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Stereocontrolled Synthesis and Pharmacological Evaluation of Azetidine-2,3-Dicarboxylic Acids at NMDA Receptors

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*The four stereoisomers of azetidine-2,3-dicaroxylic acid (*L*-trans-ADC, *L*-cis-ADC, *D*-trans-ADC, and *D*-cis-ADC) were synthesized in a stereocontrolled fashion following two distinct strategies: one providing the two* cis*-ADC enantiomers and one giving access to the two* trans*-ADC enantiomers. The four azetidinic amino acids were characterized in a radioligand binding assay ([*^{3}*H]CGP39653) at native NMDA receptors: L-trans-ADC showed the highest affinity ($K_i = 10\ \mu M$) followed by the D-cis-ADC stereoisomer (21 μM). In contrast, the two analogues L-cis-ADC and D-trans-ADC were low-affinity ligands (> 100 and 90 μM, respectively). Electrophysiological characterization of the ADC compounds at the four NMDA receptor subtypes NR1/NR2A, NR1/NR2B, NR1/NR2C, and NR1/NR2D expressed in* Xenopus *oocytes showed that L-trans-ADC displayed the highest agonist potency at NR1/NR2D ($EC_{50} = 50\ \mu M$), which was 9.4-, 3.4-, and 1.9-fold higher than the respective potencies at NR1/NR2A–C. D-cis-ADC was shown to be a partial agonist at NR1/NR2C and NR1/NR2D with medium-range micromolar potencies ($EC_{50} = 720$ and 230 μM, respectively). A subsequent in silico ligand–protein docking study suggested an unusual binding mode for these amino acids in the agonist binding site.*

Introduction

Fast excitatory neurotransmission is a neurological process during which (*S*)-glutamate (Glu, Figure 1) is released from the presynaptic membrane, diffuses across the synaptic cleft, and activates ligand-gated ion channels located at the postsynaptic membrane. These ligand-gated ion channels are known as the ionotropic glutamate receptors (iGluRs) and are further divided into three functional groups: *N*-methyl-D-aspartate (NMDA) receptors, 2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl)propionate (AMPA) receptors, and kainate receptors (Figure 1).[1] The NMDA receptors play pivotal roles in fast glutamatergic neurotransmission and are critically involved in many important neuronal functions including frequency encoding of information,[2] synaptic plasticity,[3] and neuronal development.[4] Under a variety of acute conditions such as ischemia, seizures, or traumatic brain injury, the release of excess glutamate and the resultant NMDA receptor-mediated Ca^{2+} flux into the cell may be of sufficient magnitude to promote neuronal death (excitotoxicity).[5] Under chronic conditions of enhanced neuronal susceptibility, as in Parkinson's, Huntington's, and Alzheimer's diseases, the potential involvement of NMDA receptor-mediated excitotoxicity may be of a slower process.[6–9] For these reasons, there has been an extensive interest in understanding the structure, function, localization, and regulation of NMDA receptors with the goal of designing new therapeutic strategies for a number of diseases.

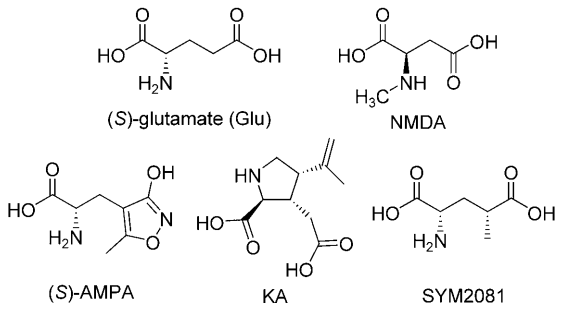

Figure 1. (*S*)-Glutamate (Glu) and ionotropic glutamate receptor (iGluR) ligands: *N*-methyl-D-aspartic acid (NMDA), 2-amino-3-(3-hydroxy-5-methyl-4-isoxazolyl)propionic acid (AMPA), kainic acid (KA), and (2*S*,4*R*)-methylglutamate (SYM2081).

Functional NMDA receptors are assembled from two NR1 subunits and two NR2 subunits and are activated by the simultaneous binding of glycine and glutamate to the NR1 and NR2 subunits, respectively.[10] One NR1 subunit and four different

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NR2 subunits (NR2A, NR2B, NR2C, and NR2D) have been identified, and these different NR2 subunits determine the physiological role of the NMDA receptor subtype.[11] In response to agonist binding, NMDA receptors undergo conformational changes that open a cation-conducting channel pore. The time course of these conformational changes differs considerably among the NMDA receptor subtypes (NR1/NR2A, NR1/NR2B, NR1/NR2C, and NR1/NR2D), and these differences influence the amplitude and time course of excitatory postsynaptic currents at glutamatergic synapses. Progress toward understanding this functional variation among the NMDA receptor subtypes and the increasingly precise anatomical localization of NR2 subunits have strengthened the therapeutic interest in the development of subunit-selective NMDA receptor agonists of which only few exist.

Herein we present the stereoselective synthesis of the four stereoisomers of azetidine-2,3-dicarboxylic acid, subsequent radioligand binding experiments, and electrophysiological recordings at recombinant NMDA receptors. Finally, we conducted an in silico study and put forward new hypotheses as to how the rational design of potentially subtype-selective NMDA receptor agonists may be attained.

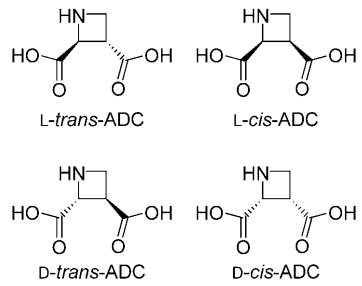
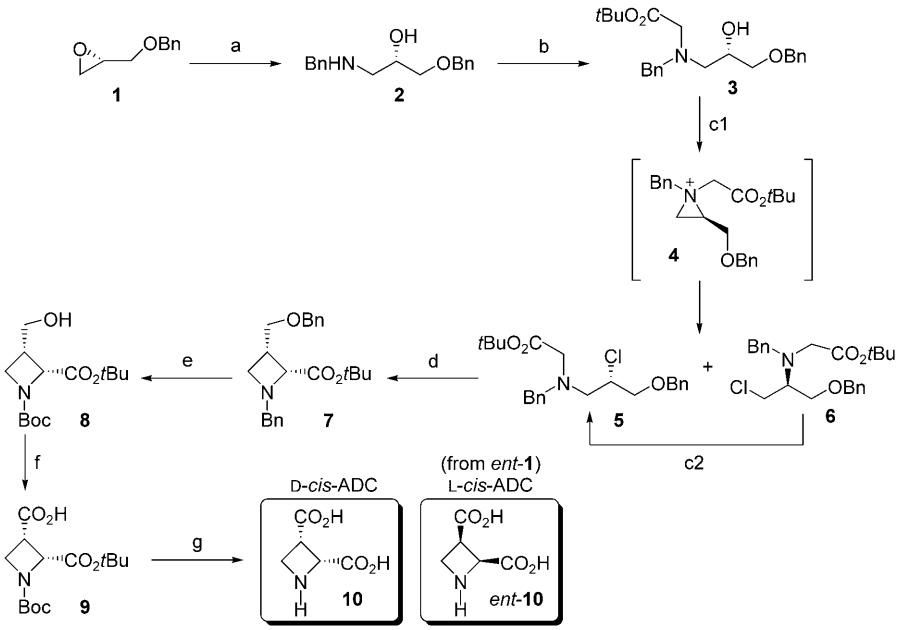


Figure 2. The four stereoisomers of azetidine-2,3-dicarboxylic acid: L-*trans*-ADC, L-*cis*-ADC, D-*trans*-ADC and D-*cis*-ADC.



