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Original article

The search for TCP analogues binding to the low affinity PCP receptor sites in the rat cerebellum

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Abstract – With the aim of obtaining selective ligands of the low affinity binding sites of [^{3}H]-1-[1-(2-thienyl)cyclohexyl]piperidine ([^{3}H]TCP) in the rat cerebellum, oxygen and sulfur atoms were introduced in the TCP structure and derivatives to obtain analogues with a lowered lipophilicity. These compounds, and others already obtained, were assayed comparatively to determine their affinities for three sites labeled with [^{3}H]TCP: one in the forebrain, the originally described PCP receptor, and two in the rat cerebellum. Lowering the lipophilicity and modifying the hetero-aromatic moiety yielded some ligands with increased affinity for the low affinity sites in the rat cerebellum and decreased affinity for the high affinity sites in the forebrain. Particularly, two compounds displaying both a high affinity and a good selectivity might be valuable tools to elucidate the pharmacology of the low affinity PCP sites labeled with [^{3}H]TCP in the rat cerebellum. © Elsevier, Paris

TCP / TCP analogues / PCP receptor subsites / rat cerebellum

1. Introduction

[^{3}H]-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*, *d*]cyclohepten-5,10-imine ([^{3}H]MK-801) and [^{3}H]-1-[1-(2-thienyl)cyclohexyl]piperidine ([^{3}H]TCP) are the most frequently used ligands for the labeling of the non-competitive antagonists binding site within the N-methyl-D-aspartate (NMDA) receptor associated Ca^{2+} channel (the PCP receptor) [1, 2]. However, competition data analysis using a two-site model revealed that both ligands labeled at least two different binding sites in the rat brain: (i) high affinity sites, well represented in the forebrain and corresponding to the initially discovered PCP receptor and (ii) lower affinity sites, more abundant in the hindbrain and particularly in the cerebellum [3–8]. The nature and the role of the low affinity binding sites are poorly documented mostly because selective ligands are not available. Particularly, their possible role in neuronal protection is unknown whereas the high affinity sites is a well-established target for neuroprotective agents [6]. Thus, the finding of ligands binding selectively and potently to the low affinity sites might be crucial for their pharmacological characterization. For clarity, the different binding sites discussed here will be marked PCP_1 (the [^{3}H]TCP high affinity binding sites in the forebrain, i.e., the PCP receptor within the NMDA receptor associated ionic channel), PCP_2 (the [^{3}H]TCP high affinity binding sites in the cerebellum), and PCP_3 ([^{3}H]TCP low affinity binding sites in the cerebellum).

We have previously introduced oxygen and sulfur atoms in the cyclohexyl and piperidinyl moieties of the TCP structure to obtain analogues with a lowered lipophilicity. In rat forebrain membranes, the affinity of these analogues for the PCP_1 sites labeled with [^{3}H]TCP was decreased as a function of lipophilicity: the lower lipophilicity was, the lower affinity was [7]. However, their affinity for PCP_2 and PCP_3 sites in rat cerebellum membranes was not studied. The possibility that lowering the lipophilicity decreased the affinity for PCP_1 in the forebrain while increasing the affinity for PCP_3 in the cerebellum was attractive since the lipophilic TCP and MK-801 (log P = 4.56 ± 0.30 and 3.71 ± 0.39 respectively) displayed very low affinities for PCP_3 *(table I)*. Thus, we have decided to investigate this hypothesis by:

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Table I. Inhibition of [^{3}H]TCP binding in rat forebrain and cerebellum membranes by TCP and MK-801. The mean of at least three independent determinations was analyzed according to a single-site model in the forebrain and cerebellum (IC_{50} [a], nM, Hill's number) or a two-site model in the cerebellum. The two-site model was statistically more probable in the cerebellum: the proportion of PCP_2 and PCP_3 sites (%) and affinities (IC_{50}, nM) are given. SEM are in brackets.

Compound	Forebrain single-site model		Cerebellum single-site model		Cerebellum two-site model			
	IC_{50} (PCP_1)	n_H	IC_{50}	n_H	IC_{50} (PCP_2)	% (PCP_2)	IC_{50} (PCP_3)	% (PCP_3)
TCP	9.3 [b]	1.00 [b]	188 (63)	0.56 (0.05)	59 (17)	72.6 (3.6)	3716 (1195)	29.8 (4.5)
MK-801	3.67 (0.65)	0.95 (0.04)	995 (458)	0.33 (0.04)	9.3 (4.6)	45.3 (4.9)	11125 (3179)	57.2 (5.8)

[a] IC_{50}: concentration of unlabeled drug that inhibited 50% of specific [^{3}H]TCP binding on specified sites; [b] from [20].

(i) preparing TCP analogues with varied lipophilicities by means of O, S or hydroxyl substitution; (ii) using these new TCP analogues and some of those previously obtained for competition measurements in rat forebrain and cerebellum membranes labeled with [^{3}H]TCP; (iii) determining the more probable model of interaction (single- or two-site) in the cerebellum and comparing the affinities of compounds for PCP_1, PCP_2, and PCP_3.

2. Chemistry

Two different synthetic strategies were used to obtain TCP analogues according to the presence or absence of a methyl substitution in the cyclohexyl or hetero-cyclohexyl moiety (X, Y-substituted ring, see *table II*). The unsubstituted compounds were easily obtained by means of a Bruylants reaction [9]. It consists in the replacement of a cyano group by an aryl- or hetero-aryl group by the reaction of an α-aminonitrile with an aryl- or hetero-arylmagnesium halide *(figure 1)*. The suitable α-aminonitrile resulted from a Strecker-like synthesis in an organic or aqueous medium [10, 11]. When the hetero-aryl moiety was a 2-furyl (**8**, **9**), 4-methyl-2-thienyl (**15**) or a 5-methyl-2-thienyl (**14**) group, the Grignard reagent was best obtained by means of a magnesium/Lithium exchange reaction [12, 13] between $MgBr_2$ and the suitable 2-Li derivative. The pure (e.e. > 99%) enantiomers of the 3-methyl-piperidine derivatives **10** and **11** were obtained according to the same pathway *(figure 1)* but starting from optically active 3-methyl-piperidines. The optical resolution of the 3-methyl-piperidine racemate was achieved by means of a crystallization procedure with (+)- and (–)-mandelic acid in ethyl acetate resulting in enantiomeric purities up to 98–99% [14, 15]. It should be noticed that we have previously described **10**-(–) and **10**-(+) obtained by a different strategy [15] but with very similar enantiomeric purities.

