Bioorganic & Medicinal Chemistry 17 (2009) 3456–3462

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

# NMDA receptor affinities of 1,2-diphenylethylamine and 1-(1,2-diphenylethyl)piperidine enantiomers and of related compounds

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ARTICLE INFO

*Article history:*
Received 13 February 2009
Accepted 13 March 2009
Available online 19 March 2009

*Keywords:*
NMDA receptor
[$^3$H]MK-801 binding
1,2-Diphenylethylamine
1-(1,2-Diphenylethyl)piperidine
Spermine

ABSTRACT

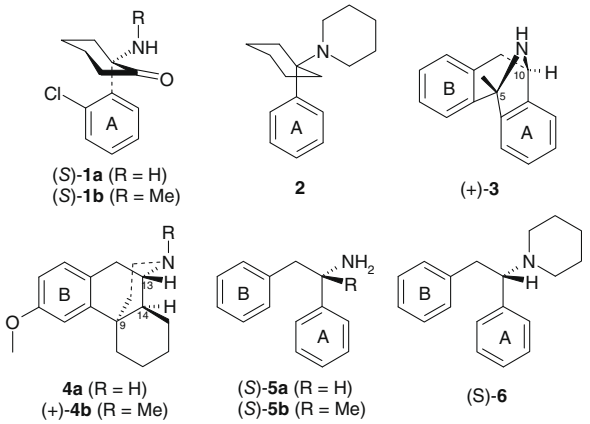
We resolved 1,2-diphenylethylamine (DPEA) into its (*S*)- and (*R*)-enantiomer and used them as precursors for synthesis of (*S*)- and (*R*)-1-(1,2-diphenylethyl)piperidine, flexible homeomorphs of the NMDA channel blocker MK-801. We also describe the synthesis of the dicyclohexyl analogues of DPEA. These and related compounds were tested as inhibitors of [$^3$H]MK-801 binding to rat brain membranes. Stereospecificity ranged between factors of 0.5 and 50. Some blockers exhibited stereospecific sensitivity to the modulator spermine. Our results may help to elucidate in more detail the NMDA channel pharmacophore.



## 1. Introduction

The *N*-methyl-D-aspartate (NMDA) receptor is the most abundant receptor for the principal excitatory neurotransmitter glutamate and consists of 4 protein subunits surrounding a central channel, with various binding sites at the extracellular domain regulating permeability to $Na^+$ and $Ca^{2+}$.[1] Its involvement in learning and memory, but also in pathologic conditions such as stroke, epilepsy and neurodegenerative diseases makes this receptor an attractive target of drug development.[2] Analogues of the agonist glutamate itself suffer from their poor ability to cross the blood brain barrier, because they have to mimic an acidic amino acid with 3 charged functional groups. A direct attack of the ion channel by more lipophilic channel blockers appears more promising, however the best known representatives of this class are Ketamine (**1b**) and Phencyclidine (**2**), two widely abused compounds inducing psychotic side effects, and the experimental high affinity channel ligand (5*S*,10*R*)-(+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine, (+)-MK-801 [(+)-**3**].[3] Examples for moderate potency NMDA channel blockers tolerated with much less psychotic side effects are 3,5-dimethyladamantan-1-amine (Memantine)[4] and Dextromethorphan [(+)-**4b**]. To learn more about SARs at this channel binding site, we resolved racemic 1,2-diphenylethylamine (DPEA, **5a**) and used its enantiomers to prepare (*R*)- and (*S*)-1-(1,2-diphenylethyl)piperidine (DEP, **6**).[5] Until now, both **5a** and **6** had only been studied as racemic mixtures.[5]

The polyamines spermine and spermidine increase frequency and burst length of NMDA-induced currents in rat hippocampal neurons, by relieve from a partial block by protons, even at physiological pH.[6] As a consequence, the channel is more readily accessible not only for the cations constituting its current, but also for the channel ligand MK-801 (as a secondary amine positively charged at physiological pH), thus increasing the affinity of the radioligand [$^3$H]MK-801. Channel blockers that act similarly to MK-801 profit from increased channel accessibility to a similar extent.[7] Here we demonstrate that the affinity of some of the studied




(*S*)-**1a** (R = H)
(*S*)-**1b** (R = Me)

**2**

(+)-**3**

**4a** (R = H)
(+)-**4b** (R = Me)

(*S*)-**5a** (R = H)
(*S*)-**5b** (R = Me)

(S)-**6**

**Chart 1.** Phenyl rings in NMDA receptor channel blockers.

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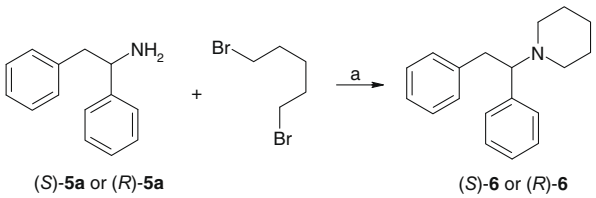
doi:10.1016/j.bmc.2009.03.025




**Scheme 1.** Reagents and conditions: (a) $K_2CO_3$, $CH_3CN$; 64%/54%.

stereoisomers was not influenced in the same way by spermine than that of the radioligand, suggesting that they acted in a way different from MK-801.

## 2. Results

### 2.1. Chemistry

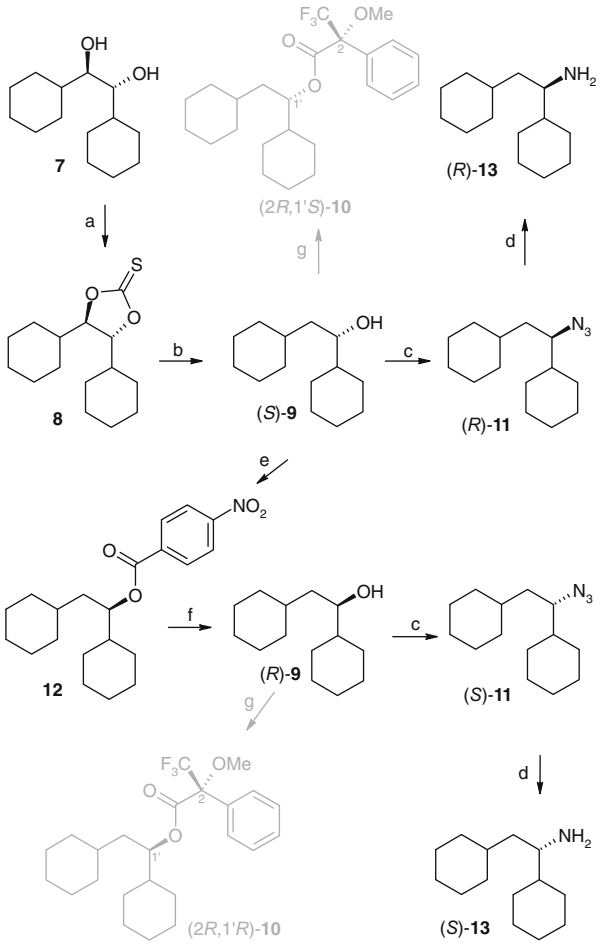
Commercially available (±)-**5a** was resolved by the method of Shinohara et al.,[8] which was modified, into (+)- and (−)-enantiomers (*S*)- and (*R*)-**5a**, respectively. The piperidine analogues **6** were prepared from the respective **5a** enantiomers with 98% ee by double alkylation[9] with 1,5-dibromopentane (Scheme 1). Under optimised conditions (3 equiv of alkylating reagent, 6 equiv of $K_2CO_3$) in dry acetonitrile at ambient temperature for 3 days, the yields for the (+)-isomer (*S*)-**6** and the (−)-isomer (*R*)-**6** were 64% and 54%, respectively. The oily bases were converted to the hydrochlorides, which crystallised as monohydrates (the racemic hydrochloride has been described, without crystal water).[10]

