completely.

In the next step, the trifluoromethylsulfonyl moiety of ketone 11 was cleaved off by elimination of trifluromethanesulfinate (F3CSO2)$^-$ using K2CO3.\[^{37,40}\] The resulting iminoketone 12 was reduced with NaBH4 to form the $\beta$-aminoalcohol 13, which was alkylated with 1-chloro-4-phenylbutane or reductively alkylated with 4-(piperidin-1-yl)benzaldehyde and NaBH(OAc)$_2$.\[^{41}\] to provide the N-alkylated alcohols 14a and 14b, respectively. The iminoketone 12 could be isolated for the first time as an intermediate of this elimination reaction as it is stabilized by the additional methyl group at the double bond.

During the NaBH4 reduction of the iminoketone 12, two diastereomeric amino alcohols 13 were formed. After alkylation of the secondary amine 13, the diastereomeric alcohols were separated by flash chromatography (FC). The cis-configured diastereomers were isolated as main diastereomers: cis-14a: 62%, cis-14b: 48%. The relative configuration of the diastereomers 14 was determined by $^1$H nuclear magnetic resonance (NMR) spectroscopy. 9-H of cis-14a resonates at 5.03 ppm as a broad singlet, whereas a doublet at 4.37 ppm with a large coupling constant ($J = 6.0$ Hz) appears for 9-H of trans-14a. Comparable chemical shifts and coupling constants were observed for cis- and trans-configured tetrahydro-3-benzazepines 3b.\[^{34}\] The 4-phenylbutyl and 4-piperidinobenzyl moieties were selected due to the rather high GluN2B affinity of the corresponding benzoazolone analogs 5 without methyl moiety.\[^{37}\] The 4-piperidinobenzyl substituted benzoazolone 5c bearing the N-allyl moiety showed a GluN2B affinity of 194 nM.\[^{37}\] 

In the last step, the allyl protective group of the diastereomeric oxazolobenzazepines cis-14a,b, and trans-14a,b was cleaved off by isomerization of the allyl group into a prop-1-en-1-yl group with RhCl3 and subsequent hydrolysis of the resulting enamine with 3 M HCl\[^{42,43}\]. The oxazolobenzazepines cis-6a,b and trans-6a,b were isolated in 17%–46% yield. Although the deallylation reaction worked well, isolation of the zwitterionic products 6 (acidic oxazolone, basic amine) was difficult.

### 2.2. Receptor affinity

The affinities of methylated oxazolobenzazepines 6 and 14 towards the ifenprodil and phencyclidine binding sites of NMDA receptors as well as their $\sigma$ receptor affinities are summarized in Table 1 and compared with the affinity data of lead compounds 5 and reference compounds.

#### 2.2.1. Affinity towards GluN2B-NMDA receptors

In the GluN2B assay, the test compounds and the radioligand $[^{3}H]$ifenprodil compete for the ifenprodil binding sites in a receptor preparation from mouse fibroblast Ltk$^{-}$-cells.\[^{45}\] The oxazolobenzazepine 5c without CH$_3$ moiety in 8-position but with the allyl protective group at the oxazolone ring and the piperidinobenzyl moiety at N-7 exhibited promising GluN2B affinity ($K_i = 194$ nM).\[^{37}\] The corresponding piperidinobenzyl derivatives 14b with allyl protective group and additional 8-CH$_3$ moiety revealed lower GluN2B affinity than 5c. Removal of the allyl protective group led to elimination of the interactions with the ifenprodil binding site for both the nonmethylated derivative 5b as well as for the methylated derivatives cis-6b and trans-6b.
The oxazolobenzazepine 5a with a 4-phenylbutyl side chain at N-7 exhibited a moderate GluN2B affinity of 422 nM. Introduction of an additional methyl moiety in the 8-position slightly increased the GluN2B affinity. However, the GluN2B affinity of the cis- and trans-configured diastereomers, for example, cis-6a and trans-6a, as well as the GluN2B affinity of allylated and nonallylated compounds, for example, cis-14a and cis-6a, are very similar. It can be concluded that introduction of an additional 8-CH₃ moiety slightly increased the GluN2B affinity of cis-6a and trans-6a compared to 5a. However, the presence or absence of the N-allyl protective group and the relative configuration only marginally influenced the GluN2B affinity.

**SCHEME 1** Synthesis of methylated oxazolo-benzazepines 6. Reagents and reaction conditions: (a) DIAD, Ph₃P, THF, 0°C → rt, 16 h, 77%. (b) Allyl bromide, K₂CO₃, CH₃CN, 50°C, 45 min. (c) LiOH·H₂O, THF/H₂O (7:3), 0°C, 1 h, 89%. (d) P₄O₁₀ (3 equiv., portion wise), CH₂Cl₂, (62 ml/mmol), rt (60 h) → 40°C (24 h), aqueous work-up of P₄O₁₀, 43%. (e) 1. Iodo-4-phenylbutane, CH₃CN, K₂CO₃, 80°C, 16 h, 62% cis-14a, 17% trans-14a. (h) 4-(Piperidin-1-yl)benzaldehyde, NaBH(OAc)₃, CH₂Cl₂, rt, 16 h, 48% cis-14b, 11% trans-14b. (i) 1. RhCl₃·3H₂O (0.1 equiv.), isopropanol, 85°C, 2.5–29 h: 2. HCl (3 M), 85°C, 3 h, 17%–46%. *Only one enantiomer of racemic compounds 14 and 6 is shown.
2.2.2 | Receptor selectivity

Up to a concentration of 1 µM, the tricyclic oxazolobenzazepines 6 and 14 did not compete with the radioligand \([3^H]^{(+)}MK801\) indicating only very low affinity towards the phencyclidine binding site of the NMDA receptor.