Scheme 1. Reagents and conditions: a) $BnNH_2$, H_2O (cat.), 60 °C, 5 h (80%); b) *tert*-butylbromoacetate, $NaHCO_3$, NaI, DMF, RT, 5 h (70%); c) 1) $SOCl_2$, CH_2Cl_2, reflux, 3 h, 2) DMF, 65 °C (90%); d) LiHMDS, THF/HMPA, −78→0 °C (60%); e) H_2 (15 bar), Pd/C (20% wt. cat.), EtOH, Boc_2O (96%); f) $RuCl_3$ (cat.), $NaIO_4$, MeCN/CCl_4/H_2O (1:1:1) (71%); g) TFA/CH_2Cl_2, RT, 12 h then Dowex 50WX8-200 (90%).

Results and Discussion

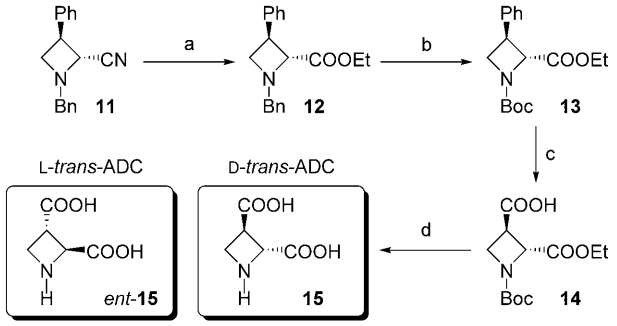
Design of azetidines

Azetidine-2,3-dicarboxylic acids (ADCs, Figure 2) can be envisaged as highly conformationally restricted analogues of NMDA and are thus potential agonists at NMDA receptors. Whereas azetidinic α-amino acids have been prepared through several diverse strategies,[12] the class of ADCs has only been synthesized by a stereoselective strategy,[13] and by an approach in which an electrocyclic reaction was the key step.[14] Furthermore, the pharmacology of this class of amino acids has only been investigated briefly at native excitatory amino acid transporters (EAATs) at which only the L-*trans*-ADC stereoisomer showed affinity in the mid-micromolar range.[13]

Chemistry

We developed two distinct synthetic strategies for the synthesis of the *cis* stereoisomers and the *trans* stereoisomers (Schemes 1 and 2, respectively). The stereospecific synthesis of D-*cis*-ADC (Scheme 1) commenced with the reaction of (*R*)-*O*-benzylglycidol **1** with benzylamine to give amino alcohol **2**.[13] This compound was N-alkylated with *tert*-butylbromoacetate, and the resulting alcohol **3** was chlorinated with thionyl chloride. As previously described with similar substrates,[12] this chlorination gave a mixture of regioisomeric chlorides **5** and **6** via aziridinium intermediate **4** that were next equilibrated by heating in DMF to afford the more stable secondary chloride **5** in good overall yield. This sequence has been shown to occur without racemization with similar substrates.[15] Upon treatment with LiHMDS, this chloride was closed to the corresponding 2,3-*cis*-azetidine **7** with complete diastereoselectivity and a fair yield of 60%. Azetidine **7** was subsequently N- and O-debenzylated and *N*-Boc protected in one step to afford **8** in very good yield. The free primary alcohol was next oxidized into the corresponding carboxylic acid[16] to give **9**, which, upon treatment with TFA followed by ion-exchange chromatography, gave a good yield of the target amino acid **10**. The same strategy was applied to the synthesis of *ent*-**10** starting from *ent*-**1**, with similar yields.

The D-*trans*-ADC isomer was synthesized by following a different pathway, depicted in Scheme 2. The known cyanoazetidine **11**,[17] easily prepared from (*R*)-phenylglycinol as the



Scheme 2. Reagents and conditions: a) H_2SO_4, EtOH, reflux, 12 h (89%); b) H_2 (1 bar), Pd/C (10% wt. cat.), Boc_2O, EtOH, 6 days (quant.); c) $NaIO_4$, $RuCl_3$ (cat.), $MeCN/CCl_4/H_2O$ (1:1:1), RT, 48 h (75%); d) NaOH, $MeOH/H_2O$ (1:1), RT, 48 h then HCl (1 M), RT, 12 h then Dowex 50WX8-200 (72%).

source of chirality, was converted into the ethyl ester **12** by treatment with concentrated sulfuric acid in ethanol, followed by aqueous workup. No epimerization was observed during these quite harsh conditions. Next, the *N*-benzyl protecting group was cleaved, with in situ reprotection as a *tert*-butyl carbamate to give **13**. The 3-carboxylic acid functionality was unveiled by oxidation of the phenyl ring with sodium periodate in the presence of a catalytic amount of ruthenium chloride[18] to give the expected acid **14** in 75% yield. The final deprotection to give D-*trans*-ADC **15** was effected in one pot: Saponification of the ethyl ester with sodium hydroxide followed by acidification with 1 M hydrochloric acid gave the amino diacid in its protonated form, and the zwitterionic amino acid was obtained in good yield after ion-exchange chromatography. The same sequence was conducted by starting with *ent*-**11** (prepared from (*S*)-phenylglycinol) and gave *ent*-**15** with similar yields.

Pharmacology

First, the affinities of the four stereoisomeric ADCs for the glutamate binding site of native NMDA receptors were determined in radioligand binding assays (rat brain synaptosomes). Summarized in Table 1, L-*trans*-ADC and L-*cis*-ADC displayed affinities in the low-micromolar range (10 and 21 μM, respectively), whereas D-*trans*-ADC and L-*cis*-ADC had essentially no affinity.