Synthesis of the dicyclohexyl analogues **13** was started from (*R*,*R*)-1,2-dicyclohexylethane-1,2-diol (**7**, Scheme 2). The diol was converted to the thiocarbonate **8** in high yield (89%) using thiocarbonyl diimidazole in refluxing toluene.[11] The crystalline product was deoxygenated to (*S*)-**9** with 60% yield using $Bu_3SnH$/AIBN in refluxing toluene. With tris(trimethylsilyl)silane instead of the tin hydride, yield dropped to 18%.[12] To determine the ee, a sample was converted to the (*R*)-Mosher ester (2*R*,1′*S*)-**10**;[13] its $^{19}F$ NMR spectrum indicated ee >98%. The alcohol (*S*)-**9** was transformed into the azide (*R*)-**11** smoothly using the Mitsunobu reaction ($S_N2$ mechanism),[14] but subsequent reduction to the amine was not trivial. Neither catalytic hydrogenation (Pd–C, $H_2$ in EtOH containing some HCl, 3 atm) nor hydrolysis of the iminophosphorane, which formed very slowly and incompletely from the azide with triphenylphosphine in a Staudinger reaction, gave the desired amine. However, after a moderately successful attempt with Li-$BEt_3H$ (yield only 40%), reduction[15] with $LiAlH_4$ gave the amine (*R*)-**13** as an oil with 88% yield, which was finally converted to the crystalline hydrochloride.

For synthesis of (*R*)-**9**, the configuration of alcohol (*S*)-**9** was inverted by esterification with $Ph_3P$, 4-nitrobenzoic acid and diisopropyl azodicarboxylate (DIAD) (Mitsunobu reaction) (Scheme 2, e).[14] Although the starting material was consumed, the yield of the 4-nitrobenzoate **12** was low (45%) and could not be improved. Elimination with formation of isomeric alkenes predominated, because the stereogenic centre is encumbered, which retarded nucleophilic substitution. The yield dropped to 22%, when the less acidic benzoic acid was used instead of 4-nitrobenzoic acid. Transesterification of **12** with MeONa in MeOH furnished the alcohol (*R*)-**9** [ee >96%; by $^{19}F$ NMR spectroscopy of (*R*)-Mosher ester (2*R*,1′*R*)-**10**], which was transformed into amine (*S*)-**13** and its hydrochloride as described above.

### 2.2. Pharmacology

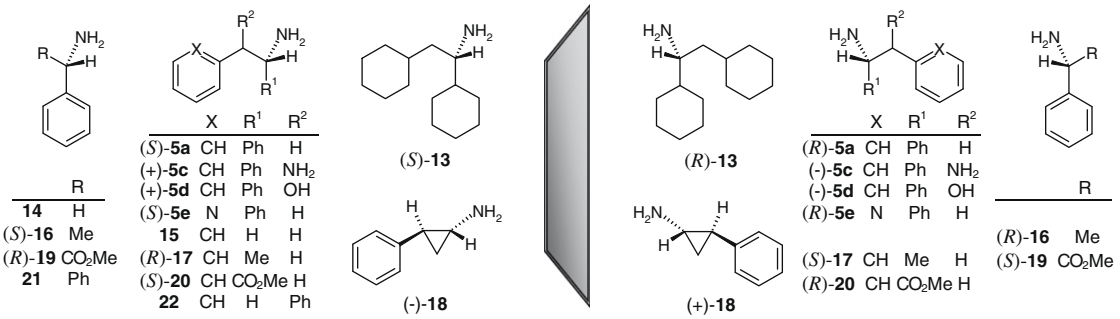
In search for common features of NMDA channel blockers as diverse as MK-801, Phencyclidine and Dextromethorphan, we




**Scheme 2.** Reagents and conditions: (a) thiocarbonyl diimidazole, toluene, reflux, 89%; (b) $Bu_3SnH$, AIBN, toluene, reflux, 60%; (c) $Ph_3P$, DIAD, $HN_3$, toluene, rt, 87%; (d) $LiAlH_4$, 88%; (e) 4-nitrobenzoic acid, DIAD, $Ph_3P$, toluene, 45%; (f) 0.2 M MeONa, MeOH, 87%; (g) (*S*)-MTPA-Cl, $CH_2Cl_2$, pyridine; in grey: synthesised for analytic reasons only.

distinguished Ph rings A and B according to their distance from the amino group (Chart 1). Although not all rings A (respectively B) can be expected to interact with the same amino acid residue(s) of the receptor protein, this approach allows the exploration of a common pharmacophore starting from simpler precursors. Already benzylamine (**14**) and phenylethylamine (**15**) inhibited [$^3$H]MK-801 binding with $K_i$ 1.45 and 0.75 mM, respectively (Table 1). Replacing an aliphatic hydrogen atom of **14** by a methyl group results in (*S*)- and (*R*)-1-phenylethylamine (**16**), and similar substitution of **15** in (*R*)- and (*S*)-Amphetamine (**17**). This modification increased the potency of **14** by a factor of two, without any stereoselectivity. In the case of **15**, an increase in potency (also by a factor of two) was only observed for (*S*)-**17** (D-Amphetamine), not for (*R*)-**17** (as already described).[16] Incorporation of the methyl group of Amphetamine into a cyclopropyl ring leads to the antidepressant and MAO inhibitor Tranylcypromine. We obtained both *trans* isomers (three times more potent as MAO inhibitors than the *cis* isomers) (1*R*,2*S*)-(−)- and (1*S*,2*R*)-(+)-2-phenylcyclopropylamine (**18**); (+)-**18** is more potent than (−)-**18** as MAO inhibitor by a factor of 4.[17] These isomers of Tranylcypromine were slightly more potent than the respective isomers of Amphetamine, and also their inhibition was stereoselective (Table 1). On the other hand, introduction of a methoxycarbonyl instead of a methyl group into **14** and **15**, giving (*R*)- and (*S*)-phenylglycine methylesters (**19**), and