In the \(\sigma_2\) assay using the radioligand \([3^H]dihydroxyguanidine\) in the presence of \((+)^{Pentazocine\,to\,mask\,\sigma_1\,receptors,\,the\,tricyclic}

![Scheme 2](image.png)

**Scheme 2** Formation of side product tetrahydroisoquinoline 16 by intramolecular alkylation via the iminium ion 15.

**Table 1** Interactions of oxazolobenzazepines 6 and 14 with the ifenprodil and phencyclidine binding sites of the NMDA receptor and with both \(\sigma\) receptor subtypes.

| Compd. | \(R^1\) | Configuration | \(K_i\) ± SEM (nM) (n = 3)\(^a\) |
|--------|--------|---------------|---------------------------------|
|        |        |               | GluN2B  | PCP     | \(\sigma_1\) | \(\sigma_2\) |
| 5a\(^b\) | \(-(CH_2)_4Ph\) | - | 422\(^b\) | 34% | 32% | 576 |
| 5b\(^b\) | \(-H_2C=\)- | - | 38% | 47% | 136 | 284 |
| 5c\(^b\) | \(-H_2C=\)- | - | 194 ± 21 | 51% | 238 | 591 |
| cis-6a | \(-(CH_2)_4Ph\) | cis | 256 ± 17 | 22% | 41 ± 10 | 43% |
| Trans-6a | \(-(CH_2)_4Ph\) | trans | 326 | 31% | 249 | >1000 |
| cis-6b | \(-H_2C=\)- | cis | 46% | 14% | 30 ± 12 | >1000 |
| trans-6b | \(-H_2C=\)- | trans | 49% | 43% | 472 | >1000 |
| cis-14a | \(-(CH_2)_4Ph\) | cis | 219 ± 24 | 0% | 26 ± 10 | 401 |
| trans-14a | \(-(CH_2)_4Ph\) | trans | 318 ± 4 | 24% | 69 ± 11 | 31% |
| cis-14b | \(-H_2C=\)- | cis | 489 ± 181 | 0% | 67 ± 24 | 49% |
| trans-14b | \(-H_2C=\)- | trans | 643 ± 130 | 37% | 37% | 10% |
| (1R,2R)-ifenprodil | \(\pm\) | - | 5.8 ± 1.3 | - | 125 ± 24 | 98 ± 34 |
| 3 (WMS-1410)\(^b\) | \(\pm\) | - | 84 ± 18 | - | 123 | 32 ± 18 |
| Eliprodil | \(\pm\) | - | 13 ± 2.5 | - | - | - |
| (+)-Pentazocine | \(\pm\) | - | 5.4 ± 0.5 | - | - | - |
| Haloperidol | \(\pm\) | - | 6.3 ± 1.6 | 78 ± 2.3 | - | - |
| Di-hydroxyguanidine | \(\pm\) | - | 89 ± 29 | 57 ± 18 | - | - |

Abbreviation: PCP, 1-(1-phenylcyclohexy1)piperidine.

\(^a\)Values in % reflect the removal of the radioligand at a test compound concentration of 1 µM.

\(^b\)Due to low GluN2B affinity, \(K_i\) values were recorded only twice and the mean is given.
oxazolobenzazepines 6 and 14 did not show remarkable affinity. The allylated phenylbutyl derivative cis-14a is the only compound displaying $\sigma_2$ affinity below 1 $\mu$M ($K_i = 400$ nM).

As the pharmacophores of $\sigma_2$ and GluN2B ligands are very similar, we always test the $\sigma_2$ affinity of GluN2B ligands in competitive radioligand receptor binding studies with $[^3H]$(+)-pentazocine as a radioactive competitor. In this assay, the methylated oxazolobenzazepines 6 and 14 showed remarkable affinity towards $\sigma_2$ receptors, which even exceeded their GluN2B affinity. Particular high $\sigma_2$ affinity was found for cis-configured derivatives, for example, cis-6a ($K_i = 41$ nM), cis-14a ($K_i = 26$ nM). Thus, the $\sigma_2$ receptor clearly prefers cis-configured oxazolobenzazepines over trans-configured analogs, but does not discriminate between allylated and nonallylated compounds, for example, cis-6a/cis-14a. Moreover, the substituent at N-7 did not influence the $\sigma_2$ affinity considerably. As example, the $\sigma_2$ affinity of the phenylbutyl derivative cis-6a ($K_i = 41$ nM) and the piperidinobenzyl derivative cis-6b ($K_i = 30$ nM) are very similar.