The new compounds obtained according to *figure 1* (**6**–**9**, **11**–**15**) were all checked by ^{13}C-NMR spectroscopy of the hydrochloride salts *(table III)*. In these series indeed, the hydrochlorides solutions are stabilized in almost homogeneous conformations: the aromatic or hetero-aromatic rings are essentially restrained to the axial position. Consequently, the conformational trapping induced by the protonation allows for structural comparisons (see below) between similar (axial aromatic rings) conformations [16–19].

Diastereomers **16** and **17,** bearing a methyl substitution at the hetero-cyclohexyl ring *(table IV)*, were prepared using the azide synthesis shown in *figure 2* [15, 20]. The *cis*/*trans* configurations were attributed from the ^{13}C-NMR spectra of their HCl salts. The chemical shifts were attributed by comparison with the spectra of GK-11 and GK-12 hydrochlorides, two analogues whose diastereomeric configurations have been previously characterized [20, 21]. The *cis* (Me/Pip) configuration was attributed to compound **16** where the specific γ-interaction due to the axial methyl substitution causes a clear upfield shift of carbon 5 *(table IV)*.

The synthesis of non-commercial ketonic or piperidinic starting materials was required. Briefly, according to reference [22], alkylation of ethyl 2-sulfanylacetate with ethyl 4-chlorobutanoate gave a 76% yield of ethyl 4-[(2-ethoxy-2-oxoethyl)sulfanyl]butanoate which was submitted to a Dieckmann cyclization to afford a 54%

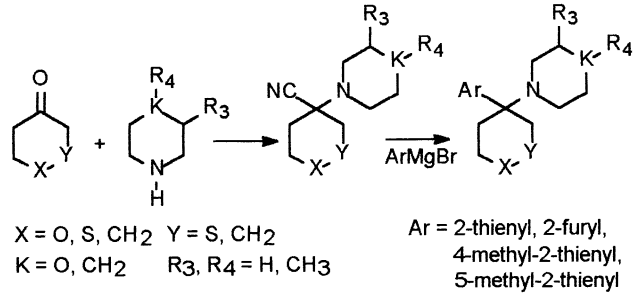
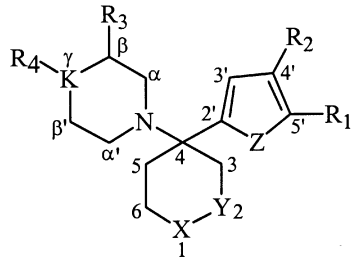


Figure 1.

Table II. TCP derivatives and analogues unsubstituted at the cyclohexyl or heterocyclohexyl ring and their calculated log *P*.



Compound	X	Y	Z	K	R_1	R_2	R_3	R_4	log *P* [a]
TCP	CH_2	CH_2	S	CH_2	H	H	H	H	4.56 ± 0.30
1	S	CH_2	S	CH_2	H	H	H	H	3.66 ± 0.50
2	O	CH_2	S	CH_2	H	H	H	H	2.85 ± 0.39
3	CH_2	CH_2	S	O	H	H	H	H	3.02 ± 0.39
4	S	CH_2	S	O	H	H	H	H	2.11 ± 0.56
5	O	CH_2	S	O	H	H	H	H	1.31 ± 0.41
6	SO_2	CH_2	S	CH_2	H	H	H	H	1.99 ± 0.40
7	CH_2	S	S	CH_2	H	H	H	H	3.52 ± 0.53
8	CH_2	CH_2	O	CH_2	H	H	H	H	4.05 ± 0.29
9	S	CH_2	O	CH_2	H	H	H	H	3.14 ± 0.52
10	CH_2	CH_2	S	CH_2	H	H	CH_3	H	5.06 ± 0.30
11	S	CH_2	S	CH_2	H	H	CH_3	H	4.15 ± 0.53
12	CH_2	CH_2	S	CH_2	H	H	CH_2OH	H	3.35 ± 0.37
13	CH_2	CH_2	S	CH_2	H	H	CH_3	OH	3.21 ± 0.37
14	S	CH_2	S	CH_2	CH_3	H	H	H	4.12 ± 0.53
15	S	CH_2	S	CH_2	H	CH_3	H	H	4.12 ± 0.53

[a] Calculated with the ACD/Log *P* program (ACD, Inc.) (95% confidence, octanol/water).

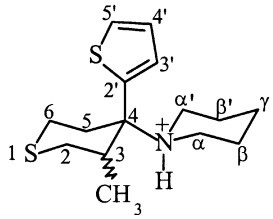
yield of ethyl 3-oxotetrahydro-2*H*-thiopyran-2-carboxylate. A decarboxylation in a 10% sulfuric acid solution gave 54% of pure dihydro-2*H*-thiopyran-3(4*H*)-one *(figure 3)*. 3-Methyltetrahydro-4*H*-thiopyran-4-one was obtained by reacting tetrahydro-4*H*-thiopyran-4-one with methyl-iodide in THF in the presence of one equivalent of LDA at the temperature of –80 °C. 3-Methyl-4-piperidinol was obtained essentially as its

Table III. ^{13}C-NMR [a] chemical shifts [b] (hydrochloride in $CDCl_3$, δ ppm from TMS).

Carbon	**6** [c]	**7**	**8**	**9**	**11**	**12**	**13**	**14**	**15**
1	–	22.7	23.4	–	–	22.2	22.5	–	–
2	*48.0*	–	*22.5*	24.5	24.9	23.0	30.8	24.7	25.2
3	30.5	33.4	29.8	30.2	33.4	33.4	32.8	32.8	33.6
4	66.6	68.0	67.6	66.8	68.9	64.2	69.0	68.8	69.4
5	30.5	32.4	29.8	30.2	33.2	33.0	32.8	32.8	33.6
6	*48.0*	26.8	*22.5*	24.5	24.9	23.0	30.8	24.7	25.2
α	*47.3*	47.0	47.0	46.7	52.3	49.7	50.6	46.5	47.2
α'	*47.3*	47.0	47.0	46.7	46.2	46.8	45.3	46.5	47.2
β	22.8	25.5	*22.3*	22.0	28.2	36.6	35.4	22.3	22.8
β'	22.8	25.5	*22.3*	22.0	30.5	25.3	32.8	22.3	22.8
γ	21.5	23.9	21.7	21.3	22.1	24.0	70.0	21.7	21.1
R	–	–	–	–	18.9	69.7	15.1	14.7	15.6
CAr	132.9–128.4	134.6–128.6	146.6–110.7	145.2–110.7	134.5–127.9	135.8–128.0	135.1–127.5	142.9–125.8	138.9–123.9

[a] For carbon atom numbering see *table IV*; [b] italicized chemical shifts may be exchanged; [c] DMSO-d_6.