Next, we evaluated the four ADC analogues in a functional assay for agonist activity at the four different heteromeric NMDA receptor subtypes (NR1/NR2A–D). The NMDA receptor subtypes were expressed in *Xenopus* oocytes, and responses in current to the application of various concentrations of the compounds were recorded using two-electrode voltage-clamp electrophysiology. Of the four stereoisomeric ADC analogues, only L-*trans*-ADC was able to (partially) activate NR1/NR2A. Activation of subtypes NR1/NR2B, NR1/NR2C, and NR1/NR2D was also observed, with the highest potency at NR1/NR2D. With respect to subtype selectivity, L-*trans*-ADC showed a notable 9.4-fold preference for NR2D over NR2A, whereas the EC_{50} values at NR2B and NR2C were within the same range as that of NR2D (170, 95, and 50 μM, respectively). In contrast, L-*cis*-ADC was unable to activate NR1/NR2A and NR1/NR2B, and only at high concentrations (1000 μM) could some activation of NR1/NR2C and NR1/NR2D subtypes be detected. D-*trans*-ADC and D-*cis*-ADC activated NR1/NR2B, NR1/NR2C, and NR1/NR2D subtypes, with the highest potency at NR1/NR2D. All active compounds for which concentration–response data were obtained were partial agonists at the NMDA receptor subtypes, as they display sub-maximal activation relative to glutamate. The concentration–response data are shown in Figure 3, and the EC_{50} values and maximal responses relative to glutamate are summarized in Table 2.

Table 1. Binding affinities of azetidines at native NMDA receptors (rat brain synaptosomes).

Compound	K_i [μM][a]	Mean pK_i ± SEM[a]
SYM2081[b]	5.9	–
NMDA[c]	6.2	–
L-*trans*-ADC	10	5.04 ± 0.10
L-*cis*-ADC	> 100	–
D-*trans*-ADC	90	4.05 ± 0.03
D-*cis*-ADC	21	4.74 ± 0.13

[a] Radioligand ([^{3}H]CGP39653) binding data are reported the as mean of 3–5 individual experiments. [b] Value for SYM2081 taken from reference [19]. [c] Value for NMDA taken from reference [20].

In silico docking study

To better understand the binding mode and subtype activity of these NMDA receptor agonists, we performed a docking study using the recently published X-ray crystal structure of the agonist binding domain (ABD) of NR2A.[22] In this structure (Figure 4A), Glu connects the upper and lower domain of the ABD. A grid of the ABD was created around glutamate excluding all water molecules, and the ligands displaying activity were docked (flexibly) in this grid. The best-scoring binding modes were subsequently minimized with the ABD, leaving the ligand and amino acid side chains flexible, but restricting the movements of the protein backbone. This minimization did not significantly alter the position of the side chains or the ligand. The binding modes are shown together with that of Glu in Figure 4. In the crystal structure, the α-carboxylic acid of Glu makes a bidentate electrostatic interaction with R518 (Figure 4B). This is a general characteristic of all amino acid analogues that have been crystallized with ABDs of GluRs.[10] The distal carboxylic acid group of Glu is bound by hydrogen bonds. Surprisingly, the three ligands docked inversely to Glu, with the distal carboxylic acid group in a bidentate interaction with R518 (Figure 4B–D) and the α-carboxylic acid of the ADC ligands overlaid with the distal carboxylic acid group of Glu. We also docked the ligands including two water molecules im-

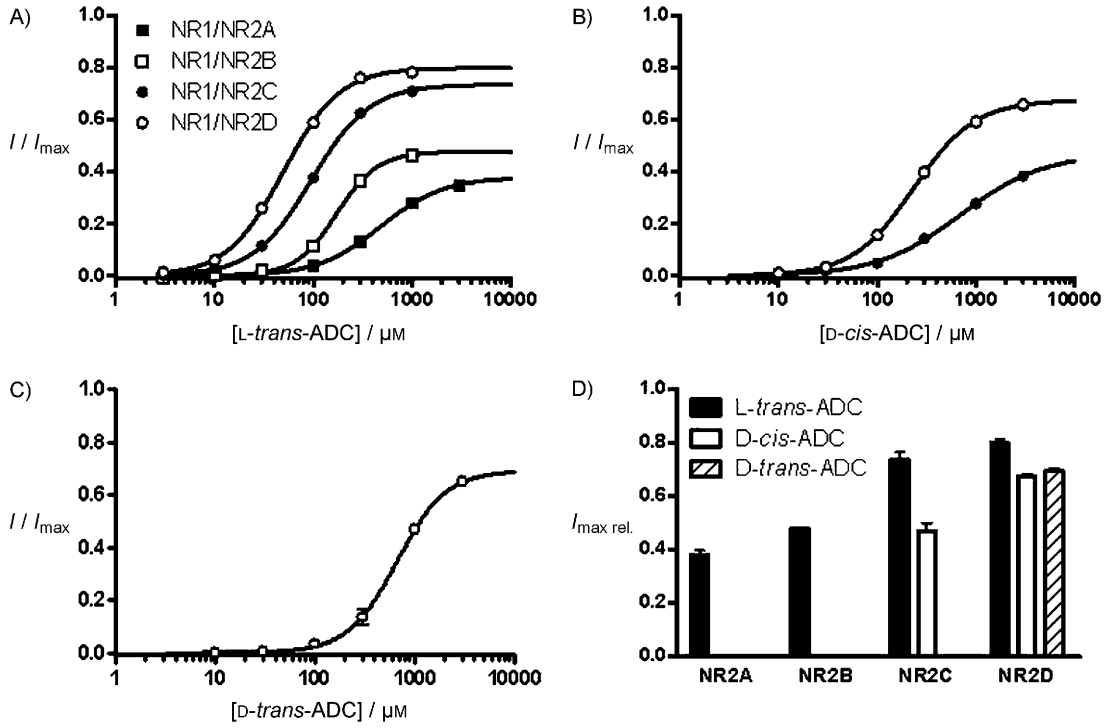


Figure 3. Mean concentration–response curves for ADC analogues: A) L-*trans*-ADC, B) D-*cis*-ADC, and C) D-*trans*-ADC determined using two-electrode voltage-clamp recordings on *Xenopus* oocytes expressing NMDA receptor subtypes NR1/NR2A–D. The curves are normalized to the maximal current response (I_{max}) to glutamate in the same recording. Data points are represented as mean ± SEM. All EC_{50} values are listed in Table 2. D) Comparison of maximal currents induced by D-*trans*-ADC, D-*cis*-ADC, and L-*trans*-ADC relative to glutamate at NR1/NR2A–D subtypes. All bars are represented as mean + SEM. All relative I_{max} values are listed in Table 2.