3 | CONCLUSION

The $\beta$-aminoalcohol substructure of ifenprodil (1) and traxoprodil (2) contain an additional methyl moiety in $\beta$-position. To mimic these negative allosteric GluN2B-NMDA receptor modulators, the benzoxazolone biososteres 5 should be provided with an additional methyl moiety in 8-position.

The synthesis of methylated oxazolobenzazepines 6 followed the same strategy as the synthesis of 5, but started with N-trifly protected alanine methyl ester instead of glycine methyl ester. During the intramolecular Friedel–Crafts acylation of glycine derivatives, the trifly protective group inhibited completely the formation of tetrahydroisoquinoline side products. Due to better stabilization of the intermediate iminium ion 15 by the additional methyl moiety, the reaction conditions for the intramolecular Friedel–Crafts acylation of acid 10 had to be optimized carefully to obtain the tricyclic ketone 11 in 43% yield. However, the formation of tetrahydroisoquinoline side product 16 could not be avoided completely.

Due to additional stabilization by the methyl group, the iminoketone 12 resulting from K$_2$CO$_3$ induced trifluoromethanesulfinate elimination from 11 could be isolated and characterized for the first time.

After alkylation or reductive alkylation of the secondary amine 13, cis- and trans-configured alcohols were formed and separated by FC. The cis-configured alcohols cis-14a and cis-14b represent the major diastereomers.

The additional methyl moiety of cis-6a and trans-6a increased the GluN2B affinity of the nonmethylated analog 5a almost twofold. Unexpectedly, the allyl derivatives cis-14a and trans-14a showed almost the same GluN2B affinity as the nonallylated analogs cis-6a and trans-6a. Obviously, the absence or presence of the allyl protective group and the relative configuration do not influence the GluN2B affinity of tricyclic oxazolobenzazepines considerably.

Although the oxazolobenzazepines 6 and 14 show high selectivity for GluN2B-NMDA receptors over the phencyclidine binding site of the NMDA receptor and $\sigma_2$ receptors, the selectivity over the $\sigma_2$ receptor subtype is very poor. For some compounds, the $\sigma_2$ affinity is even higher than the GluN2B affinity.

4 | EXPERIMENTAL

4.1 | Chemistry

4.1.1 | General remarks

Unless otherwise noted, moisture-sensitive reactions were conducted under dry nitrogen. Thin-layer chromatography: Silica gel 60 F$_{254}$ plates (Merck), FC: Silica gel 60, 40–64 $\mu$m (Merck); parentheses include: diameter of the column (d), fraction size (v), eluent, R$_f$ value. Melting point: Melting point apparatus SMP3 (Stuart Scientific), uncorrected. MS: MAT GCQ (Thermo-Finnigan); EI, MAT LCQ (Thermo Finnigan); ESI. $^1$H NMR (400 MHz), $^{13}$C NMR (100 MHz) (see the Supporting Information for the original spectra): Mercury Plus AS 400 NMR spectrometer (Varian); $\delta$ in ppm related to tetramethylsilane; coupling constants are given with 0.5 Hz resolution; the assignments of $^{13}$C and $^2$H NMR signals were supported by 2D NMR techniques. IR: IR spectrophotometer 480Plus FT-ATR-IR (Jasco) or FT/IR Prestige 21 (Shimadzu). Elemental analysis: CHNOS-Elementar Analyser Vario EL III (Elementar).

The InChI codes of the investigated compounds, together with some biological activity data, are provided as Supporting Information.

4.1.2 | High-performance liquid chromatography methods to determine the purity

High-performance liquid chromatography (HPLC) method 1: HPLC: Merck Hitachi Equipment; UV detector: L-7400; autosampler: L-7200; pump: L-7100; degasser: L-7614; column: LiChrospher® 60 RP-select B (5 $\mu$m); LiChroCART® 250–254 mm cartridge; flow rate: 1.0 ml/min; injection volume: 5.0 $\mu$l; detection at $\lambda$ = 210 nm; solvents: A: water with 0.05% (vol/vol) trifluoroacetic acid; B: acetonitrile with 0.05% (vol/vol) trifluoroacetic acid; gradient elution: (A%): 0–4 min: 90%, 4–29 min: 90% → 0%, 29–31 min: 0%, 31–31.5 min: 0 → 90%, 31.5–40 min: 90%. Data acquisition: HSM software; manual integration.

HPLC method 2: HPLC: Merck Hitachi Equipment; UV detector: L-7400; autosampler: L-7200; pump: L-7100; degasser: L-7614; column: LiChrospher® 60 RP-select B (5 $\mu$m); LiChroCART® 250–254 mm cartridge; Guard Column: LiChrospher® 60 RP-select B (5 $\mu$m), LiChroCART® 4–4 mm cartridge; flow rate: 1.0 ml/min; injection volume: 5.0 $\mu$l; detection at $\lambda$ = 210 nm; solvents: A: water with 0.05% (vol/vol) trifluoroacetic acid; B: methanol with 0.05% (vol/vol) trifluoroacetic acid; gradient elution: (A%): 0–1 min: 80%, 1–22 min: 80 → 0%, 22–30 min: 0%, 30–31.5 min: 0 → 80%,
31.5–40 min: 80%. Data acquisition: HSM software, manual integration.