**Table 1**
Inhibition of [$^3$H]MK-801 binding by structural analogues of benzylamine (**14**) and phenylethylamine (**15**)




| Nr | Config | $K_i$ (μM) | $n$ | $K_i$ (spm)/$K_i$ | $n$ | Stereoselectivity | $n$ | Nr | Config | $K_i$ (μM) | $n$ | $K_i$ (spm)/$K_i$ | $n$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| (*S*)-**1a** | *S* | 4.18, 4.46 | 2 | 2.91, 2.82 | 2 | 14.4, 12.3 | 2 | (*R*)-**1a** | *R* | 60, 55 | 2 | 1.29, 1.57 | 2 |
| (*S*)-**1b** | *S* | 0.96 ± 0.21 | 5 | 2.70 ± 0.09** | 4 | 5.6 ± 0.5 | 4 | (*R*)-**1b** | *R* | 5.68 ± 1.52 | 4 | 1.02 ± 0.10 | 4 |
| **2** | | 0.27 ± 0.01 | 3 | 0.78 ± 0.06 | 3 | | | | | | | | |
| (+)-**3** | 10*R* | 0.009, 0.012 | 2 | 0.53, 0.51 | 2 | 5.32, 4.81 | 2 | (−)-**3** | 10*S* | 0.043 ± 0.011 | 4 | 0.54 ± 0.29 | 4 |
| **4a** | 9*S* | 1.87, 1.70 | 2 | 0.85, 1.15 | 2 | | | | 9*R* | n.a. | | | |
| (+)-**4b** | 9*S* | 4.54 ± 1.42 | 5 | 0.70 ± 0.21 | 4 | 0.65 ± 0.28 | 3 | (−)-**4b** | 9*R* | 3.36 ± 0.50 | 3 | 0.66 ± 0.19 | 3 |
| (*S*)-**5a** | *S* | 0.70 ± 0.21 | 5 | 2.59 ± 0.43** | 3 | 42.6 ± 15.8 | 4 | (*R*)-**5a** | *R* | 25.1 ± 3.4 | 4 | 1.93, 1.45 | 2 |
| (*S*)-**5b** | *S* | 0.79 ± 0.14 | 4 | 2.24 ± 0.96* | 3 | 9.6 ± 0.7 | 3 | (*R*)-**5b** | *R* | 8.08 ± 0.47 | 3 | 0.58, 0.37 | 2 |
| (+)-**5c** | 1*R* | 4.12 ± 1.75 | 3 | 2.07 ± 0.60* | 3 | 15.4 ± 10.7 | 3 | (−)-**5c** | 1*S* | 51 ± 6 | 3 | 1.61 ± 0.28 | 3 |
| (+)-**5d** | 2*R* | 15.7 ± 6.5 | 4 | 2.13 ± 0.77* | 3 | 11.4 ± 1.42 | 3 | (−)-**5d** | 2*S* | 142 ± 2 | 3 | 1.25, 1.10 | 2 |
| (*S*)-**5e** | *S* | 14.0 ± 5.8 | 3 | 1.50 ± 0.49 | 3 | 6.3 ± 1.24 | 3 | (*R*)-**5e** | *R* | 84 ± 15 | 3 | 0.85 ± 0.13 | 3 |
| (*S*)-**6** | *S* | 0.13, 0.12 | 2 | 0.83, 0.81 | 2 | 40.3, 57.4 | 2 | (*R*)-**6** | *R* | 5.25, 7.02 | 2 | 1.49, 0.99 | 2 |
| (*S*)-**13** | *S* | 271 ± 31 | 3 | 0.81 ± 0.23 | 3 | 0.49, 0.79 | 2 | (*R*)-**13** | *R* | 138 ± 51 | 3 | 0.47, 1.15 | 2 |
| **14** | | 1830, 1063 | 2 | 1.09, 1.17 | 2 | | | | | | | | |
| **15** | | 753 ± 152 | 3 | 1.45, 1.19 | 2 | | | | | | | | |
| (*S*)-**16** | *S* | 659, 622 | 2 | 1.24, 0.86 | 2 | 1.00, 1.08 | 2 | (*R*)-**16** | *R* | 659, 674 | 2 | 1.28, 0.74 | 2 |
| (*R*)-**17** | *R* | 625 ± 158 | 4 | 1.61 ± 0.20 | 3 | 0.58, 0.61 | 2 | (*S*)-**17** | *S* | 300 ± 65 | 4 | 1.47 ± 0.36 | 4 |
| (−)-**18** | 1*R* | 544 ± 164 | 3 | 1.26 ± 0.33 | 3 | 0.49 ± 0.08 | 3 | (+)-**18** | 1*S* | 269 ± 98 | 4 | 1.78 ± 0.67 | 4 |
| (*R*)-**19** | *R* | 1372 ± 921 | 4 | 1.07 ± 0.15 | 4 | 1.66 ± 0.76 | 4 | (*S*)-**19** | *S* | 1978 ± 846 | 4 | 1.25 ± 0.12 | 4 |
| (*S*)-**20** | *S* | 701 ± 452 | 5 | 1.94 ± 0.43* | 4 | 1.23 ± 0.13 | 5 | (*R*)-**20** | *R* | 855 ± 517 | 5 | 1.48 ± 0.35 | 4 |
| **21** | | 64 ± 17 | 3 | 1.22 ± 0.14 | 3 | | | | | | | | |
| **22** | | 152, 145 | 2 | 1.09, 1.21 | 2 | | | | | | | | |

Compounds isosteric[a] to (*S*)-**5a** on the left, isosteric to (*R*)-**5a** on the right side of the table; $K_i$ ± SD (or both values if $n = 2$). See also Chart 1 for structures.
Config, configuration at the carbon atom bearing the amino group; $n$, number of experiments; $K_i$ (spm), $K_i$ in presence of 100 μM spermine; stereoselectivity is the mean of $K_i$ ratios obtained in individual experiments (not necessarily identical to the ratio of the mean $K_i$ values); n.a., the (*R*)-isomer was not available.

[a] Depending on the priority of the carbon atoms surrounding the chiral centre(s), compounds sharing the same steric configuration do not necessarily share the same stereochemical descriptor.

* Significantly higher than values ≤0.85.

** Significantly higher than values ≤1.26 (ANOVA, Newman-Keuls).

(*S*)- and (*R*)-phenylalanine methylesters (**20**), respectively, was without influence.