Table 2. Characterization of ADC analogues at recombinant NMDA receptor subtypes NR1/NR2A–D expressed in *Xenopus* oocytes using electrophysiological recordings.[a]

Compound	NR1/NR2A		NR1/NR2B		NR1/NR2C		NR1/NR2D	
	EC_{50} [μM] (pEC_{50})	I/I_{max}	EC_{50} [μM] (pEC_{50})	I/I_{max}	EC_{50} [μM] (pEC_{50})	I/I_{max}	EC_{50} [μM] (pEC_{50})	I/I_{max}
NMDA[b]	75 (4.13±0.07)	0.90±0.04	22 (4.66±0.03)	0.77±0.01	23 (4.63±0.02)	0.73±0.02	8.3 (5.08±0.04)	0.80±0.02
L-*trans*-ADC	470 (3.32±0.03)	0.38±0.02	170 (3.76±0.01)	0.48±0.01	95 (4.02±0.02)	0.73±0.03	50 (4.30±0.02)	0.80±0.01
L-*cis*-ADC	NR[c]	ND[d]	NR	ND	>1000	ND	>1000	ND
D-*trans*-ADC	NR	ND	>1000	ND	>1000	ND	660 (3.18±0.02)	0.69±0.01
D-*cis*-ADC	NR	ND	>3000	ND	720 (3.15±0.06)	0.47±0.03	230 (3.63±0.01)	0.67±0.01

[a] Mean pEC_{50} ± SEM (shown in parentheses) and the corresponding EC_{50} values as well as the mean relative maximal response (I/I_{max} ± SEM) (relative to maximal response to glutamate; 1.0) were calculated from full concentration–response curves. For all agonist data, the Hill coefficients were between 1.1 and 2.1, and the number of oocytes was between 5 and 10. [b] Data from reference [21]. [c] NR indicates no response up to 1000 μM. [d] ND indicates that the relative maximal response was not determined.

portant for Glu binding.[22] However, the general binding mode with the amino group in the same position as that of Glu (Figure 4E) was only observed for D-*cis*-ADC. An X-ray crystallographic study of these new ligands in the ABD of the NMDA receptors would further address the validity of suggested inverse binding modes.

The inverse binding mode is probably due to a close contact (~3 Å for D-*cis*-ADC and L-*trans*-ADC, and 5 Å for D-*trans*-ADC) between the positively charged amino group of the ADC ligands and Y730 (Figure 4F), which further mediates an interdomain contact to E413. Y730 is also proximal to V715, which is part of a short sequence containing several nonconserved residues among NR2A–D. Thus, the altered conformation of Y730 could affect V715 and may explain the activity variance observed at these subtypes.

In a recent study, mutagenesis of Y730 to phenylalanine in NR2A decreased the potency of glutamate by 45-fold, thereby supporting the idea that an interdomain contact predicted

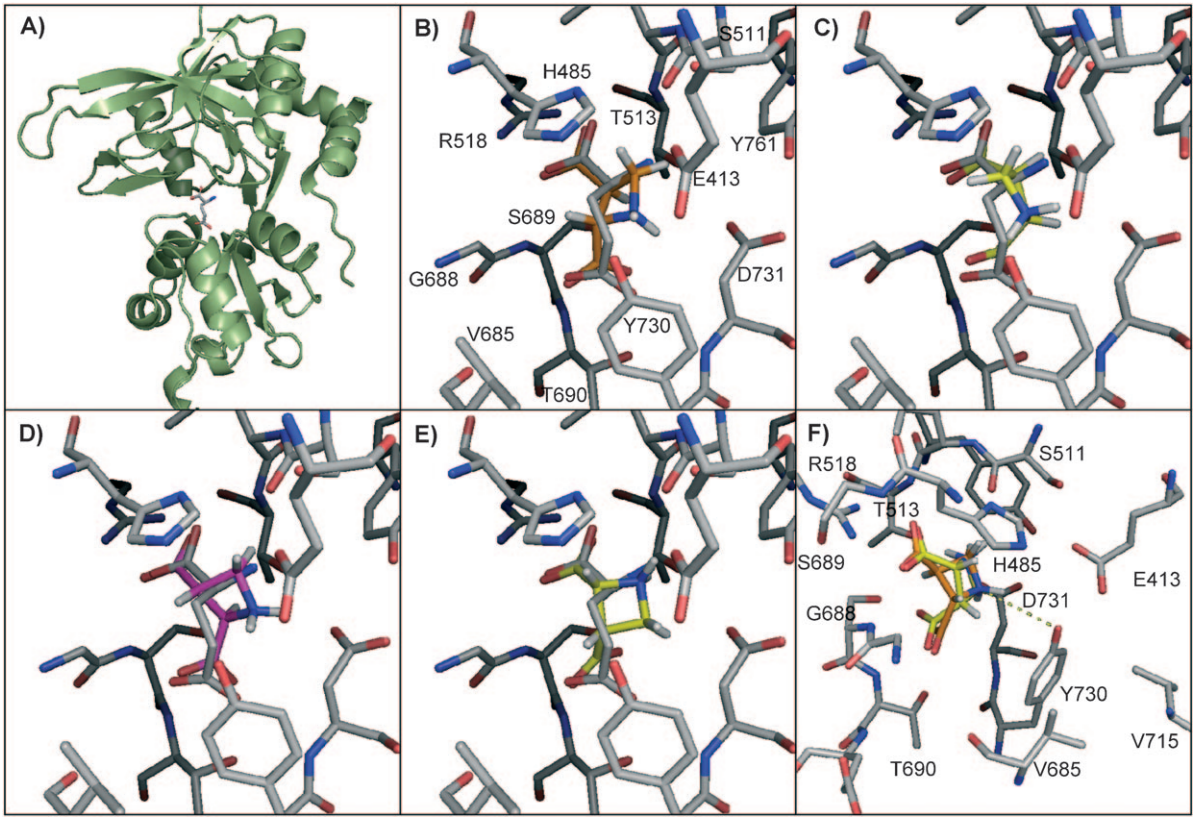


Figure 4. A) X-ray crystal structure of ABD of NR2A with Glu bound. Proposed binding mode of B) L-*trans*-ACD (orange), C) D-*cis*-ADC (yellow), and D) D-*trans*-ADC (purple) in the NR2A agonist binding site relative to Glu (gray). E) Alternative binding mode of D-*cis*-ADC (yellow) when docked with two water molecules in the binding site. F) View of proposed binding mode of L-*trans*-ACD (orange) and D-*cis*-ADC (yellow) rotated 90° to the right, highlighting a possible interaction with Y730.

from molecular dynamics simulations between E413 and Y730 in NR2A contributes to agonist potency.[23] In the same study, the decrease in glutamate potency was not observed when this potential interdomain contact was disrupted in NR2D (Y732F mutation in NR2D). In fact, the potency of glutamate was slightly increased in NR2D(Y732F). Furthermore, the glutamate analogue (2*S*,4*R*)-4-methylglutamate (SYM2081) displays a 46-fold higher potency at NR1/NR2D over NR1/NR2A that can be explained by steric clash between the methyl group of SYM2081 and Y730 in NR2A.[23] These data argue that the E413:Y730 interdomain hydrogen bonds can stabilize the active conformation of the ABD and thus influence the energetics underlying agonist binding to the NR2A subunit. It is possible that the close contact between the positively charged amino group of the ADC ligands and Y730 in NR2A disrupts the conformation of Y730 and possibly the predicted E413:E730 interdomain contact as well, thereby decreasing the ability of the ADC ligands to activate NR1/NR2A.