Unless otherwise noted, the purity of all test compounds is >95% according to one of these HPLC methods.

4.1.3 | Synthetic procedures

4.1.3.1 | Methyl 2-[(2-oxo-2,3-dihydrobenzoxazol-5-yl)ethyl]-N-(trifluoromethylsulfonyl)aminopropionate (8)

Benzoazalone 7 (179 mg, 1 mmol), methyl 2-(trifluoromethylsulfonyl)amino propionate (8) (202 mg, 1 mmol), and Ph3P (263 mg, 1 mmol) were dissolved in tetrahydrofuran (THF) (7 ml) under N2 atmosphere and the solution was cooled down to 0°C. Disospropyl azodicarboxylate (DIAD) (202 mg, 1 mmol) was added drop-wise during 30 min. Then, the mixture was stirred for another 1 h at 0°C. After that the cooling bath was removed and the reaction was completed by stirring overnight at room temperature (rt). The solvent was removed under reduced pressure giving a pale yellow oil as a crude product which was purified by flash column chromatography (Ø 2 cm, 20 cm, ethyl acetate/n-hexane 1:2, fraction size 10 ml, Rf = 0.41). Colorless solid, yield 304 mg (77%). Melting point 102–103°C. Purity: HPLC method 1: tR = 19.6 min, purity 98.8%. C16H15F2NO2S (M+ = 396.3). MS (EI): m/z (%) = 396 (M+, 18), 161 (M–N(SO2CF3)–CH(CH3)–CO2H–H–, 100), 148 (M–CH2–N(SO2–CF3)–CH(CH3)–CO2Cl–N=N–, 16). 1H NMR (CDCl3): δ [ppm] = 1.58 (d, J = 7.3 Hz, 3H, N–CH3–), 2.98 (m, 2H, Ph–CH2–CH–N–), 3.48 (m, 1H, Ph–CH2–CH2–N–), 3.63 (m, 1H, Ph–CH2–CH2–N–), 3.79 (s, 3H, OCH3), 4.65 (q, J = 7.3 Hz 1H, N–CH–), 6.94 (m, 2H, 4-Hphenyl, 6–Hphenyl). 7.14 (d, J = 8.7 Hz 1H, 7-Hphenyl). A signal for the NH-proton is not seen in the 1H NMR spectrum. IR (neat): ν [cm⁻¹] = 2985 (w, νC=H aliph.), 1758 (s, νC=O oxazolone), 1687 (m, νC=O ester), 1632 (w)/1525 (w)/1468 (m, νC=C arom.), 1389 (m)/1174 (s, νC=Namide), 1131 (s, νC=N). 1H NMR (CDCl3): δ [ppm] = 1.68 (d, J = 7.5 Hz, 3H, N–CH3–), 3.01 (dd, J = 15.1/5.9 Hz, 1H, Ph–CH2–CH2–N–), 3.13 (ddd, J = 15.1/11.9/6.7 Hz, 1H, Ph–CH2–CH2–N–), 3.71 (m broad, 1H, Ph–CH2–CH2–N–), 4.02 (m broad, 1H, Ph–CH2–CH2–N–), 4.41–4.52 (m, 2H, N–CH2–CH2–C), 4.73 (q, J = 7.5 Hz 1H, N–CH–), 5.24–5.36 (m, 2H, N–CH2–CH2–C), 5.84–5.95 (m, 1H, N–CH2–CH2–C), 6.81 (s, 1H, 4-Hphenyl), 7.44 (s, 1H, 7-Hphenyl). IR (neat): ν [cm⁻¹] = 2959 (w, νC–H aliph.), 1770 (s, νC=O oxazolone), 1677 (m, νC=O ketone), 1627 (w)/1611 (w)/1495 (m, νC=C arom.), 1387 (s)/1180 (s, νC=Namide), 1141 (s, νC=N). 3-Allyl-8-methyl-7-(trifluoromethylsulfonyl)-5,6,7,8-tetrahydrooxazolo[4.5-h][3]benzazepine-2,9(3H)-dione (11) and 3-allyl-8-methyl-7-(trifluoromethylsulfonyl)-5,6,7,8-tetrahydrooxazolo[4.5-g]-isooquinolin-2(3H)-one (16)

Under N2 atmosphere, acid 10 (4.76 g, 11.3 mmol) was dissolved in dry CH2Cl2 (700 ml) and P2O5 (4.81 g, 33.9 mmol) was added under vigorous stirring. Stirring was continued for 60 h at rt. The transformation was completed by stirring at 40°C for an additional 24 h. P2O10 was filtered off and the solvent of the filtrate was removed completely giving a brown-colored oil as crude product, which was recrystallized with ethyl acetate under the addition of petroleum ether giving pure 3-benzazepine 11. Then, H2O (50 ml) was added to the residual solid matter of P2O5 on the filter and the suspension was stored for 3 d at rt. The aqueous suspension of P2O5 was extracted with CH2Cl2 (3x), the combined organic layers were dried (Na2SO4) and the solvent was removed under reduced pressure giving a dark oil as crude product, which was recrystallized with ethyl acetate under addition of petroleum ether giving pure 11. The solvent of the mother liquor was removed completely under reduced pressure giving a dark oil as crude product, which was recrystallized with CH2Cl2 to afford pure 16.