Table IV. Comparative ^{13}C- (up) and ^{1}H-NMR (down) chemical shifts of GK11, GK12, and compounds **16, 17** (hydrochloride in $CDCl_3$, δ ppm from TMS).



Carbon	GK11 (*cis*)	*GK12 (trans)*	**17** *(trans)*	**16** *(cis)*
1	17.8	22.5	–	–
2	30.2	*30.0* [a]	*29.8* [a]	33.2
3	35.4	36.3	34.7	34.8
4	72.9	74.5	73.3	72.4
5	26.5	*30.7* [a]	*29.6* [a]	27.0
6	22.6	22.0	22.7	24.9
CH_3	15.8	17.1	16.8	15.1
α	48.9	48.2	48.6	48.9
α’	46.7	47.9	47.2	46.3
β	22.3	21.72	21.9	22.0
β’	22.1	21.69	21.6	22.0
γ	22.5	21.9	21.2	22.5
2’	137.1	136.4	135.8	134.8
3’	127.6	126.8	127.3	128.3
4’	127.2	126.4	127.0	127.8
5’	130.1	130.6	130.3	131.1
δ-CH_3	1.6	1.1	1.2	1.8
J (Hz)	6.9	6.6	6.2	6.7

[a] Italicized chemical shifts may be exchanged.

trans isomer by a 4-step synthesis starting from benzamide and ethyl acrylate *(figure 4)* [23]. The configuration was attributed by comparison with the ^{13}C-NMR spectra of *cis*- and *trans*-2-methylcyclohexanol [24].

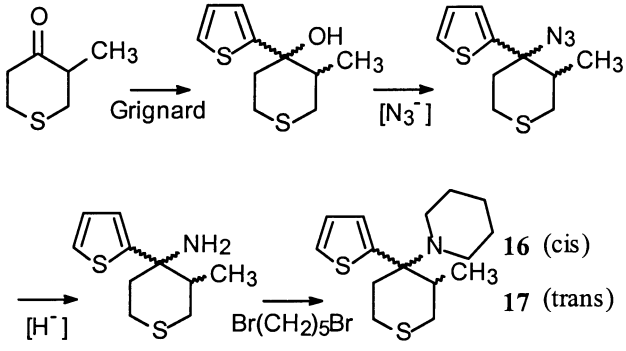


Figure 2.

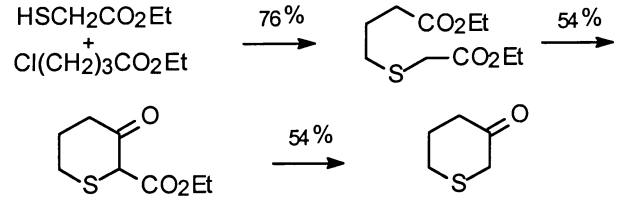


Figure 3.

3. Pharmacology

The binding assays in rat forebrain and cerebellum membranes and the data analysis are described in experimental protocols (Section 5.2). We have first checked that a two-site model was more probable than a single-site model to describe the competition of TCP and MK-801 in the rat cerebellum membranes labeled with [^{3}H]TCP *(table I)*. The results were consistent with those previously reported in membranes [3, 4] as well as in cultured cerebellum cells [5]. The same treatment was applied to 21 compounds derived from the TCP structure; the results are presented in *table V*.

4. Results and discussion

In forebrain homogenates, [^{3}H]TCP inhibition curves were better fitted to a single-site model (PCP_1-sites) *(table V)*. This result was likely since Hill numbers were most generally close to unity. However, in cerebellum homogenates, Hill numbers were mostly lower than unity and the inhibition curves were better fitted to a two-site

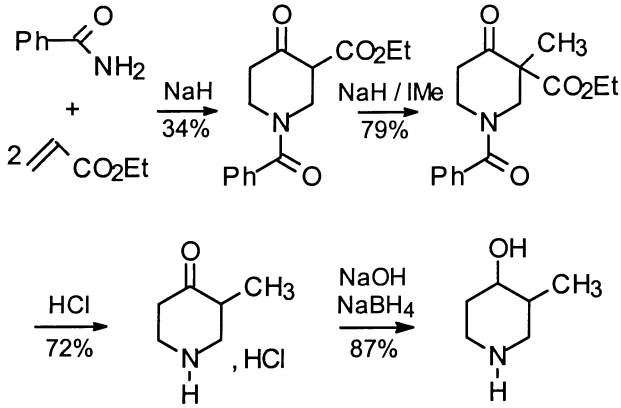


Figure 4.

Table V. Inhibition of [^{3}H]TCP binding in rat forebrain and cerebellum membranes. The mean of at least three independent determinations was analyzed according to a single-site model in the forebrain and cerebellum (IC_{50} [a], nM, Hill's number) or a two-site model in the cerebellum (SEM in brackets). When the two-site model was more probable, the proportion of PCP_2 and PCP_3 sites (%) and affinities (IC_{50} [a], nM) are given. Very low affinities precluded the two-site computation (n.d.).