Conclusions

We have synthesized the four stereoisomers of ACD (L-*trans*-ADC, L-*cis*-ADC, D-*trans*-ADC, and D-*cis*-ADC) in a stereocontrolled fashion by following distinct strategies for the *cis*-ADC and the *trans*-ADC enantiomers. The four ACDs were investigated in a radioligand binding assay ([^{3}H]CGP39653) at native NMDA receptors and subsequently characterized as potential agonists at the four NMDA receptor subtypes NR1/NR2A, NR1/NR2B, NR1/NR2C, and NR1/NR2D. Most notable was L-*trans*-ADC, which showed the highest potency at NR1/NR2D (EC_{50} = 50 μM), with a 9.4-, 3.4-, and 1.9-fold preference over NR1/NR2A–C, respectively. Subsequent in silico ligand–protein docking suggested an unusual binding mode for these amino acids in the agonist binding site.

Experimental Section

Chemistry

General: ^{1}H and ^{13}C NMR spectra (in $CDCl_3$ unless otherwise stated) were recorded on a Bruker AC 200 or 300 spectrometer at 200, 300 (^{1}H); 50.3, 75.5 MHz (^{13}C). Chemical shifts are reported in ppm from tetramethylsilane. Optical rotations were determined with a PerkinElmer 241 instrument. All reactions were carried out under argon. Column chromatography was performed on silica gel 230–400 mesh by using various mixtures of diethyl ether (Et_2O), ethyl acetate (EtOAc), cyclohexane (*c*Hex), and petroleum ether (PE). Thin-layer chromatography (TLC) was carried out with Merck Kieselgel 60 F_{254} plates. Melting points are uncorrected. THF was distilled from sodium/benzophenone ketyl. Dichloromethane and triethylamine were distilled from calcium hydride. Hexamethylphosphoramide (HMPA) was distilled before use. Other reagents were used as purchased. Mention of "usual workup" means: 1) decantation of the organic layer, 2) extraction of the aqueous layer with Et_2O,

3) washing the combined organic layers with brine and drying of the combined organic phases over $MgSO_4$, and 4) solvent evaporation under reduced pressure. Compositions of stereoisomeric mixtures were determined by NMR analysis of crude products before any purification. HRMS was performed at the "Service Central d'Analyses du CNRS" (Vernaison, France). Mass spectra were recorded on a GC–MS HP MS 5989B spectrometer at the University of Versailles.

(2*R*)-[Benzyl-(3-benzyloxy-2-hydroxypropyl)amino]acetic acid *tert*-butyl ester 3 and *ent*-3. *tert*-Butylbromoacetate (3.1 mL, 20.66 mmol) was added dropwise to a solution of the β-amino alcohol **2**[24] (2.8 g, 10.33 mmol), NaI (3.1 g, 20.66 mmol), and $NaHCO_3$ (1.73 g, 20.66 mmol) in *N*,*N*-dimethylformamide (DMF, 50 mL). The suspension was stirred at room temperature for 12 h and was then poured into a 1:1 mixture of H_2O and Et_2O. Usual workup gave a residue that was purified by flash chromatography (PE/EtOAc 85:15). Compound **3** was obtained as a colorless oil. Yield: 2.8 g (70%); $R_f = 0.40$ (PE/EtOAc 85:15); $[\alpha]_D^{20} = -30.9$ ($c = 0.4$, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$, Me_4Si): $\delta = 1.51$ (s, 9H, *t*Bu), 2.73 (dd, $J =$ 13 and 9.3 Hz, 1H, NC*H*HCHOH), 2.94 (dd, $J = 13$ and 3.6 Hz, 1H, NCH*H*CHOH), 3.29 (s, 2H, NCH_2CO_2tBu), 3.56 (app. d, $J = 4.9$ Hz, 2H, CH_2OBn), 3.81 (d, $J = 13.5$ Hz, 1H, NC*H*HPh), 3.94 (d, $J = 13.5$ Hz, 1H, NCH*H*Ph), 4.02 (quint, $J = 4.7$ Hz, 1H, C*H*OH), 4.61 (s, 2H, OCH_2Ph), 7.27–7.47 (m, 10H, Ar); ^{13}C NMR (75 MHz, $CDCl_3$, Me_4Si): $\delta = 28.2$ (CH_3), 55.8, 58.0, 58.9 (CH_2), 67.6 (CH), 72.5, 73.5 (CH_2), 81.3 (Cq), 127.6, 127.7, 127.9, 128.4, 128.5, 128.7, 128.8, 129.0 (CH Ar), 138.3, 138.5 (Cq Ar), 171.1 (CO); MS (CI, NH_3): *m*/*z* (%) 386 (100) $[M+H]^+$, 330 (10), 284 (20), 234 (14), 178 (7); HRMS (ESI) calcd for $C_{23}H_{32}NO_4$ $[M+H]^+$: 386.2331, found: 386.2328. Starting from *ent*-**2** and following the same procedure, *ent*-**3** was obtained in 78% yield; $[\alpha]_D^{20} = +30.4$ ($c = 0.4$, $CHCl_3$).

(2*R*)-[Benzyl-(3-benzyloxy-2-chloropropyl)amino]acetic acid *tert*-butyl ester 5 and *ent*-5. Thionyl chloride (415 μL, 5.33 mmol) was added dropwise to a solution of amino alcohol **3** (1 g, 2.6 mmol) in CH_2Cl_2 (10 mL) cooled to 0 °C. The resulting mixture was warmed to room temperature and held at reflux for 2 h. The reaction was then cooled to 0 °C and treated with the dropwise addition of a saturated aqueous solution of $NaHCO_3$ (10 mL). The aqueous layer was extracted with Et_2O (2×15 mL), and usual workup gave a residue (mixture of regioisomeric chlorides) that were rapidly filtered on a short pad of silica gel eluted with PE/EtOAc (1:1). After evaporation of the eluent, the residue (1 g) was taken up in DMF and heated under Ar at 65 °C for 64 h. At this time, DMF was removed in vacuo, and the residue was purified by flash chromatography (PE/EtOAc 9:1) to give chloride **5** as a thick oil. Yield: 960 mg (96%); $R_f = 0.70$ (PE/EtOAc 9:1); $[\alpha]_{578}^{20} = +1$ ($c = 0.4$, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$, Me_4Si): $\delta = 1.54$ (s, 9H, *t*Bu), 3.13 (dd, $J = 14.3$ and 6.4 Hz, 1H, NC*H*HCHCl), 3.26 (dd, $J = 14.3$ and 6.9 Hz, 1H, NCH*H*CHCl), 3.35 (s, 2H, NCH_2CO_2tBu), 3.75 (dd, $J = 10.4$ and 5.6 Hz, 1H, C*H*HOBn), 3.83 (dd, $J = 10.4$ and 4.6 Hz, 1H, CH*H*OBn), 3.96 (s, 2H, NCH_2Ph), 4.12 (quint, $J = 6.6$ Hz, 1H, C*H*Cl), 4.62 (s, 2H, OCH_2Ph), 7.27–7.48 (m, 10H, Ar); ^{13}C NMR (75 MHz, $CDCl_3$, Me_4Si): $\delta = 28.3$ (CH_3), 55.7, 57.9, 58.8 (CH_2), 58.9 (CH), 71.9, 73.3 (CH_2), 81.1 (Cq), 127.3, 127.7, 127.8, 128.4, 128.9 (CH Ar), 137.9, 139.5 (Cq Ar), 170.7 (CO); MS (CI, NH_3): *m*/*z* (%) 404 (100) $[M+H]^+$, 348 (15), 312 (50), 303 (40); HRMS (ESI) calcd for $C_{23}H_{31}NO_3Cl$ $[M+H]^+$: 404.1992, found: 404.2014. Starting from *ent*-**3** and following the same procedure, *ent*-**5** was obtained in 80% yield; $[\alpha]_{578}^{20} = -0.9$ ($c = 0.4$, $CHCl_3$).