Compound 11: Colorless crystals, total yield 1.96 g (43%). Melting point 165–166°C. Purity: HPLC method 1: tR = 20.6 min, purity 99.8%. C26H25F2N4O2S (M+ = 404.4). MS (EI): m/z (%) = 404 (M+, 30), 271 (M–SO2–CF3–, 100). 1H NMR (CDCl3): δ [ppm] = 1.68 (d, J = 7.5 Hz, 3H, N–CH3–), 3.01 (dd, J = 15.1/5.9 Hz, 1H, Ph–CH2–CH2–N–), 3.13 (ddd, J = 15.1/11.9/6.7 Hz, 1H, Ph–CH2–CH2–N–), 3.71 (m broad, 1H, Ph–CH2–CH2–N–), 4.02 (m broad, 1H, Ph–CH2–CH2–N–), 4.41–4.52 (m, 2H, N–CH2–CH2–C), 4.73 (q, J = 7.5 Hz 1H, N–CH–), 5.24–5.36 (m, 2H, N–CH2–CH2–C), 5.84–5.95 (m, 1H, N–CH2–CH2–C), 6.81 (s, 1H, 4-Hphenyl), 7.44 (s, 1H, 7-Hphenyl).
5.13 (m, broad, 1H, 8-H), 5.27-5.35 (m, 2H, N-CH2-CH=N=CH2), 5.83-5.94 (m, 1H, N-CH2-CH=N=CH2), 6.71 (s, 1H, 4-H), 6.93 (s, 1H, 9-H). IR (neat): \( \tilde{\nu} \) [cm\(^{-1}\)] = 3005 (w, vC=H arom.), 2918 (w, vC=H aliph.), 1772 (s, vC=O oxazolone), 1618 (m)/1495 (s, vC=O arom.), 1379 (s)/1186 (s, vSulfonamide).

*Deviating from the IUPAC rules the numbering of the isoquinoline ring was performed in analogy to the numbering of the oxazolobenzepinone system.

4.1.3.4 | 3-Allyl-8-methyl-5,6-dihydrooxazolo[4,5-h]-[3]benzepin-2,9(3H)-dione (12) Under N\(_2\) atmosphere, triflylketone 11 (50.3 mg, 0.12 mmol) was dissolved in dry acetonitrile (1.5 ml) and K\(_2\)CO\(_3\) (51.0 mg, 0.37 mmol) was added. The suspension was stirred at 60°C for 3 h. Then H\(_2\)O (5 ml) was added and the solution was extracted with CH\(_2\)Cl\(_2\) (3x). The combined organic layers were dried (Na\(_2\)SO\(_4\)) and the solvent was removed under reduced pressure giving a pale yellow oil as crude product, which was purified by flash column chromatography (Ø 2 cm, 16 cm, ethyl acetate/n-hexane 2:3 + 0.5%, N,N-dimethylethylamine, fraction size 10 ml, R\(_f\) (cis-14a) = 0.07, R\(_f\) (trans-14a) = 0.33).

cis-14a (R\(_f\) = 0.07): Colorless crystals, yield 126 mg (62%). Melting point 110–111°C. Purity: HPLC method 1; \( t_f \) = 17.8 min, purity 99.2%. C\(_{25}\)H\(_{30}\)N\(_2\)O\(_3\) (M\(_r\) = 406.5). MS (EI): m/z (%) = 406 (M, 7), 388 (M-H\(_2\)O, 6), 287 (M-CH\(_2\)-CH\(_2\)-CH\(_2\)H\(_4\), 44), 190 (Ar-CH\(_2\)Ar-NH-CH=CH\(_2\)), 100, 91 (Ar-CH\(_2\), 12). \(^1\)H NMR (CDCl\(_3\)): \( \delta \) [ppm] = 2.57 (s, 3H, CH\(_3\)), 2.70-2.76 (m, 2H, 6-H, 2H, 5-H), 4.45 (dt, J = 5.5/1.5 Hz, 2H, N-CH\(_2\)-CH=CH\(_2\)), 5.27-5.35 (m, 2H, N-CH\(_2\)-CH=CH\(_2\)), 5.84-5.95 (m, 1H, N-CH=CH=CH\(_2\)), 6.78 (s, 1H, 4-H), 7.75 (s, 1H, 10-H). IR (neat): \( \tilde{\nu} \) [cm\(^{-1}\)] = 3222 (w, vC=O ketone), 1611 (m)/1580 (w)/1496 (s, vC=C arom.).