Compound	Forebrain single-site model		Cerebellum single-site model		Cerebellum two-site model			
	IC_{50} (PCP_1)	n_H	IC_{50}	n_H	IC_{50} (PCP_2)	% (PCP_2)	IC_{50} (PCP_3)	% (PCP_3)
1	71.6 (10.5)	1.05 (0.05)	178 (40)	0.68 (0.10)	586 (96)	75.2 (8.8)	8.5 (4.5)	25.2 (7.2)
2	1223 (143)	0.98 (0.05)	2396 (475)	0.58 (0.12)	5840 (1690)	80.3 (6.5)	35 (18)	15.5 (5.7)
3	294 (48)	0.77 (0.02)	1001 (408)	0.69 (0.09)	183 (65)	64.3 (3.4)	2127 (1273)	33.0 (1.1)
4	8253 (320)	0.78 (0.06)	29700 (8022)	0.72 (0.02)	n.d.	–	n.d.	
5	> 100 μM	_	63800 (10400)	0.60 (0.08)	n.d.	–	n.d.	–
6	97700 (19066)	0.96 (0.18)	198500 (8500)	0.77 (0.22)	n.d.	–	n.d.	–
7	73.4 (1.4)	1.00 (0.05)	228 (43)	0.65 (0.02)	659 (206)	77.8 (3.7)	8.0 (4.8)	23.4 (5.0)
8	47.8 (1.1)	0.83 (0.06)	150 (78)	0.70 (0.11)	187 (42)	66.3 (12.0)	5.6 (3.5)	30.7 (10.0)
9	133 (20)	0.87 (0.06)	0.55 (0.03)	0.55 (0.03)	1015 (262)	68.3 (2.8)	13.8 (3.6)	32.3 (4.4)
10-(±)	5.5 (1.3)	1.09 (0.07)	122 (38)	0.80 (0.13)	47.3 (5.2)	68.5 (9.6)	1450 (590)	33.7 (8.1)
10-(+)	5.2 (1.0)	1.04 (0.06)	155 (10)	0.62 (0.08)	28.3 (17.1)	52.5 (10.5)	808 (47)	51.0 (11.8)
10-(–)	158 (20)	1.08 (0.09)	452 (53)	0.90 (0.08)	– [b]	–	– [b]	
11-(±)	132 (39)	1.14 (0.11)	265 (80)	0.69 (0.07)	265 (101)	69.1 (9.8)	2850 (433)	31.6 (11.1)
11-(+)	24 (8.2)	0.84 (0.03)	231 (71)	0.59 (0.04)	53.2 (15.8)	66.3 (1.1)	2076 (747)	34.6 (2.5)
11-(–)	529 (50)	0.93 (0.05)	996 (314)	0.85 (0.13)	– [b]	–	– [b]	–
12	28.4 (6.6)	0.96 (0.09)	467 (158)	0.61 (0.06)	2240 (330)	69.0 (4.0)	12.4 (5.7)	31.0 (5.0)
13	2406 (218)	1.07 (0.04)	3362 (1013)	1.02 (0.09)	– [b]	–	– [b]	–
14	462 (88)	0.97 (0.07)	1170 (242)	0.63 (0.06)	532 (20)	83 (3.5)	> 100 μM	15.3 (6.9)
15	77.8 (9.3)	0.93 (0.06)	762 (335)	0.62 (0.06)	772 (202)	76.4 (2.6)	5.4 (3.4)	21.8 (3.8)
16	26.8 (4.5)	0.89 (0.01)	206 (30)	0.71 (0.07)	1355 (733)	62.8 (7.3)	17.6 (6.6)	34.7 (9.5)
17	17233 (1417)	0.94 (0.09)	50467 (2663)	0.60 (0.06)	n.d.	–	n.d.	–

[a] IC_{50}: concentration of unlabeled drug that inhibited 50% of specific [^{3}H]TCP binding on specified sites; [b] a one-site model was more probable.

model (PCP_2- and PCP_3-sites) which produced a significant reduction in the sum of squares ($P < 0.05$, Student's *t*-test) and a Durbin–Watson coefficient between 1.5 and 2.5 (see Experimental protocols). The results presented in *table V* confirmed that [^{3}H]TCP labeled two sites in the cerebellum in a 70:30 mean relative proportion (PCP_2/PCP_3). Interestingly, most compounds were more or less able to interact with these binding sites. However compounds **4, 5, 6,** and **17** displayed a very low affinity whatever the binding sites and thus their affinity and selectivity could not be evaluated. Compounds **10**-(–), **11**-(–), and **13** displayed Hill numbers close to unity and their interaction was best described by a single-site model.

As previously shown, the affinity for PCP_1-sites was reduced with the decrease of lipophilicity [7] in homogeneous groups of structures. Indeed, TCP and derivatives displayed greater affinities for PCP_1 than their thio- or oxa-analogues: TCP had a higher affinity for the PCP_1-sites than **1** or **2** *(tables I* and *V)*, similarly **10** was a higher affinity ligand than **11** whatever the chirality is *(table V)*. The same behavior was revealed when comparing TCP and **8**. Interestingly, **1** and **7** were equipotent ligands for the PCP_1-sites although their sulfur atoms (in the six atoms ring) are located in two different positions.

Affinity for PCP_2-sites could be determined for 16 compounds only *(table V)*. Among them, **3**, **11**-(±), **11**-(+), **13**, and **14** were unable to discriminate significantly PCP_1- and PCP_2-sites since they displayed statistically close affinities for both sites. At the contrary, the 12 remaining compounds had affinities significantly different at PCP_1- and PCP_2-sites. These affinities were apparently linearly correlated ($P < 0.004$, $r = 0.76$, see *figure 5*). The lower affinity of these compounds for PCP_2-sites might be related to the distribution of NMDA NR_2 subunits. Indeed NR_{2A} and NR_{2B} subunits are highly expressed in the forebrain while NR_{2C} subunit is mainly expressed in the cerebellum [25, 26]. NMDA receptors involving NR_{2C} subunits are less sensitive to MK-801 blockade than those comprising NR_{2A} and/or NR_{2B} subunits [27]. These results are confirmed by competition experiments with [^{3}H]MK-801 [4, 5] and consistent with MK-801 affinities in *table I*. Thus PCP_1- and PCP_2-sites are likely to represent two different states of the NMDA receptor discriminated by some of the new molecules.