(2*R*,3*S*)-*tert*-Butyl-1-benzyl-3-[(benzyloxy)methyl]azetidine-2-carboxylate 7. A solution of lithium hexamethyldisilazanide (LiHMDS, 1 M solution in THF, 1.5 mL, 1.5 mmol) was added dropwise to a solution of chloride **5** (403 mg, 1 mmol) in a mixture of THF/HMPA (5 mL + 0.5 mL, respectively) at −78 °C. The reaction was monitored by TLC and was warmed gradually to 0 °C (2 h) and then quenched by the addition of an aqueous saturated solution of NH_4Cl (5 mL). Addition of Et_2O and H_2O was followed by usual workup. The crude residue was purified by flash chromatography (PE/EtOAc 4:1) to give azetidine **7** as an oil. Yield: 220 mg (60%); $R_f = 0.50$ (PE/EtOAc 4:1); $[\alpha]_{578}^{20} = +23.1$ ($c = 0.13$, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$, Me_4Si): $\delta = 1.38$ (s, 9H, *t*Bu), 2.82–2.97 (m, 1H, H-3), 3.06 (app. t, $J = 7.3$ Hz, 1H, H-4), 3.26 (dd, $J = 7.0$ and 2.5 Hz, 1H, H-4′), 3.63–3.90 (m, 5H, H-2, NCH_2Ph, OCH_2CH), 4.54 (s, 2H, OCH_2Ph), 7.24–7.42 (m, 10H, Ar); ^{13}C NMR (75 MHz, $CDCl_3$, Me_4Si): $\delta = 28.0$ (CH_3), 33.6 (C-3), 53.4 (C-4), 61.9 (CH_2), 65.6 (CH), 69.8, 73.3 (CH_2), 81.8 (Cq), 127.6, 127.8, 128.3, 128.4, 129.3 (CH Ar), 137.2, 138.3 (Cq Ar), 170.2 (CO); MS (CI, NH_3): *m*/*z* (%) 368 (100) $[M+H]^+$, 312 (21), 266 (30), 91 (10); HRMS (ESI) calcd for $C_{23}H_{30}NO_3$ $[M+H]^+$: 368.2226, found: 368.2239. Starting from *ent*-**5** and following the same procedure, *ent*-**7** was obtained in 58% yield; $[\alpha]_{578}^{20} = -22.9$ ($c = 0.1$, $CHCl_3$).

(2*R*,3*S*)-Di-*tert*-butyl-3-hydroxymethylazetidine-1,2-dicarboxylate 8. $[Pd(OH)_2]$ (20% wt. on carbon, 1.5 g) was added to a solution of azetidine **7** (2.25 g, 6.13 mmol) and di-*tert*-butyldicarbonate (2.67 g, 12.4 mmol) in absolute EtOH (15 mL). The suspension was hydrogenated at room temperature at 15 bar (218 psi) of H_2 for 36 h. The reaction mixture was then filtered over Celite, concentrated and dried in vacuo. The residue was purified by flash chromatography (PE/EtOAc 1:1) to give **8** as a thick oil. Yield: 1.7 g (96%); $R_f =$ 0.35 (PE/EtOAc 1:1); $[\alpha]_D^{20} = +43.8$ ($c = 0.27$, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$, Me_4Si): $\delta = 1.36$ (s, 9H, *t*Bu), 1.44 (s, 9H, *t*Bu), 2.54 (br s, 1H, O*H*), 2.97 (app. sext, $J = 7.1$ Hz, 1H, H-3), 3.58–3.77 (m, 3H, H-4, CH_2OH), 3.84 (app. t, $J = 8.3$ Hz, 1H, H-4′), 4.52 (d, $J = 9.1$ Hz, H-2); ^{13}C NMR (75 MHz, $CDCl_3$, Me_4Si): $\delta = 27.9$, 28.2 (CH_3), 33.8 (C-3), 49.5 (C-4), 61.4 (CH_2), 63 (C-2), 79.8, 82.2 (Cq), 155.3, 169.1 (CO); MS (ESI): *m*/*z* (%) 310 (100) $[M+Na]^+$; HRMS (ESI) calcd for $C_{14}H_{25}NO_5Na$ $[M+H]^+$: 310.1630, found: 310.1639. Starting from *ent*-**7** and following the same procedure, *ent*-**8** was obtained in 92% yield; $[\alpha]_D^{20} = -44.2$ ($c = 0.3$, $CHCl_3$).