The potency of **14** was increased more than 20-fold by introduction of a second Ph substituent (aminodiphenylmethane, **21**). Into **15**, a second Ph residue can be introduced in three different ways: Introduction in vicinity to the first Ph group resulted in the (achiral) 2,2-diphenylethylamine (**22**) 5 times more potent than **2**. In vicinity to the primary amino group, two stereoisomers are obtained, (*S*)- and (*R*)-DPEA (**5a**), the first one 1000 times, the second one 30 times more potent than **15**. (*S*)-(+)- and (*R*)-(−)-1,2-Diphenyl-2-propylamines (**5b**) are 1-methyl derivatives of **5a** and desglycine metabolites of (±)-Remacemide. They act as NMDA channel blockers in whole cell voltage-clamp recordings from cultured rat hippocampal neurons and in binding studies with [$^3$H]MK-801 in rat forebrain membranes.[18] (*S*)-**5b** was equipotent to (*S*)-**5a**, but the high stereospecificity of the pair (*S*)-**5a**/(*R*)-**5a** (stereo-factor 43) was not reproduced in the pair (*S*)-**5b**/(*R*)-**5b** (factor 10; Table 1). Potency was reduced by introduction of an amino or a hydroxyl group at position 2: (*R*,*R*)-(+)-1,2-diphenylethylenediamine [(+)-**5c**] was six times weaker, and (1*S*,2*R*)-(+)-2-amino-1,2-diphenylethanol [(+)-**5d**] was 22 times weaker than (*S*)-**5a**; their (*S*,*S*)-(−)- and (1*R*,2*S*)-(−)-isomers (−)-**5c** and (−)-**5d** revealed stereo-factors of 15 and 11, respectively; thus, in spite of reduced potency, stereospecificity was not reduced further. Exchange of Ph ring B in **5a** for a pyridinyl ring resulted in (*S*)-(+)- and (*R*)-(−)-1-phenyl-2-(pyridin-2-yl)ethylamines (**5e**). (*S*)-**5e** is an experimental drug known as AR-R15896AR, with blocking properties similar to those of Ketamine and Memantine.[19] Potency was reduced by this Ph/pyridinyl exchange by a factor of 20, and also stereoselectivity was strongly reduced (from 43 to 6; Table 1). Incorporation of the nitrogen atom of the primary amino group of (*S*)-**5a** into a piperidine ring resulted in the highly potent NMDA receptor channel blocker (*S*)-**6**, five times more potent than (*S*)-**5a**, with comparable stereoselectivity (factor 49). Finally, changing in **5a** Ph to cyclohexyl resulted in (*S*)- and (*R*)-1,2-dicyclohexylethylamines (**13**), with potency reduced by a factor of 385 and stereoselectivity totally lost.

Inhibition of [$^3$H]MK-801 binding by the stereoisomers of Ketamine (**1b**) and its main metabolite Norketamine (**1a**) has been described.[20] In agreement with the published data, we found (*S*)-**1b** moderately more potent than the (*R*)-isomer (factor 5.6), and its demethylated variant (*S*)-**1a** by a factor of 4.5 less potent than (*S*)-**1b**, with somewhat higher stereospecificity (factor 13). The potency of (*S*)-**1b** was similar to that of (*S*)-**5a**. Phencyclidine (**2**, an achiral compound) with its amino group incorporated into a piperidine ring (as in DEP) was 3.5 times more potent than *N*-methylated (*S*)-**1b**, and by a factor of 16 in comparison with the primary amine (*S*)-**1a** (Table 1). Other differences (carbonyl

group, Cl substituent) may have contributed to this change. (−)-MK-801 [(−)-**3**], the enantiomer of (+)-**3**, was five times less potent than (+)-**3**, in agreement with the literature.[3] In strict terms, in Table 1 the more potent isomer (+)-**3** should be listed on the right side, since the orientation of its amino group appears isosteric to (*R*)-**5a** rather than to (*S*)-**5a** (see Chart 1). Other structural features than amino group orientation might be responsible for the (moderate) stereoselectivity of MK-801. Finally, the NMDA channel blocker Dextromethorphan [(+)-**4b**] blocked [$^3$H]MK-801 binding with slightly weaker potency than (*S*)-**5a** and (*S*)-**1b**; here, demethylation [to (+)-**4a**] resulted in a moderate increase in potency [not in a decrease as after demethylation of (*S*)-**1b**]. Levomethorphan [(−)-**4b**] yielded values similar to Dextromethorphan (although 100 times more potent at opiate receptors).[21]

Inhibition of [$^3$H]MK-801 binding by these compounds was estimated also in the presence of the polyamine modulator spermine. As a concomitant of increased accessibility of the NMDA receptor channel, the affinity of [$^3$H]MK-801 to rat brain membranes was strengthened in the presence of 100 μM spermine from 14.3 ± 2.6 nM ($K_D$) to 4.70 ± 1.24 nM (mean ± SD, $n$ = 12). The $K_i$ of several test compounds was also reduced by spermine [**2**, (*R*)-**5b**, (+)-**4b**, (−)-**4b**, ratio $K_i$ (spm)/$K_i$ <1, Table 1]. On the other side, many $K_i$ values were considerably increased by spermine, especially those of (*S*)-**1a** (by a factor of 2.87), (*S*)-**1b** (2.70), and (*S*)-**5a** (2.59; Table 1). Similar results had been obtained for Memantine[22] and for tryptamine.[16] These influences of spermine were often stereoselective: for example, the potency of (*S*)-**5b** was decreased, but that of (*R*)-**5b** was increased by spermine, and inhibitions by (*R*)-**1a** and (*R*)-**1b** were not influenced by spermine, in contrast to the results obtained with their respective (*S*)-enantiomers (Table 1).

## 3. Discussion

Stereoselectivity is a characteristic feature of drug interaction with a target in a geometrically restricted micro-environment. The high stereo-factors (>30) of DPEA (**5a)** and DEP (**6**) in blocking [$^3$H]MK-801 binding as demonstrated in this article strongly suggest the interaction with such a restricted target. Similar enantioselectivity has been reported for several 1-aryl-1,2,3,4-tetrahydroisoquinoline derivatives.[23] NMR spectroscopy of (*S*)-**5a** × HCl in $D_2O$ has shown that the Ph residues preferred an antiperiplanar conformation pointing into opposite directions, but also that the central ethane linkage rotated freely.[24] The absolute configurations of (*S*)-Ketamine [(*S*)-**1b**][25] and of its metabolite (*S*)-Norketamine [(*S*]-**1a**][26] have been revealed by X-ray analysis. However, the target of NMDA channel blockers has not yet been precisely defined. The extracellular part of the NMDA receptor complex, with its agonist and regulatory sites, has been crystallised and subjected to X-ray structural analysis down to 1.35 Å resolution,[27] but for the channel itself only tentative models have been proposed based on site-directed mutagenesis and accessibility studies. Nevertheless, evidence is accumulating that not all channel blockers interact with the channel in the same way. Combinations of NR1 with NR2C or NR2D responded weaker to MK-801 than combinations of NR1 with NR2A or NR2B; for Phencyclidine, these differences were smaller, whereas Ketamine did not differentiate at all.[28] The potency of all channel blockers is affected by mutating asparagine residues at the selectivity filter in the middle of the pore.[29] But an alanine residue on subunit NR1 slightly above this position seems to be more important for inhibition by MK-801 than by Phencyclidine,[30] and plays also a role for inhibition by Memantine.[31] On the other hand, the aromatic residue W563 still higher on NR1, if mutated to a non-aromatic residue, weakened the affinity of MK-801 at least 20-fold, without affecting that of Memantine.[31]