trans-14a (R\(_f\) = 0.33): Colorless oil, yield 35.3 mg (17%). Purity: HPLC method 1; \( t_f \) = 17.9 min, purity 98.8%. C\(_{25}\)H\(_{30}\)N\(_2\)O\(_3\) (M\(_r\) = 406.5). MS (EI): m/z (%) = 406 (M, 10), 388 (M-H\(_2\)O, 7), 287 (M-Ar-CH\(_2\)-CH\(_2\)-CH\(_2\)H\(_4\), 37), 190 (Ar-CH\(_2\)-NCH=CH\(_2\)), 100, 91 (Ar-CH\(_2\), 12). \(^1\)H NMR (CDCl\(_3\)): \( \delta \) [ppm] = 0.67 (d, J = 6.8 Hz, 3H, CH\(_3\)), 1.54-1.63 (m, 2H, N-CH\(_2\)-CH=CH\(_2\)), 1.65-1.74 (m, 2H, N-CH\(_2\)-CH=CH\(_2\)-CH=CH\(_2\)Ph), 2.51-2.60 (m, 3H, 6-H, N-CH\(_2\)-CH=CH\(_2\)-CH=CH\(_2\)Ph), 2.63-2.71 (m, 4H, 5-H/6-H, N-CH\(_2\)-CH=CH\(_2\)-CH=CH\(_2\)Ph), 3.17-3.39 (m, 2H, 5-H/8-H), 4.37 (d, J = 6.0 Hz, 1H, 9-H), 4.39-4.44 (m, 2H, N-CH\(_2\)-CH=CH\(_2\)), 5.25-5.32 (m, 2H, N-CH\(_2\)-CH=CH\(_2\)), 5.84-5.94 (m, 1H, N-CH=CH=CH\(_2\)), 6.66 (s, 1H, 4-H), 6.98 (s, 1H, 10-H). 7.17-7.22 (m, 3H, 2H, 4-H/5-H/Pheny1). A signal for the OH proton is not found in the \(^1\)H NMR spectrum. IR (neat): \( \tilde{\nu} \) [cm\(^{-1}\)] = 3364 (w, vC=O ketone), 1678 (s, vC=O oxazolone), 1619 (w)/1603 (w)/1491 (s, vC=C arom.).

4.1.3.5 | (8RS,9RS)-3-Allyl-9-hydroxy-8-methyl-7-[4-phenylbutyl]-3,5,6,7,8,9-hexahydrooxazolo[4,5-h]-[3]benzepin-2-one (cis-14a) and (8RS,9SR)-3-allyl-9-hydroxy-8-methyl-7-[4-phenylbutyl]-3,5,6,7,8,9-hexahydrooxazolo[4,5-h]-[3]benzepin-2-one (trans-14a)

Under the N\(_2\) atmosphere, triflylketone 11 (202 mg, 0.50 mmol) was dissolved in CH\(_2\)CN (25 ml) and K\(_2\)CO\(_3\) (208 mg, 1.50 mmol) was added. The suspension was stirred at 65°C for 2 h. Then H\(_2\)O (20 ml) was added and the mixture was extracted with CH\(_2\)Cl\(_2\) (3x). The combined organic layers were dried (Na\(_2\)SO\(_4\)) and the solvent was removed under reduced pressure giving a pale yellow oil. To the crude intermediate (13) a solution of 1-iodo-4-phenylbutyrate in acetonitrile (10 ml) was added, which was prepared as follows:

1-Chloro-4-phenylbutyrate (127 mg, 0.75 mmol) and Nal (134.9 mg, 0.90 mmol) were dissolved in acetonitrile (2 ml) and the mixture was irradiated in the microwave (220 W, 90°C, 4 bar, 20 min). Then, the mixture was filtered and the solvent was removed completely under reduced pressure giving 1-iodo-4-phenylbutyrate as crude product, which was dissolved in acetonitrile (10 ml) and the solution was added to the reaction mixture. After addition of K\(_2\)CO\(_3\) (208 mg, 1.50 mmol), the suspension was stirred at 80°C overnight. Then, H\(_2\)O (15 ml) was added and the solution was extracted with CH\(_2\)Cl\(_2\) (3x). The combined organic layers were dried (Na\(_2\)SO\(_4\)) and the solvent was removed under reduced pressure giving a pale yellow oil as crude product, which was purified by flash column chromatography (Ø 2 cm, 16 cm, ethyl acetate/n-hexane 2:3 + 0.5%, N,N-dimethylethylamine, fraction size 10 ml, R\(_f\) (cis-14b) = 0.07, R\(_f\) (trans-14b) = 0.33).

4.1.3.6 | (8RS,9RS)-3-allyl-9-hydroxy-8-methyl-7-[4-(piperidin-1-yl)-benzyl]-3,5,6,7,8,9-hexahydrooxazolo[4,5-h]-[3]benzepin-2-one (cis-14b) and (8RS,9SR)-3-allyl-9-hydroxy-8-methyl-7-[4-(piperidin-1-yl)-benzyl]-3,5,6,7,8,9-hexahydrooxazolo[4,5-h]-[3]benzepin-2-one (trans-14b)