(2*R*,3*S*)-1,2-Di-(*tert*-butoxycarbonyl)azetidine-3-carboxylic acid 9. $RuCl_3 \cdot H_2O$ (15 mg, 0.07 mmol) was added to a suspension of $NaIO_4$ (165 mg, 0.77 mmol) in a mixture of $MeCN/CCl_4/H_2O$ (1:1:1, 5 mL), and the mixture was stirred at room temperature for 45 min. Alcohol **8** (200 mg, 0.7 mmol) dissolved in MeCN (3 mL) was added to this mixture, followed by the addition of a second portion of $NaIO_4$ (150 mg, 0.7 mmol). The resulting mixture was stirred at room temperature for 0.5 h, then filtered through a pad of Celite and thoroughly washed with EtOAc. The combined filtrates were dried over $MgSO_4$ and concentrated to give a residue that was purified by flash chromatography ($CHCl_3$/MeOH 9:1). Compound **9** was obtained as a low-viscosity oil. Yield: 150 mg (71%); $R_f = 0.25$ ($CHCl_3$/MeOH 9:1); $[\alpha]_D^{20} = +29.9$ ($c = 0.85$, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$, Me_4Si): $\delta = 1.36$ (s, 9H, *t*Bu), 1.39 (s, 9H, *t*Bu), 3.57 (app. quart, $J = 9.0$ Hz, 1H, H-3), 3.93 (app. t, $J = 7.5$ Hz, 1H, H-4), 4.21 (app. t, $J =$ 8.4 Hz, 1H, H-4′), 4.60 (d, $J = 9.4$ Hz, H-2); ^{13}C NMR (75 MHz, $CDCl_3$, Me_4Si): $\delta = 27.9$, 28.3 (CH_3), 35.3 (C-3), 49.5 (C-4), 63.6 (C-2), 80.7, 82.5 (Cq), 167.8, 174.8 (CO); MS (ESI): *m*/*z* (%) 324 (100) $[M+Na]^+$; HRMS (ESI) calcd for $C_{14}H_{23}NO_6Na$ $[M+H]^+$: 324.1423, found: 324.1433. Starting from *ent*-**8** and following the same procedure, *ent*-**9** was obtained in 70% yield; $[\alpha]_D^{20} = -30.5$ ($c = 0.8$, $CHCl_3$).

(2*R*,3*S*)-Azetidine-2,3-dicarboxylic acid 10. Trifluoroacetic acid (TFA, 3 mL) was added to a solution of *N*-Boc-azetidine **9** (160 mg, 0.53 mmol) in CH_2Cl_2 (3 mL), and the solution was stirred at room temperature overnight. Upon completion, the reaction mixture

was concentrated in vacuo, and the residue was triturated with small portions of dry acetone, which were removed in vacuo. The obtained trifluoroacetate salt was dissolved in a minimal quantity of H_2O and deposited on an ion-exchange resin (Dowex 50WX8-200, 7 g) previously washed with H_2O until neutrality. Elution with H_2O was followed by elution with a solution of aqueous NH_3 (1%). The ninhydrin-positive fractions were lyophilized to give the title compound as a hygroscopic foam. Yield: 70 mg (90%); $R_f = 0.1$ (EtOH/[30% aq. NH_3]/H_2O 9:3:1); mp: 189–90 °C (Ref. [6]: 187 °C); $[\alpha]^{20}_{578} = +736.6$ ($c = 0.06$, H_2O); $[\alpha]^{20}_{365} = +636.4$ ($c = 0.06$, H_2O); ^{1}H NMR (300 MHz, D_2O): $\delta = 3.52$ (td, $J = 5.7$ and 3.2 Hz, 1H, H-3), 3.84 (dd, $J = 10.6$ and 5.5 Hz, 1H, H-4), 4.031 (app.t, $J = 9.6$ Hz, 1H, H-4′), 4.81 (d, $J = 9.6$ Hz, H-2); ^{13}C NMR (75 MHz, D_2O): $\delta = 41.1$ (C-3), 45.4 (C-4), 60.7 (C-2), 171.5, 175.6 (CO); MS (ESI): *m/z* (%) 168 (100) $[M+Na]^+$; HRMS (ESI) calcd for $C_5H_8NO_4Na$ $[M+H]^+$: 146.0453, found: 146.0488. Starting from *ent*-**9** and following the same procedure, *ent*-**10** was obtained in 85% yield; $[\alpha]^{20}_{578} = -727$ ($c = 0.08$, H_2O).

(2*R*,3*R*)-1-Benzyl-2-ethoxycarbonyl-3-phenylazetidine 12. H_2SO_4 (9.5 mL, 40 equiv) was added to a solution of cyanoazetidine **11** (1.1 g, 4.43 mmol) in absolute EtOH (150 mL) at 0 °C. The mixture was then heated at reflux for 24 h, and was then poured carefully into a large beaker containing a saturated solution of sodium bicarbonate (300 mL) precooled at 0 °C. After the evolution of gas had ceased and the solution was verified to be below pH 7, the aqueous phase was extracted with CH_2Cl_2 (3×100 mL) and the combined organic layers were dried over $MgSO_4$ and concentrated in vacuo. The title compound was obtained as a clear oil, which was used without further purification. An analytical sample was purified by flash chromatography using (Et_2O/*c*Hex 20:80). Yield: 1.2 g (89%); $R_f = 0.49$ (Et_2O/*c*Hex: 20/80); $[\alpha]^{20}_D = -6.0$ ($c = 1.00$, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 1.21$ (t, $J = 7.2$ Hz, 3H, H-8), 3.14 (dd, $J = 6.2$ and 8.5 Hz, 1H, H-4), 3.70 (A of an AB system, $J = 12.5$ Hz, 1H, H-5), 3.78 (dd, $J = 6.2$ and 7.9 Hz, 1H, H-4′), 3.81 (d, $J = 7.9$ Hz, 1H, H-2), 3.91 (B of an AB system, $J = 12.5$ Hz, 1H, H-5′), 3.87–3.95 (m, 1H, H-3), 4.05–4.22 (m, 2H, H-7), 7.22–7.36 (m, 5H, Ph); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 14.3$ (C-8), 39.9 (C-3), 57.4 (C-4), 60.9 (C-7), 62.6 (C-5), 71.3 (C-2), 126.9–129.3 (CH Ar), 137.0 and 140.4 (*Cipso*-Ph), 172.1 (C-6); Elemental analysis calcd for $C_{19}H_{21}NO_2$: C 77.26, H 7.17, N 4.74, found: C 77.14, H 7.44, N 4.55. Starting from *ent*-**11** and following the same procedure, *ent*-**12** was obtained in 96% yield; $[\alpha]^{20}_D = +6.4$(*c*=0.8, $CHCl_3$).