Sensitivity of NMDA channels to inhibition by MK-801 is increased eight times by splicing in 'exon 5' into the *N*-terminus of subunit NR1, but sensitivity to inhibition by Ketamine only two times.[32] Exon 5 is rich in positively charged amino acid residues and acts as an intrinsic positive modulator via the polyamine regulatory site. Most NMDA receptors in adult tissue (as used in our study) do not contain this exon,[33] but respond to spermine in a similar way. Here we show that accessibility of the channel is changed by spermine to the disadvantage of (*S*)-Ketamine as compared to MK-801, in agreement with data obtained with exon 5 containing receptors. The mechanism by which polycationic exon 5 and spermine increase opening frequency and burst length of NMDA channels is still not fully understood. Although most researchers agree that this mechanism includes the relieve from a block by ambient $H_3O^+$ ions ('proton block'), it is not exactly clear how this is accomplished. Site-directed mutagenesis studies point to the extracellular *N*-terminal domain of the NR1 subunit (harbouring the splice site for exon 5) as the major mediator of this stimulatory effect. However, a high number of amino acid residues lining the inner wall of the channel appear to be involved as well,[34,35] either as a direct target or mediating proton block down to the narrow constriction of the channel. Detailed SAR studies with several lipophilic amines have shown that spermine sensitivity of inhibition of [$^3$H]MK-801 binding depended on a primary amino group[36]—with the notable exception of the secondary amine (*S*)-Ketamine. It may, thus, be speculated that spermine and certain channel blocking (mostly) primary amines share common targets at the inner channel wall.

Although (*S*)-Ketamine and Memantine have similar effects on [$^3$H]MK-801 binding ($K_i$, sensitivity to spermine), their clinical properties differ: the former is narcotic and psychotomimetic, the latter neuroprotective without severe side-effects. It has been suggested that the block by Memantine is more easily reversible because it binds to a second site located at a higher level and escapes more readily than Ketamine from being trapped in the channel.[37] The special binding mode of the adamantane derivative Memantine might be related to its non-aromatic nature. Fluoro-substitution of aromatic residues seems to mitigate psychotic side effects as exemplified by 3,3-bis(3-fluorophenyl)-propylamine,[38] possibly due to lowering the density of π-electrons in the aromatic nuclei. NMDA receptor inhibition potencies of inhaled aromatic drugs of abuse correlate with their abilities to engage in cation–π interactions.[39] Our first attempt to create a channel blocker with similar favourable properties as Memantine failed, because our non-aromatic dicyclohexyl analogues **13** were virtually inactive.

## 4. Conclusion

Resolution of the NMDA channel blocker (±)-DPEA, and stereospecific synthesis of (+)- and (−)-DEP, resulted in enantiomers with highly differing potencies (stereo selectivity >30). The more potent DEP-isomer (*S*)-**6** behaved similarly to the high affinity channel ligand MK-801, whereas the more potent DPEA-isomer (*S*)-**5a** exhibited, like (*S*)-Ketamine and various primary amine channel blockers, a high degree of spermine sensitivity. Our detailed SAR study on enantiomers may help at refining a pharmacophore for clinically favourable NMDA receptor channel blockers.

## 5. Experimental

### 5.1. General procedures

$^1$H, $^{13}$C (*J* modulated), and $^{19}$F NMR spectra were measured in $CDCl_3$ on a Bruker Avance DRX 400, at 400.13, 100.63, and 376.5 MHz, respectively. Chemical shifts were referenced to

residual $CHCl_3$ ($\delta_H$ = 7.24) and $CDCl_3$ ($\delta_C$ = 77.00). IR spectra were run on a Perkin–Elmer 1600 FT-IR spectrometer; liquid samples were measured as films on a silicon disc.[40] Optical rotations were measured at 20 °C on a Perkin–Elmer 351 polarimeter in a 10 cm cell. TLC was carried out on 0.25 mm Silica Gel 60 $F_{254}$ plates (Merck). Flash (column) chromatography was performed with Merck Silica Gel 60 (230–400 mesh). Spots were visualised by UV and/or dipping the plate into a solution of $(NH_4)_6Mo_7O_{24} \times 4H_2O$ (23.0 g) and $Ce(SO_4)_2 \times 4H_2O$ (1.0 g) in 10% aqueous $H_2SO_4$ (500 mL), followed by heating with a heat gun. Melting points were determined on a Reichert Thermovar instrument and were uncorrected.

### 5.2. Compound sources

[$^3$H]MK-801 was obtained from New England Nuclear (Vienna); unlabelled (+)-MK-801 [(+)-**3**] and (−)-MK-801 [(−)-**3**] from Tocris Cookson Ltd (Bristol, UK). (*R*)- and (*S*)-Amphetamine (**17**) were obtained from Smith, Kline & French; (1*R*,2*S*)-(−)- and (1*S*,2*R*)-(+)-Tranylcypromine (**18**) from Procter & Gamble Germany (Darmstadt); (*S*)-(+)- and (*R*)-(−)-1,2-diphenyl-2-propylamine (**5b**), and (*S*)-(+)- and (*R*)-(−)-1-phenyl-2-(pyridin-2-yl)ethylamine (**5e**) from Astra Charnwood; (*S*)-(+)- and (*R*)-(−)-Ketamine (**1b**), (+)- and (−)-Norketamine (**1a**) from Gödecke, Parke-Davis; and (9*S*,13*S*,14*S*)-(+)-3-Methoxymorphinan (**4a**) and (9*R*,13*R*,14*R*)-(−)-Levomethorphan (−)-**4b**) from Hoffmann-La Roche (Basel). (*R*,*R*)-1,2-Dicyclohexylethane-1,2-diol (**7**) from Aldrich was crystallised from 1,2-dichloroethane to ee >98%. All other compounds were used as obtained either from Aldrich or from Sigma.

### 5.3. Resolution of (±)-DPEA into its (*S*)-(+)- and (*R*)-(−)-enantiomers [(*S*)-5a and (*R*)-5a]

The dextrorotary salt formed from (−)-tartaric acid and (*S*)-**5a** was obtained[8] by allowing a warm (35 °C) solution of (−)-tartaric acid (4.03 g, 26.9 mmol) and (±)-**5a** (5.3 g, 26.9 mmol) in dry MeOH (40 mL) to cool very slowly down to +4 °C. It was recrystallised (again with very slow cooling) twice from dry MeOH, treated with 2 N NaOH, and the free (*S*)-(+)-amine was extracted with $CH_2Cl_2$ and bulb to bulb distilled (82 °C, 0.06 mbar) to give a colourless oil; $[\alpha]_D^{20} = +10.6$ (*c* 1.45, $CHCl_3$), lit.[8] $[\alpha]_D^{20} = +10.7$ (*c* 1.05, $CHCl_3$); ee >99%; $[\alpha]_D^{20} = +47.7$ (*c* 1.58, EtOH). The mother liquors were concentrated, treated with 2 N NaOH and the recovered amine was resolved with (+)-tartaric acid to give the (*R*)-(−)-**5a**; $[\alpha]_D^{20} = -10.4$ (*c* 1.40, $CHCl_3$), lit.[8] $[\alpha]_D^{20} = -11.1$ (*c* 1.03, $CHCl_3$); ee >99%; $[\alpha]_D^{20} = -45.5$ (*c* 1.48, EtOH), lit.[41]: $[\alpha]_D^{20} = -45$ (*c* 2.0, EtOH). The ee of 98% for both samples was determined by $^1$H NMR spectroscopy using (*R*)-(−)-*O*-acetylmandelic acid as a chiral solvating agent (molar ratio of amine/chiral solvating agent 1/3).[42]