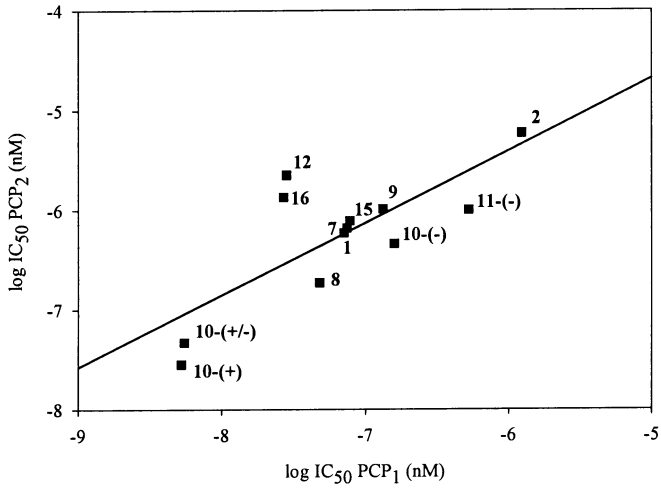


Figure 5. Linear relationships between affinities for PCP_1- and PCP_2-sites ($r = 0.76$, $p < 0.004$) for 12 compounds discriminating significantly both sites.

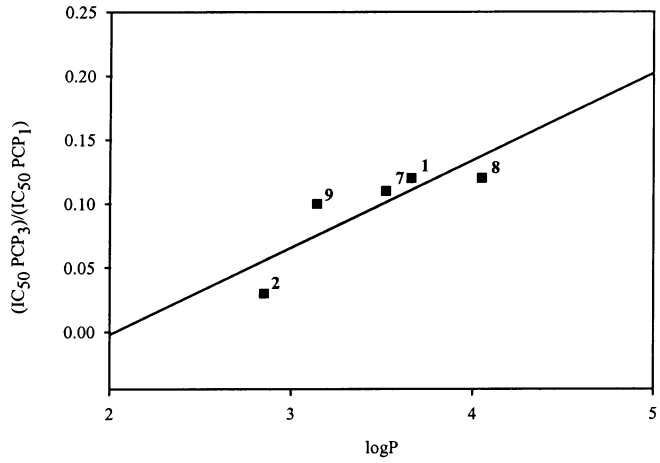


Figure 6. Relationships between selectivity (PCP_3/PCP_1) and log *P* ($r = 0.84$, $p < 0.08$) for molecules differing from the TCP model only by substitution of one heteroatom in the cyclohexyl ring and/or in the hetero-aromatic moiety.

The affinities for the PCP_3-sites could not be correlated with those for PCP_1- or PCP_2-sites and they appeared very sensitive to structure. Indeed, molecules tested appeared grossly separated into two groups: (i) molecules differing from TCP only by substitution of one heteroatom in the cyclohexyl ring and/or in the hetero-aromatic moiety, (ii) molecules differing from TCP by introduction of a methyl substitution in any of the constitutive rings or by specific modifications of the piperidine ring. Comparatively to the TCP model structure, lowering the lipophilicity *(table II)* clearly directed the first group of molecules (**1**, **2**, **7**, **8**, **9**) toward the PCP_3-sites since they displayed higher affinities for these sites (5.6 to 35 nM) than for PCP_1- (47.8 to 1223 nM) or PCP_2-sites (187 to 5840 nM) *(table V)*.Moreover, plotting IC_{50} PCP_3/IC_{50} PCP_1 against log *P* confirmed this tendency: the lower log *P* was, the higher the selectivity for PCP_3 was *(figure 6)*, in line with our hypothesis. A similar tendency was found with regard to PCP_2 (not shown). Finally, this group of compounds revealed interesting selectivities for PCP_3 when compared to PCP_2 and PCP_1. **2**, the less potent (35 nM) and the less lipophilic (log $P = 2.85$) in this group, displayed a high selectivity for the PCP_3-sites when compared to the PCP_1- and PCP_2-sites ($PCP_3/PCP_1 < 0.026$; $PCP_3/PCP_2 < 0.006$).

In the second group, changing the piperidine for a morpholine ring decreased considerably the affinities (**3**) or precluded the two-site computation given very low affinities (**4**, **5**). Substitution of a methyl group in the piperidine ring gave compounds with low affinities for PCP_3-sites: **13**, **10**-(±), **10**-(+), and their thio-analogues **11**-(±), **11**-(+). Interestingly, the affinities of **10**-(–), **11**-(–), and **13** were better described by a single-site model in the cerebellum. Compound **12** bearing an hydroxymethyl substitution in the piperidine ring exhibited a high affinity for both PCP_3- and PCP_1-sites with a high selectivity with regard to PCP_2-sites. The methyl substitution in the thiopyranyl ring gave a high affinity *cis*-compound (**16**) equipotent at the PCP_1- and PCP_3-sites and an inactive *trans*-compound (**17**). The difference between *cis* and *trans* diastereomers in the forebrain was higher than previously observed in the cyclohexyl homologue series [15, 20] although affinities were lower. Finally, the position of a methyl group in the hetero-aromatic moiety was crucial. In the α position from the sulfur atom (**14**), it lowered the affinity for PCP_3 and at the contrary, in the β position (**15**), it increased the affinity for these sites. Moreover **15** displayed a good selectivity with regard to both PCP_1- and PCP_2-sites ($PCP_3/PCP_1 < 0.07$; $PCP_3/PCP_2 < 0.007$). This is a confirmation of the very important role played by the aromatic or hetero-aromatic ring in the arylcyclohexylamines selectivity of binding [28]. In this second group of molecule steric interactions due to substitutions are likely to influence more selctivity than lipophilicity.

It is now well admitted that the NMDA receptor is an hetero-oligomeric protein composed with 5 sub-units differently expressed in forebrain, cerebellum, and spinal cord [29–31]. The resulting diversity of NMDA receptors

in the CNS, and consequently the diversity of the PCP receptors, is responsible for the heterogeneity of pharmacological responses [32]. The present study confirms the binding sites heterogeneity since TCP analogues were able to interact differently with subsites labeled with [^{3}H]TCP in the rat cerebellum. In this region interestingly NMDA receptors display particular properties when compared with NMDA receptors found in the forebrain [26]. Some of the structures we have prepared and tested might be valuable tools to clarify the pharmacological role of the low affinity sites in the rat cerebellum (PCP_3). Indeed, compounds **2** and **15** possess both a high affinity and a good selectivity for these sites. Such molecules might also be leads for new compounds able to interact with this specific target.