(2*R*,3*R*)-3-Phenylazetidine-1,2-dicarboxylic acid 1-*tert*-butyl ester 2-ethyl ester 13. The *N*-benzyl-protected azetidine **12** (1.09 g, 3.69 mmol) was dissolved in absolute EtOH (50 mL). Boc_2O (1.6 g, 7.39 mmol, 2 equiv) was added, followed by $[Pd(OH)_2]$ (10% wt. on carbon, 300 mg). The suspension was stirred under H_2 atmosphere for 6 days, then filtered over Celite and concentrated in vacuo. The residue was purified by flash chromatography (Et_2O/*c*Hex 20:80), and gave **13** as a colorless oil. Yield: 1.13 g (quant.); $R_f = 0.6$ (Et_2O/*c*Hex 20:80); $[\alpha]^{20}_D = -13.8$ ($c = 9.35$, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 1.22$ (t, $J = 7.11$ Hz, 3H, CH_2CH_3), 1.37 (s, 9H, *t*Bu), 3.55–3.62 (m, 1H, H-3), 3.89 (dd, $J = 5.8$ and 8.1 Hz, 1H, H-4), 4.18 (q, $J = 7.11$ Hz, 2H, CH_2CH_3), 4.32 (t, $J = 8.1$ Hz, 1H, H-4′), 4.52 (d, $J = 5.4$ Hz, 1H, H-2), 7.19–7.28 (m, 5H, Ph); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 14.3$ (CH_3), 28.3 (CH_3, *t*Bu), 38.4 (C-3), 54.3 (C-4), 61.3 (CH_2), 67.8 (C-2), 80.3 (Cq *t*Bu), 126.8, 127.5, 128.9 (CH Ar), 140.2 (Cq Ar), 155.5 (CO), 170.7 (CO); HRMS (ESI) calcd for $C_{19}H_{22}NO_4$ $[M+H]^+$: 328.1549, found: 328.1543. Starting from *ent*-**12** and following the same procedure, *ent*-**13** was obtained in 92% yield; $[\alpha]^{20}_D = +14.3$(*c*=1.5, $CHCl_3$).

(2*R*,3*R*)-Azetidine-1,2,3-tricarboxylic acid 1-*tert*-butyl ester 2-ethyl ester 14. $RuCl_3 \cdot H_2O$ (7 mg) was added to a suspension of $NaIO_4$ (823 mg, 3.85 mmol, 20 equiv) in a mixture of MeCN/CCl_4/H_2O (1:1:1, 6 mL), and the mixture was stirred at room temperature for 30 min. Compound **13** (58.8 mg, 0.19 mmol) dissolved in MeCN (3 mL) was added to this suspension. The reaction was stirred at room temperature for 24 h, and was then diluted with H_2O and extracted with EtOAc (4×20 mL). The organic layers were dried over $MgSO_4$ and concentrated to give a dark residue that was purified by flash chromatography ($CHCl_3$/MeOH 9:1) to give **14** as a clear oil. Yield: 39.3 mg (75%); $R_f = 0.5$ ($CHCl_3$/MeOH 9:1); $[\alpha]^{20}_D = +4.3$ ($c = 1.1$, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 1.24$ (t, $J = 7.11$ Hz, 3H, CH_2CH_3), 1.36 (s, 9H, *t*Bu), 3.27 (dt, $J = 5.6$ and 10.4 Hz, 1H, H-3), 3.98 (dd, $J = 5.6$ and 8.1 Hz, 1H, H-4), 4.11–4.23 (m, 3H, H-4′ and CH_2CH_3), 4.73 (d, $J = 5.4$ Hz, 1H, H-2), 9.15 (br s, 1H, COO*H*); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 14.1$ (CH_3), 28.2 (CH_3, *t*Bu), 36.2 (C-3), 50.1 (C-4), 61.7 (CH_2), 62.8 (C-2), 81.1 (Cq *t*Bu), 155.4 (CO), 169.8 (CO), 175.1 (CO_2H); HRMS (ESI) calcd for $C_{10}H_{21}NO_6Na$ $[M+H]^+$: 274.1267, found: 274.1278. Starting from *ent*-**13** and following the same procedure, *ent*-**14** was obtained in 74% yield; $[\alpha]^{20}_D = -5$ ($c = 0.9$, $CHCl_3$).

(2*R*,3*R*)-Azetidine-2,3-dicarboxylic acid 15. NaOH (23 mg dissolved in 1 mL H_2O) was added to a solution of *N*-Boc-azetidine **14** (52.1 mg, 0.19 mmol) in MeOH (1 mL). The solution was stirred at room temperature for 48 h, during which time HCl (2 mL, 1 M) was added. The solution was stirred for 5 h at room temperature, and was then concentrated in vacuo. The white residue was dissolved in a minimal quantity of H_2O and deposited on an ion-exchange resin (Dowex 50WX8-200, 7 g) previously washed with H_2O until neutrality. Elution with H_2O was followed by elution with a solution of aqueous NH_3 (1%). The ninhydrin-positive fractions were lyophilized to give the title compound as a white powder. Yield: 72% (20 mg); $[\alpha]^{20}_D = +45.6$ ($c = 0.09$, H_2O); ^{1}H NMR (300 MHz, D_2O): $\delta = 3.50$ (dt, $J = 7.7$ and 9.2 Hz, 1H, H-3), 4.01–4.17 (m, 2H, H-4), 4.83 (d, $J = 7.7$ Hz, 1H, H-2); ^{13}C NMR (75 MHz, D_2O): $\delta = 42.2$ (C-3), 46.4 (C-4), 62.5 (C-2), 173.3 (COOH), 176.9 (COOH); HRMS (ESI) calcd for $C_5H_8NO_4$ $[M+H]^+$: 146.0453, found: 146.0446. Starting from *ent*-**14** and following the same procedure, *ent*-**15** was obtained in 40% yield; $[\alpha]^{20}_D = -47$ ($c = 0.2$, H_2O).

Two-electrode voltage-clamp electrophysiology

cRNAs for rat NR1–1a (hereafter NR1) and NR2A, B, C, and D were synthesized in vitro and injected (5–10 ng) into *Xenopus laevis* oocytes as previously described.[25] Rat cDNAs for NR1 and NR2 subunits (GenBank numbers: NR1, U11418 and U08261; NR2A, D13211; NR2B, U11419; NR2C, M91563; NR2D, L31611 (modified according to Monyer et al.[26]) were provided by Drs. S. Heinemann (The Salk Institute), S. Nakanishi (Kyoto University), and P. Seeburg (University of Heidelberg). Two-electrode voltage-clamp current recordings were made 24–72 h post-injection as previously described.[23] Recordings from 5–10 oocytes from two different *X. laevis* were performed for all compounds. Agonist concentration–response data were pooled among oocytes, and composite dose–response data were fitted by the following equation:

$$I = I_{max} / \{1 + 10^{(\log EC_{50} - \log [A]) n_H}\}$$

for which I_{max} is the maximum current in response to the agonist, n_H denotes the Hill coefficient, [A] is the agonist concentration, and EC_{50} is the agonist concentration that produces a half-maximum response. Relative I_{max} values were calculated from a full concentration–response measurement as $I_{max(agonist)}/I_{max(Glu)}$, in which $I_{max(agonist)}$

is the fitted I_{max} value according to the Hill equation and $I_{max(Glu)}$ is the maximum current obtained from glutamate in the same recording.

NMDA receptor binding

The four stereoisomeric azetidines were evaluated for NMDA receptor binding affinity ([^{3}H]CGP39653) in native rat synaptosomes in accordance with previously described experimental procedures.[27]

Molecular modeling

The docking of the ligands was performed using essentially the same procedure as previously described,[28] the only difference being that the ligands were docked into the X-ray crystal structure of NR2A (PDB code: 2A5S) rather than a homology model of NR2B.

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