### 5.4. (*R*)-(−)- and (*S*)-(+)-DEP [(*R*)-6 and (*S*)-6]

1,5-Dibromopentane (1.083 g, 0.64 mL, 4.71 mmol, 3.0 equiv) was added dropwise to a solution of (*R*)-**5a** (0.309 g, 1.57 mmol) and $K_2CO_3$ (1.30 g, 9.42 mmol, 6.0 equiv) in dry $CH_3CN$ (12.1 mL) under argon. Stirring was continued for 3 days at room temperature (TLC: hexane/EtOAc = 5/1, $R_f$ = 0.27). The potassium salts were removed by filtration and washed with $CH_3CN$ (3 × 10 mL). The filtrate was concentrated under reduced pressure and the residue was purified by flash chromatography (hexane/EtOAc = 5/1) to give (*R*)-(−)-**6** (0.265 g, 64%) as a colourless oil; $[\alpha]_D^{20} = -87.3$ (*c* 0.92, EtOH). Similarly, (*S*)-**5a** (0.174 g, 0.882 mmol) was converted to (*S*)-(+)-**6** (0.127 g, 54%); $[\alpha]_D^{20} = +87.6$ (*c* 0.93, EtOH).

IR (Si): $\nu_{max}$ = 3027, 2932, 2793, 1495, 1452, 1114 $cm^{-1}$; $^1$H NMR: $\delta$ = 7.39–7.18 (m, 8H, $H_{ar}$), 7.12 (m, 2H, $H_{ar}$), 3.72 (dd, *J* = 9.4, 5.3 Hz, 1H, CHN), 3.44 (dd, *J* = 13.4, 5.3 Hz, 1H, $CH_2CN$), 3.13 (dd, *J* = 13.4, 9.4 Hz, 1H, $CH_2CH$), 2.56 (m, 4H, $H_{pip}$), 1.69 (m, 4H, $H_{pip}$), 1.49 (m, 2H, $H_{pip}$); $^{13}$C NMR: $\delta$ = 140.0, 139.4, 129.4 (2C), 128.9 (2C), 127.8 (2C), 127.6 (2C), 126.8, 125.6, 72.3, 51.4 (2C), 39.2, 26.3 (2C), 24.6. Anal. Calcd for $C_{19}H_{23}N$ (265.97): C, 85.99; H, 8.74; N, 5.28. Found for (*R*)-**6**: C, 86.49; H, 8.73; N, 5.12.

### 5.5. Hydrochlorides of (*R*)- and (*S*)-6

Concd HCl (0.4 mL) was added to a warm (40 °C) mixture of (*R*)-**6** (0.200 g, 0.754 mmol) and water (5 mL). On slowly cooling to +4 °C crystals formed, which were collected and recrystallised from a small amount of water containing HCl. The warm solution was allowed to cool slowly from 40 °C to +4 °C after seeding. The colourless crystals were collected and dried to give (*R*)-**6** × HCl × $H_2O$ (0.205 g, 85%); mp 220–221 °C (phase transitions at 100 °C and 160 °C); $[\alpha]_D^{20} = -83.5$ (*c* 0.86, EtOH). Similarly, (*S*)-**6** (0.110 g, 0.414 mmol) was converted to (*S*)-**6** × HCl × $H_2O$ (0.110 g, 83%); mp 222–224 °C; $[\alpha]_D^{20} = +82.6$ (*c* 0.82, EtOH). We also obtained the racemic piperidine derivative prepared in analogy to the enantiomers in 50% yield: (±)-**6** × HCl × $H_2O$, mp 207–209 °C (phase transitions at 100 °C and 155 °C) (lit.[43] 207 °C, without crystal water; lit.[10] 207–209 °C, without crystal water).

$^1$H NMR: $\delta$ = 12.40 (br s, 1H, NHCl), 7.35 (br s, 5H, $H_{ar}$), 7.10–6.97 (m, 5H, $H_{ar}$), 4.18 (ddd, *J* = 12.1, 4.1, 3.3 Hz, 1H, CHN), 3.99 (dd, *J* = 12.1, 3.0 Hz, 1H, $CH_2CN$), 3.64–3.46 (m, 2H, $H_{pip}$), 3.42 (t, *J* = 12.1 Hz, 1H, $CH_2CN$), 2.60–2.37 (m, 3H, $H_{pip}$), 2.34–2.21 (m, 1H, $H_{pip}$), 1.87–1.73 (m, 3H, $H_{pip}$), 1.78 (s, 2H, $H_2O$), 1.30–1.17 (m, 1H, $H_{pip}$); $^{13}$C NMR: $\delta$ = 135.8, 131.1, 130.2 (2C), 130.0, 129.3 (2C), 129.2 (2C), 128.4 (2C), 126.8, 72.9, 53.4, 48.8, 36.8, 22.7, 22.7, 22.3. Anal. Calcd for $C_{19}H_{24}ClN \times H_2O$ (319.87): C, 71.34; H, 8.19; N, 4.38. Found for (*R*)-**6** × HCl × $H_2O$: C, 71.32; H, 8.22; N, 4.36; found for (±)-**6** × HCl × $H_2O$: C, 71.20; H, 8.17; N, 4.54.

### 5.6. (*R*,*R*)-(+)-4,5-Dicyclohexyl-[1,3]dioxolane-2-thione (8)

A mixture of (*R*,*R*)-1,2-dicyclohexylethane-1,2-diol (**7**) (1.223 g, 5.40 mmol, ee >98%) and thiocarbonyl diimidazole (1.29 g, 7.29 mmol, 1.35 equiv) in dry toluene (31.9 mL) was refluxed for 3 h, then allowed to cool and concentrated in vacuo. Water (15 mL) was added and the mixture was extracted with EtOAc (3 × 20 mL). The combined organic layers were dried ($Na_2SO_4$) and concentrated under reduced pressure. The residue was purified by flash chromatography (hexane/EtOAc = 5/1; $R_f$ = 0.80, hexane/EtOAc = 3/1) to give thiocarbonate **8** (1.289 g, 89%) as colourless crystals; mp 161–162 (EtOAc, hexane), $[\alpha]_D^{20} = +75.0$ (*c* 0.90, acetone).