5. Experimental protocols

5.1. Chemistry

Melting points (uncorrected) were determined with a Büchi–Tottoli apparatus. Yields were not optimized. Elemental analysis was performed at the CNRS Microanalytical Section in Montpellier on the hydrochloride salts and were within ±0.4% of theoretical values. ^{1}H- and ^{13}C-NMR spectra were obtained on a Brucker AC 200 spectrometer at 200.13 and 50.32 MHz respectively in 5-mm sample tubes in the FT mode. For some ^{13}C-signal assignments, a spin-echo sequence (Jmod) was used. Chemical shifts are reported in (δ) ppm downfield from TMS. Enantiomeric purities were determined on a Shimadzu HPLC equipment (LC-1O AD pump, SPD-6A UV spectrometer), computer-controlled by the Class LC-10 program. Analysis were made on a Chiralcel-OD column (10 mm, 4.6 × 250 mm) (Daicel Chemical Industries) in heptane (0.6 mL /min) at 36 °C. UV-detection was made at 240 nm. Typical injection volumes were 5 mL of a 30 μM solution of base compound in heptane. Optical rotations were obtained in methanol with a Perkin-Elmer 241 polarimeter in a 1-dm microcell at 20 °C. For NMR and in vitro experiments, compounds were used as their hydrochloride salts; salts were precipitated by adding a dry HCl ethereal solution in ether to a solution of base in ether. After filtration, the solids collected were dried in vacuum.

5.1.1. Synthesis of dihydro-2H-thiopyran-3(4H)-one

5.1.1.1. Ethyl 4-[(2-ethoxy-2-oxoethyl)sulfanyl]butanoate

Sodium (4.6 g, 200 mmol, 1 eq.) was added cautiously by portion in a nitrogen atmosphere to ethanol (100 mL). After the solid was consumed, the mixture was cooled to 0 °C and ethyl 2-sulfanylacetate (22 mL, 200 mmol, 1 eq.) then ethyl 4-chlorobutanoate (30.1 g, 200 mmol, 1 eq.) was added slowly. The resulting mixture was stirred for 20 h at room temperature. The NaCl precipitate was filtered, the filtrate concentrated in vacuum, the oil obtained was diluted in water (100 mL) and extracted with ether (3 × 80 mL). The combined organic layers were dried over $MgSO_4$ and concentrated in vacuum. The resulting yellow oil was distilled to yield 35.6 g (76%) of a colorless oil.

5.1.1.2. Ethyl 3-oxotetrahydro-2H-thiopyran-2-carboxylate

Ethyl 4-[(2-ethoxy-2-oxoethyl)sulfanyl]butanoate in anhydrous ether (200 mL) was added dropwise in a nitrogen atmosphere to a solution of sodium ethanolate (20.7 g, 0.3 mol, 2 eq.) at 0 °C, stirred at 0 °C for 45 min and at room temperature for 3 h. The mixture was hydrolyzed with a water/acetic acid (80:20) solution, the aqueous phase was separated and extracted with ether (3 × 50 mL), the combined organic layers were dried over $MgSO_4$ and concentrated in vacuum. The resulting yellow oil obtained was distilled under reduced pressure to yield 15.3 g (54%) of a colorless liquid.

5.1.1.3. Dihydro-2H-thiopyran-3(4H)-one

Ethyl 3-oxotetrahydro-2*H*-thiopyran-2-carboxylate (15.2 g, 80.9 mmol) was refluxed in a 15% sulfuric acid solution for 18 h then cooled to room temperature. A 10% NaOH solution in water was then added dropwise to reach pH 6. The mixture was extracted with ether (3 × 50 mL), the organic phases washed with water, dried over $MgSO_4$, and concentrated in vacuum. The resulting oil was distilled under reduced pressure to yield 5.1 g (54%) of a colorless oil.

5.1.2. Synthesis of 1,1-dioxo-tetrahydro-1λ^6-thiopyran-4-one

A solution of hydrogen peroxide (11.4 mL, 0.1 mol, 2 eq.) was added dropwise to a mixture of tetrahydro-4*H*-thiopyran-4-one(5.6 g, 48 mmol, 1 eq.) and acetic acid (25 mL) keeping the temperature below 30 °C. The mixture was stirred for 4 h, the acetic acid distilled under reduced pressure, and the crystallized yellow residue was filtered and washed with ether to yield 4.7 g (67%) of white crystals.

5.1.3. Synthesis of 3-methyl-4-piperidinol

5.1.3.1. Ethyl 1-benzoyl-4-oxo-3-piperidinecarboxylate

Sodium hydride (4 g, 0.1 mol, 1 eq.) was added in a nitrogen atmosphere to a solution of benzamide (12.1 g, 0.1 mol, 1 eq.) in toluene (200 mL), the mixture was refluxed for 1 h, cooled to 0 °C and ethyl acrylate (32.6 mL, 0.3mol, 3 eq.) was then rapidly added. The solution was stirred at 60 °C for 24 h, cooled to 0 °C, diluted with ice-cold water (100 mL) and stirred for 0.5 h. The aqueous phase was separated, washed with ether (50 mL), acidified until pH 3 and extracted with CH_2Cl_2 (3 × 50 mL). The combined organic layers were dried over Na_2SO_4, filtered and concentrated in vacuum. The yellow oil obtained was purified by column chromatography (SDS Chromagel 70–100 μ) in ether to yield 9.4 g (34%) of a red oil.

5.1.3.2. Ethyl 1-benzoyl-3-methyl-4-oxo-3-piperidinecarboxylate

A mixture of ethyl 1-benzoyl-4-oxo-3-piperidinecarboxylate (9.3 g, 33.8 mmol, 1 eq.) and sodium hydride (1.35 g, 34 mmol, 1 eq.) in dimethoxyethane (50 mL) was refluxed for 2 h then cooled to 0 °C. ICH_3 (5.2 mL, 84 mmol, 2.5 eq.) was added and the mixture heated at 60 °C for 40 h. The mixture, after concentration in vacuum, was diluted with water (100 mL) and extracted with CH_2Cl_2 (3 × 50 mL). The combined organic layers were washed successively with a 5% NaOH water solution (50 mL), a 5% HCl water solution (50 mL), with water (50 mL), then dried over Na_2SO_4, and concentrated in vacuum. The resulting brown oil (9.4 g) was purified by column chromatography (SDS Chromagel 70–200 μ) in ether to yield 7.7 g (79%) of a slightly yellow oil.