Under the N\(_2\) atmosphere, triflylketone 11 (203 mg, 0.50 mmol) was dissolved in CH\(_2\)CN (30 ml) and K\(_2\)CO\(_3\) (209 mg, 1.51 mmol) was added. The suspension was stirred at 65°C for 2 h. Then H\(_2\)O (15 ml) was added and the mixture was extracted with CH\(_2\)Cl\(_2\) (3x). The combined organic layers were dried (Na\(_2\)SO\(_4\)) and the solvent was removed.
removed under reduced pressure until a residue of around 3 mL. To this mixture (12) methanol (6 mL) and NaBH₄ (19.0 mg, 0.50 mmol) were added and stirring was continued at rt for 1 h. Then, H₂O (20 mL) was added and the mixture was extracted with CH₂Cl₂ (10x). The combined organic layers were dried (Na₂SO₄) and the solvent was removed completely under reduced pressure giving a pale yellow oil (13). To the crude intermediate, CH₂Cl₂ (5 mL), 4-(piperidin-1-yl)benzaldehyde (120 mg, 0.63 mmol), and NaBH₄ (OAc)₂ (212.6 mg, 1.0 mmol) were added and the mixture was stirred at rt overnight. Then, K₂CO₃ solution (20 mL, 5%) was added and the aqeous layer was extracted with CH₂Cl₂ (3x). The combined organic layers were dried (Na₂SO₄) and the solvent was removed under reduced pressure giving a pale yellow oil as crude product, which was purified by flash column chromatography (Ø 2 cm, 20 cm, ethyl acetate/n-hexane 1:2 or 0.5% N,N-dimethylaniline, fraction size 10 mL, R₀ = 0.08). Colorless crystals, yield 20.7 mg (46%). Melting point 167–168°C. Purity: HPLC method 1: t_R = 16.3 min, purity 99.00%, C₂₂H₂₂N₂O₃ (M = 366.5). MS (EI): m/z (%) = 366 (M, 5), 348 (M−H₂O, 22), 247 (M−Ar−CH₂−CH₂−CH₃), 190 (Ar−(CH₂)₂−N(CH−CH₃)(CH₃), 100), 91 (Ar−CH₂−CH₂−Ph), 2.69−2.79 (m, 2H, 5−H/6−H), 2.95−3.05 (m, 1H, 5−H), 3.07−3.14 (m, 1H, 8−H), 5.09 (broad, 1H, 9−H), 6.76 (s, 1H, 4−H), 7.15−7.21 (m, 3H, 2−H/4−H/6−Hoxazolone), 7.25−7.35 (m, 3H, 10−H, 3−H/5−Hoxazolone) Signals for the OH- and NH-protons are not seen in the H NMR-spectrum. IR (neat): v [cm⁻¹] = 3414 (v, ν_C O oxazolone), 1472 (s, ν_C=O oxazolone), 1489 (s, ν_C=C arom.), 748 (m)/699 (s, ν_C=N arom.).

4.1.3.8 | cis-9-Hydroxy-8-methyl-7-(4-phenylbutyl)-3,5,6,7,8,9-hexahydroazoxazolo[4,5-h][3]benzazepin-2-one (trans-6a)
Under N₂ atmosphere, trans-14a (47.8 mg, 0.12 mol) was dissolved in propan-2-ol (1 mL) and the mixture was heated to 85°C. A solution of RhCl₃·3H₂O (3.1 mg, 0.01 mmol) in propan-2-ol (0.5 mL) was added and stirring was continued at 85°C for 3 h. Then conc. HCl (0.5 mL) was added and the solution was heated at 85°C under stirring for 3 h. Then saturated K₂CO₃ solution was added until slightly basic reaction and the aqeous layer was extracted with CH₂Cl₂ (3x). The combined organic layers were dried (Na₂SO₄) and the solvent was removed under reduced pressure giving a brown oil as crude product, which was purified by flash column chromatography (Ø 1 cm, 12 cm, ethyl acetate/n-hexane 4:1 or 0.5% N,N-dimethylaniline, fraction size 10 mL, R₀ = 0.08). Colorless crystals, yield 20.7 mg (46%). Melting point 167–168°C. Purity: HPLC method 1: t_R = 16.3 min, purity 99.00%, C₂₂H₂₂N₂O₃ (M = 366.5). MS (EI): m/z (%) = 366 (M, 5), 348 (M−H₂O, 22), 247 (M−Ar−CH₂−CH₂−CH₃), 190 (Ar−(CH₂)₂−N(CH−CH₃)(CH₃), 100), 91 (Ar−CH₂−CH₂−Ph), 2.69−2.79 (m, 2H, 5−H/6−H), 2.95−3.05 (m, 1H, 5−H), 3.07−3.14 (m, 1H, 8−H), 5.09 (broad, 1H, 9−H), 6.76 (s, 1H, 4−H), 7.15−7.21 (m, 3H, 2−H/4−H/6−Hoxazolone), 7.25−7.35 (m, 3H, 10−H, 3−H/5−Hoxazolone) Signals for the OH- and NH-protons are not seen in the H NMR-spectrum. IR (neat): v [cm⁻¹] = 3414 (v, ν_C O oxazolone), 1472 (s, ν_C=O oxazolone), 1489 (s, ν_C=C arom.), 748 (m)/699 (s, ν_C=N arom.).
1768 (s, V=O oxazolone), 1585 (w)/1493 (s, V=C arom.,) 749 (m)/699 (s, Ymonosubst. arom.,)