IR (Si): $\nu_{max}$ = 2926, 2852, 1444, 1348, 1297, 1259, 1197, 1174 $cm^{-1}$; $^1$H NMR: $\delta$ = 4.36 (m, 2H, 2CHO), 1.82–1.54 (m, 13H, $H_{chex}$), 1.28–0.96 (m, 11H, $H_{chex}$); $^{13}$C NMR: $\delta$ = 191.7, 88.0 (2C), 41.3 (2C), 27.7 (2C), 27.0 (2C), 25.9 (2C), 25.5 (2C), 25.3 (2C). Anal. Calcd for $C_{15}H_{24}O_2S$ (268.41): C, 67.12; H, 9.01. Found: C, 67.38; H, 9.10.

### 5.7. (*S*)-(−)-1,2-Dicyclohexylethanol [(*S*)-9]

A solution of thiocarbonate **8** (0.746 g, 2.78 mmol), $Bu_3SnH$ (1.618 g, 1.5 mL, 5.56 mmol, 2 equiv), and AIBN (0.033 g, 0.20 mmol) in dry toluene (30 mL) was added dropwise to refluxing toluene (54.5 mL) under argon during 40 min. More $Bu_3SnH$ (2 × 0.75 mL) and AIBN (2 × 0.033 g) were added after 2 and 4 h. After 6.5 h the reaction mixture was cooled and treated with aqueous NaOH (10%, 27.5 mL) at 40 °C for 12 h. The organic layer was separated and the aqueous one was extracted with $Et_2O$ (3 × 30 mL). The combined organic layers were washed with water, dried ($MgSO_4$) and concentrated under reduced pressure. The crude product was purified by flash chromatography

(hexane/EtOAc = 10/1; $R_f$ = 0.33) to give alcohol (*S*)-**9** (0.353 g, 60%) as colourless crystals; mp 82–83 °C (hexane), $[\alpha]_D^{20} = -33.9$ (*c* 1.03, acetone).

IR (Si): $\nu_{max}$ = 3278, 2917, 2851, 1450 $cm^{-1}$; $^1H$ NMR: $\delta$ = 3.45 (m, 1H, CHO), 1.83–1.58 (m, 10H, $H_{chex}$ and $CH_2$), 1.43 (m, 1H, $H_{chex}$), 1.36–0.75 (m, 14H, $H_{chex}$); $^{13}C$ NMR: $\delta$ = 73.4, 44.1, 42.1, 34.6, 34.2, 32.7, 29.2, 27.6, 26.7, 26.6, 26.4, 26.4, 26.2, 26.2. Anal. Calcd for $C_{14}H_{26}O$ (210.36): C, 79.94; H, 12.46. Found: C, 80.14; H, 12.52.

### 5.8. (*R*)-Mosher esters (2*R*,1′*S*)-10 and (2*R*,1′*R*)-10

(*S*)-MTPA-Cl [0.3 mL, 0.5 M solution in dry $CH_2Cl_2$; (*S*)-α-methoxy-α-trifluoromethyl-phenylacetyl chloride, (*S*)-Mosher chloride] was added to a solution of alcohol (*S*)-**9** (21 mg, 0.1 mmol) in dry pyridine (1 mL) and dry $CH_2Cl_2$ (0.5 mL). After stirring for 18 h at room temperature, water and HCl (2 M) were added. The mixture was extracted with $CH_2Cl_2$ (3 × 10 mL). The combinded organic layers were washed with a saturated solution of $NaHCO_3$ and water, dried ($Na_2SO_4$) and concentrated under reduced pressure. The residue was purified by flash chromatography (hexane/EtOAc = 15/1) to give (*R*)-Mosher ester (2*R*,1′*S*)-**10** (42 mg, quantitative). The ester of (*R*)-**9** was prepared similarly. $^{19}F$ NMR spectra [(2*R*,1′*S*)-**10**: −71.38 ppm; (2*R*,1′*R*)-**10**: −71.57 ppm] indicated ee >98% for (2*R*,1′*S*)-**10** and >96% for (2*R*,1′*R*)-**10**.

### 5.9. (*R*)-(+)-1,2-Dicyclohexylethyl 4-nitrobenzoate (12)

Alcohol (*S*)-**9** (0.621 g, 2.95 mmol), triphenylphosphine (1.0 g, 4.13 mmol, 1.4 equiv) and 4-nitrobenzoic acid (0.690 g, 4.13 mmol, 1.4 equiv) in dry toluene (8.6 mL) was cooled at 0 °C under argon. DIAD (0.81 g, 4.13 mmol, 1.4 equiv) was added and stirring was continued for 20 h at room temperature (TLC: hexane/EtOAc = 10/1, $R_f$ = 0.79). The solvent was evaporated under reduced pressure and the residue was purified by flash chromatography (hexane/EtOAc = 15/1) to give 4-nitrobenzoate **12** (0.477 g, 45%) as needles; mp 69–70 °C (hexane); $[\alpha]_D^{20} = +31.5$ (*c* 1.06, acetone).

IR (Si): $\nu_{max}$ = 2926, 2853, 1721, 1529, 1450, 1349, 1279 $cm^{-1}$; $^1H$ NMR: $\delta$ = 8.23 (m, 4H, $H_{ar}$), 5.15 (ddd, *J* = 9.1, 5.1, 3.8 Hz, 1H, CHO), 1.87–1.43 (m, 14H, $CH_2$ and $H_{chex}$), 1.30–0.79 (m, 10H, $H_{chex}$); $^{13}C$ NMR: $\delta$ = 164.4, 150.4, 136.2, 130.7 (2C), 123.5 (2C), 77.8, 42.0, 38.9, 34.2, 34.1, 32.8, 29.2, 28.0, 26.4, 26.4, 26.2, 26.1, 26.1, 26.1. Anal. Calcd for $C_{21}H_{29}NO_4$ (359.46): C, 70.17; H, 8.13; N, 3.90. Found: C, 70.15; H, 8.39; N, 3.74.

### 5.10. (*R*)-(+)-1,2-Dicycohexylethanol [(*R*)-9]

MeONa in MeOH (0.2 M, 4.4 mL) was added dropwise to a stirred solution of the 4-nitrobenzoate **12** (0.269 g, 0.748 mmol) in dry THF (1.1 mL). The reaction mixture was stirred for 12 h at room temperature. Water (5 mL) was added and the mixture was extracted with EtOAc (3 × 10 mL). The combined organic layers were dried ($Na_2SO_4$) and concentrated under reduced pressure. The crude product was purified by flash chromatography (hexane/EtOAc = 10/1; $R_f$ = 0.28) to give alcohol (*R*)-**9** (0.137 g, 87%) as colourless crystals; mp 81–82 °C (hexane); $[\alpha]_D^{20} = +31.1$ (*c* 1.05, $CHCl_3$). The spectroscopic data were identical with those of (*S*)-**9**.