5.1.3.3. 3-Methyl-piperidin-4-one hydrochloride

A solution of ethyl 1-benzoyl-3-methyl-4-oxo-3-piperidinecarboxylate (7.7 g, 26.6 mmol, 1 eq.) in a 6 N HCl aqueous solution was refluxed for 72 h, then the benzoic acid precipitate was filtered, the filtrate washed with ether (3 × 50 mL) and concentrated in vacuum. The brown solid obtained was crystallized in ethanol to get white crystals (2.85 g, 72%).

5.1.3.4. 3-Methyl-4-piperidinol

A solution of 5% NaOH in water (7.6 mL) was added dropwise to a solution of 3-methyl-piperidin-4-one hydrochloride (2.85 g, 19 mmol, 1 eq.) in methanol (30 mL) and the mixture was stirred for 0.5 h at room temperature. A solution of $NaBH_4$ (2.46 g, 6.5 mmol, 1.4 eq.) in methanol (30 mL) was added, the mixture was stirred for 4 h at room temperature, cooled to 0 °C, made acidic with few drops of a 5% HCl aqueous solution, and concentrated in vacuum. The resulting yellow solid was dissolved in hot ethanol and precipitated at room temperature by the addition of drops of ether to yield a white solid (1.9 g, 87%).

5.1.4. Synthesis of α-aminonitriles

α-aminonitriles were obtained by means of two different synthetic methods. Since the same method was applied to various compounds we describe only one example in each case.

5.1.4.1. Method A

Acetone cyanohydrine (2.2 g, 25.8 mmol, 1 eq.) was added dropwise to a stirred mixture of tetrahydro-4*H*-thiopyran-4-one (3 g, 25.8 mmol, 1 eq.), anhydrous $MgSO_4$ (9.3 g, 77.4 mmol, 3 eq.), dimethylacetamide (2.25 g, 25.8 mmol, 1 eq.) and piperidine (4.4 g, 51.6 mmol, 2 eq.). The pasty mixture was heated at 45 °C for 48 h, cooled to room temperature, poured onto ice and stirred for 30 min. The aqueous mixture obtained was extracted with ether and the organic layer was washed with water until neutrality, dried over Na_2SO_4, filtered, and concentrated in vacuum to yield 5.3 g (98%) of an orange oil of 4-piperidinotetrahydro-2*H*-thiopyran-4-carbonitrile.

The following α-aminonitriles were similarly synthesized: 4-piperidinotetrahydro-2*H*-pyran-4-carbonitrile (79%, *F* = 46–47 °C) from piperidine and tetrahydro-4*H*-pyran-4-one; 4-(3-methylpiperidino)tetrahydro-2*H*-thiopyran-4-carbonitrile (> 98%, oil) from 3-methylpiperidine and tetrahydro-4*H*-thiopyran-4-one; R-4-(3-methylpiperidino)tetrahydro-2*H*-thiopyran-4-carbonitrile (> 98%, oil) and S-4-(3-methylpiperidino)tetrahydro-2*H*-thiopyran-4-carbonitrile (> 98%, oil) from R-(–)-3-methylpiperidine and S-(+)-3-methylpiperidine [7, 8, 21], and tetrahydro-4*H*-thiopyran-4-one; 4-(3-methylpiperidino)tetrahydro-2*H*-thiopyran-4-carbonitrile (81%, solid) from 3-hydroxymethylpiperidine and cyclohexanone.

5.1.4.2. Method B

5% HCl (a few drops) was added to a stirred mixture of 3-methyl-4-piperidinol (0.8 g, 7 mmol, 2 eq.) in 10 mL of water and cyclohexanone (2.7 g, 23.6 mmol, 1 eq.) to reach pH 3. KCN (0.47 g, 7.3 mmol, 1.05 eq.) was added (pH reached 11). The mixture was stirred at room temperature for 24 h then extracted with CH_2Cl_2, dried over Na_2SO_4, filtered, and concentrated in vacuum to yield 1.43 g (93%) of 1-(4-hydroxy-3-methylpiperidino)cyclohexanecarbonitrile as a colorless oil.

Table VI. Purification and properties of new compounds.

	Purification	Solvent (v/v)	F_{base} (°C)	F_{HCl} (°C)	Yield (%)
6	Crystallization	AcOEt	167	179–183	48
7	Column chromatograpy (Al_2O_3) [c]	Petroleum ether/ether (40:60)	58–60	184–186	83
8	Column chromatograpy (Al_2O_3)	CH_2Cl_2	Oil	166–167	76
9	Column chromatograpy (Al_2O_3)	Petroleum ether/ether (98:2)	79–81	171–173	71
11-(±)	Column chromatograpy (Al_2O_3)	Petroleum ether/ether (95:5)	Oil	168–169	57
11-(+) [a]	Column chromatograpy (Al_2O_3)	Petroleum ether/ether (95:5)	Oil	170–172	59
11-(–) [b]	Column chromatograpy (Al_2O_3)	Petroleum ether/ether (95:5)	Oil	169–171	64
12	Column chromatograpy (Al_2O_3)	Petroleum ether/ether (50:50)	Oil	176–178	56
13	Column chromatograpy (Al_2O_3)	Petroleum ether/ether (20:80)	120–122	170–172	71
14	Column chromatograpy (Al_2O_3)	Petroleum ether/ether (98:2)	93–95	160–162	67
15	Column chromatograpy (Al_2O_3)	Petroleum ether/ether (90:10)	89–91	144–146	55

[a] HPLC (chiral phase): R_t = 29.1 min, e.e. > 99%; $[\alpha_D{}^{20}]_{base}$ = +12° (*c* 1, CH_3OH); [b] HPLC (chiral phase): R_t = 32.3 min, e.e. > 99%; $[\alpha_D{}^{20}]_{base}$ = –11° (*c* 1, CH_3OH); [c] aluminium oxide 90, 2–3 Merck.

1,1-Dioxo-4-piperidinohexahydro-1λ^6-thiopyran-4-carbonitrile (64%, *F* = 148 °C) was similarly prepared from piperidine and 1,1-dioxo-tetrahydro-1λ^6-thiopyran-4-one.