4.1.3.9 | cis-9-Hydroxy-8-methyl-7-[4-(piperidin-1-yl)-benzyl]-3,5,6,7,8,9-hexahydrooxazolo[4,5-h]-[3]benzazepin-2-one (cis-6b)
Under N2 atmosphere, cis-14b (108 mg, 0.24 mol) was dissolved in propan-2-ol (5 ml) and the mixture was heated to 85°C. A solution of RhCl3·3H2O (6.4 mg, 0.02 mmol) in propan 2-ol (1 ml) was added and stirring was continued at 85°C for 29 h. Then conc. HCl (2 ml) was added and the solution was heated at 85°C under stirring for 3 h. Then saturated K2CO3 solution was added until a slightly basic reaction and the aqueous layer was extracted with CH2Cl2 (3x). The combined organic layers were dried (Na2SO4) and the solvent was removed under reduced pressure giving a brown oil as crude product, which was purified by flash column chromatography (ø 1 cm, 15 cm, ethyl acetate/n-hexane 3:1 + 0.5% N,N-dimethylethamamine, fraction size 10 ml, Rf = 0.13). Colorless crystals, yield 16.6 mg (17%). Melting point 189°C. 
Purity: Elemental analysis: Calcd. C 70.37 H 7.24 N 10.30. Compound was not stable during our standard HPLC purity analysis.

4.2 | Receptor binding studies
4.2.1 | Materials
Guinea pig brains, rat brains, and rat livers were commercially available (Harlan–Winkelmann). Pig brains were a donation from the local slaughterhouse. The recombinant L(tk–) cells stably expressing the GluN2B receptor were obtained from Prof. Dr. Dieter Steinhilber. Homogenizers: Elvejem Potter (B. Braun Biotech International) and Soniprep® 150 (MSE). Centrifuges: Cooling centrifuge Eppendorf 5424R (Eppendorf) and high-speed cooling centrifuge model Sorvall® RC-5C plus (Thermo Fisher Scientific). Multiplates: standard 96-well multiplates (Diagonal). 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).

4.2.2 | Cell culture and preparation of membrane homogenates for the GluN2B assay[45]
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 containing 10% of standardized fetal calf serum (Biochrom AG). 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 a 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), harvested by mechanical detachment, and pelleted (10 min, 5000g).

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). Subsequently, the cells were lysed by sonication (4°C, 6 × 10 s cycles with breaks of 10 s). The resulting cell fragments were centrifuged with a high-performance cool centrifuge (23,500g, 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 × 10 s cycles with a break of 10 s) and stored at −80°C.

4.2.3 | Preparation of membrane homogenates from pig brain cortex[48,49]

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 1200g for 10 min at 4°C. The supernatant was separated and centrifuged at 31,000 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 31,000g (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.

4.2.4 | General protocol for the binding assay

The test compound solutions were prepared by dissolving approximately 10 µmol (usually 2–4 mg) of the test compound in dimethyl sulfoxide (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 room temperature before use. All binding experiments were carried out in duplicates in 96-well multiplates. The concentrations given are the final concentrations in the assay. Generally, the assays were performed by addition of 50 µl of the respective assay buffer, 50 µl test compound solution in various concentrations (10⁻⁵, 10⁻⁶, 10⁻⁷, 10⁻⁸, 10⁻⁹, and 10⁻¹⁰ mol/L), 50 µl of 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 filtering 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 the scintillator at room temperature, 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 [³H]-counting protocol. The overall counting efficiency was 20%. The IC₅₀ values were calculated with the program GraphPad Prism® 3.0 (GraphPad Software) by nonlinear regression analysis. Subsequently, the IC₅₀ values were transformed into Kᵢ-values using the equation of Cheng and Prusoff.[50] The Kᵢ-values are given as mean value ± SEM from three independent experiments.

4.2.5 | Performance of the GluN2B binding assay[45]

The competitive binding assay was performed with the radioligand [³H]-ifenprodil (60 Ci/mmol; BIOTREND). 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 [³H]-ifenprodil, and Tris/EDTA-buffer (5 mM Tris/1 mM EDTA, pH 7.5) at 37°C. The nonspecific binding was determined with 10 µM unlabeled ifenprodil. The Kᵢ value of ifenprodil is 7.6 nM.

4.2.6 | Performance of the PCP-binding assay[48,49]

The assay was performed with the radioligand [³H]-(+)-MK-801 (22.0 Ci/mmol; Perkin Elmer). The thawed membrane preparation of pig brain cortex (about 100 µg of the protein) was incubated with various concentrations of test compounds, 2 nM [³H]-(+)-MK-801, and Tris/EDTA buffer (5 mM Tris/1 mM EDTA, pH 7.5) at room temperature. The nonspecific binding was determined with 10 µM unlabeled (+)-MK-801. The Kᵢ value of (+)-MK-801 is 2.26 nM.

4.2.7 | Performance of σ₁ and σ₂ receptor assays[51–53]

The interaction with σ₁ and σ₂ receptors was performed as described in Meyer and colleagues.[51–53]

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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