### 5.11. (*R*)-(+)- and (*S*)-(−)-1-Azido-1,2-dicyclohexylethane [(*R*)-11 and (*S*)-11]

A solution of alcohol (*S*)-**9** (0.243 g, 1.155 mmol) and triphenylphosphine (0.393 g, 1.50 mmol, 1.3 equiv) in dry toluene (6.85 mL) was cooled in ice bath under argon. DIAD (0.304 g, 0.296 mL, 1.50 mmol, 1.3 equiv) and $HN_3$ in toluene (1.06 mL, 1.50 mmol, 1.4 M, 1.3 equiv) were added and stirring was continued for 15 min at 0 °C and 1 h at room temperature (TLC, for azide: hexane, for precursor alcohol: hexane/EtOAc = 10/1). The solvent was evaporated under reduced pressure and the residue was purified by flash chromatography (hexane; $R_f$ = 0.75) to give azide (*R*)-**11** (0.228 g, 87%) as a colourless liquid; $[\alpha]_D^{20} = +31.9$ (*c* 0.98, acetone). Similarly, alcohol (*R*)-**9** (0.210 g, 1.0 mmol) was converted to azide (*S*)-**11** (0.191 g, 81%); $[\alpha]_D^{20} = -32.3$ (*c* 1.11, acetone).

IR (Si): $\nu_{max}$ = 2925, 2853, 2099, 1449, 1334, 1260 $cm^{-1}$; $^1H$ NMR: $\delta$ = 3.15 (ddd, *J* = 9.5, 4.9, 4.1 Hz, 1H, $CHN_3$), 1.81–1.59 (m, 10H, $H_{chex}$ and $CH_2$), 1.46–0.76 (m, 14H, $H_{chex}$); $^{13}C$ NMR: $\delta$ = 65.7, 42.8, 39.0, 34.6, 34.1, 32.5, 30.0, 28.5, 26.6, 26.4, 26.3, 26.2, 26.1, 26.1. Anal. Calcd for $C_{14}H_{25}N_3$ (235.37): C, 71.44; H, 10.71; N, 17.85. Found for (*R*)-**11**: C, 72.00; H, 11.02; N, 17.59.

### 5.12. (*R*)-(+)- and (*S*)-(−)-1,2-Dicyclohexylethylamine [(*R*)-13 and (*S*)-13]

$LiAlH_4$ (1.73 mL, 0.865 mmol, 0.5 M in dry $Et_2O$, 1.5 equiv) was added dropwise to a solution of azide (*R*)-**11** (0.136 g, 0.578 mmol) in dry $Et_2O$ (2.3 mL) and stirring was continued for 1.5 h at room temperature (TLC, for amine: hexane/EtOAc = 1/5, for azide: hexane). The mixture was cooled at 0 °C, aqueous NaOH (0.2 mL, 2 M) was added dropwise and stirring was continued for 30 min at room temperature. The solvent was decanted and the residue was extracted with $Et_2O$ (3 × 10 mL). The combined organic layers were dried ($MgSO_4$) and concentrated under reduced pressure. The crude product was purified by flash chromatography (hexane/EtOAc = 1/3; $R_f$ = 0.33) to give (*R*)-(+)-**13** (0.106 g, 88%) as a colourless oil; $[\alpha]_D^{20} = +27.8$ (*c* 0.79, EtOH). Similarly, azide (*S*)-**11** (0.177 g, 0.752 mmol) was converted to (*S*)-(−)-**13** (0.130 g, 83%); $[\alpha]_D^{20} = -27.7$ (*c* 1.2, EtOH).

IR (Si): $\nu_{max}$ = 2922, 2851, 1449 $cm^{-1}$; $^1H$ NMR: $\delta$ = 2.57 (dt, *J* = 8.9, 4.1 Hz, 1H, CHN), 1.78–1.69 (m, 3H, $CH_2$), 1.69–1.57 (m, 7H, $NH_2$ and $H_{chex}$), 1.40–0.72 (m, 17H, $H_{chex}$); $^{13}C$ NMR: $\delta$ = 52.8, 44.3, 42.8, 34.6, 34.5, 32.7, 29.7, 27.7, 26.7, 26.7, 26.6, 26.5, 26.5, 26.2.

### 5.13. Hydrochlorides of 13

A solution of HCl (0.25 mL, 8 M) in dry $Et_2O$ was added dropwise to a solution of (*R*)-**13** (0.141 g, 0.673 mmol) in $CHCl_3$ (0.25 mL) and *i*$Pr_2O$ (3 mL). The mixture was cooled from 20 °C to −18 °C. The colourless crystals were collected, washed with *i*$Pr_2O$ and dried to give hydrochloride (*R*)-**13** × HCl (0.138 g, 88%); mp 187–188 °C, $[\alpha]_D^{20} = +19.5$ (*c* 1.01, $H_2O$). Similarly, amine (*S*)-**13** (0.130 g, 0.621 mmol) was converted to (*S*)-**13** × HCl (0.130 g, 85%); mp 188–189 °C (lit.[44] 188–189 °C); $[\alpha]_D^{20} = -20.0$ (*c* 0.80, $H_2O$) [lit.[44] −22.7 (*c* 1% in $H_2O$)].

$^1H$ NMR: $\delta$ = 8.28 (br s, 3H, $NH_3$), 3.09 (br s, 1H, CHN), 1.80–1.41 (m, 14H, $CH_2$ and $H_{chex}$), 1.36–1.04 (m, 8H, $H_{chex}$), 0.97–0.76 (m, 2H, $H_{chex}$); $^{13}C$ NMR: $\delta$ = 54.6, 40.1, 37.5, 33.6, 33.4, 32.8, 28.9, 27.7, 26.4, 26.2, 26.1, 26.1 (2C), 25.8. Anal. Calcd for $C_{14}H_{28}ClN$ (245.83): C, 68.40; H, 11.48; N, 5.70. Found for (*R*)-**13** × HCl: C, 68.02; H, 11.62; N, 5.53.

### 5.14. Binding of [$^3$H]MK-801 to rat neuronal membranes

Whole cell membranes were prepared from adult rat cerebral cortex and CA1 and dentate gyrus part of hippocampus as described.[16] Binding assays were conducted in 50 mM Tris-acetate (pH 7.0) for 2 h at 23 °C, with 5 nM [$^3$H]MK-801, 10 μM glutamate, and 10 μM glycine. Under these conditions, equilibrium is reached.[45] For nonspecific binding, glutamate and glycine were replaced by 100 μM D-2-amino-5-phosphono valeric acid and 10 μM 5,7-dichlorokynurenic acid (Tocris Cookson Ltd, Bristol, UK). Nonspecific binding amounted to 10–20% of total binding. Membranes

with bound radioligand were collected by rapid filtration over polyethylenimine-soaked glass fibre filters with a 48-places harvester (Brandel, Gaithersburg, USA). Filters were immersed in toluene with PPO and POPOP, agitated for 20 min, and radioactivity quantified in a liquid scintillation counter (Tricarb 2100, Packard). $IC_{50}$ values were estimated as described[16] and converted to $K_i$ values.[46] Statistical significance of mean value differences was evaluated by analysis of variance, with post hoc Newman–Keuls test.

## Acknowledgements

For parts of this study, M.L.B. received financial support from Merck KGaA (Darmstadt, Germany). Kerstin Schöringhumer, Manuel Nagl and Tanja Werle contributed to the binding data. We thank S. Felsinger for recording the NMR spectra.

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