5.1.5. Synthesis of TCP analogues from α-amino-nitriles

Compounds **1** [7], **2** [7, 33], **3**–**5** [7] as well as compounds **10**-(±)**, 10**-(–)**, 10**-(+) [15] have been previously described. TCP analogues were obtained by means of two different synthetic methods. Since the same method was applied to various compounds we describe only one example to illustrate each strategy. Compounds purifications and properties are given in *table VI*.

*5.1.5.1. Method 1: TCP analogues with a thiophenyl hetero-aromatic ring (**6, 7, 11–13**)*

*1-[3-(2-thienyl)tetrahydro-2H-thiopyran-3-yl]piperidine **7***: An ethereal solution of 3-piperidinotetrahydro-2*H*-thiopyran-3-carbonitrile (1 g, 4.76 mmol, 1 eq.) was added dropwise at room temperature to a well stirred solution containing a Grignard reagent prepared from 2-bromo-thiophene (2.3 g, 14.3 mmol, 3 eq.) and Mg turnings (0.35 g, 14.3 mmol, 3 eq.). The mixture was refluxed for 20 h, cooled to room temperature and treated as follows: the mixture was poured carefully on an ice-cold saturated solution of NH_4Cl, stirred for 30 min, extracted with ether, the combined ether layers were extracted 3 times with 10% HCl, 20% NH_4OH was added to the aqueous phase until neutrality. The aqueous phase was extracted with ether, the organic phase washed with water, dried over Na_2SO_4, filtered, and concentrated in vacuum. The crude product obtained was purified as described in *table VI* to yield 1.05 g (83%) of **7** as a white solid.

*5.1.5.2. Method 2: TCP analogues with a furanyl (**8, 9**) or a substituted thiophenyl (**14, 15**) hetero-aromatic ring*

*1-[4-(2-furyl)tetrahydro-2H-thiopyran-4-yl]piperidine **9***: Firstly, a $MgBr_2$ solution was prepared from 1,2-dibromo-ethane (6.76 g, 36 mmol, 4 eq.) and magnesium turnings (0.88 g, 36 mmol, 4 eq.) in ether (80 mL) in a nitrogen atmosphere. Secondly, a solution of 2-furyl-Lithium was prepared in a nitrogen atmosphere by the dropwise addition of a n-butyl-lithium solution 1.6 M in hexane (28 mL, 45 mmol, 5 eq.) to a mixture of furane (3.1 g, 45 mmol, 5 eq.) and TMEDA (5.2 g, 45 mmol, 5 eq.) in anhydrous ether (100 mL) at –20 °C (without TMEDA in the synthesis of **15**). The mixture was then refluxed for 2 h, cooled to room temperature, and added dropwise to the $MgBr_2$ solution in ether. A solution of 4-piperidinotetrahydro-2*H*-thiopyran-4-carbonitrile (1.9 g, 9 mmol, 1 eq.) in ether was added dropwise at room temperature, the mixture refluxed for 16 h, cooled to room temperature, and treated as described above. The crude product was purified as described in *table VI* to yield 1.6 g (71%) of **9** as a white solid.

*5.1.6. Preparation of compounds **16** and **17***

5.1.6.1. 3-Methyltetrahydro-4H-thiopyran-4-one

n-Butyl-lithium (37.5 mL, 60 mmol, 1 eq.) was added dropwise in a nitrogen atmosphere to a stirred solution of diisopropylamine (8.4 mL, 60 mmol, 1 eq.) in THF (74 mL) and the mixture was stirred 0.5 h at room temperature, then cooled to –80 °C. After slow addition of tetrahydro-4*H*-thiopyran-4-one (6.96 g, 60 mmol, 1 eq.) and stirring for 0.5 h, ICH_3 (5.6 mL, 90 mmol, 1.5 eq.) was added, the mixture allowed to warm up to room temperature while stirred for 5 h. The mixture was diluted with a NaCl saturated 5% solution of sodium bicarbonate

in water and the organic phase separated, dried over Na_2SO_4 and concentrated in vacuum. The orange oil obtained was purified by chromatography (SDS Chromagel 70–200 μ) in petroleum ether/ethyl acetate (90:10) to yield 3.2 g (41%) of a colorless oil.

5.1.6.2. Cis- and trans-3-methyl-4-(2-thienyl)tetrahydro-2H-thiopyran-4-ol

2-Thienyl-magnesium bromide was prepared by adding dropwise in a nitrogen atmosphere a solution of 2-bromo-thiophene (6.1 g, 37.2 mmol, 1.1 eq.) in ether (100 mL) to Mg turnings (0.9 g, 37.2 mmol, 1.1 eq.). After the mixture was gently refluxed for 3 h, a solution of 3-methyltetrahydro-4*H*-thiopyran-4-one (4.2 g, 32 mmol, 1 eq.) dissolved in ether (50 mL) was added at room temperature. The mixture was refluxed for 16 h, cooled to room temperature, poured onto a saturated NH_4Cl solution in water, stirred 0.5 h. The aqueous phase was separated, extracted with ether (3 × 50 mL) and the combined organic layers extracted with 15% HCl (3 × 50 mL). 25% NH_4OH was added to the aqueous phase until neutrality and the solution extracted with ether. The final organic phase was washed with water, dried over Na_2SO_4, filtered, and concentrated in vacuum. The green oil obtained was purified by chromatography (SDS Chromagel 70–200 μ) in petroleum ether/ether (90:10) to yield 6.8 g (98%) of a slightly blue oil containing the diastereomeric alcohols.

5.1.6.3. Cis- and trans-3-methyl-4-(2-thienyl)tetrahydro-2H-thiopyran-4-azide

Sodium azide (4 g, 61.7 mmol, 2 eq.) was cautiously added to a solution of trichloro-acetic acid (15.1 g, 92.4 mmol, 3 eq.) in $CHCl_3$ (100 mL), the mixture was cooled to 10 °C, and a solution of diastereomeric alcohols 3-methyl-4-(2-thienyl)tetrahydro-2*H*-thiopyran-4-ol (6.6 g, 30.8 mmol, 1 eq.) in $CHCl_3$ (50 mL) was added. The mixture was stirred for 72 h at 10–12 °C, then a 10% NH_4OH solution was added until neutrality and the aqueous phase extracted with CH_2Cl_2 (3 × 100 mL). The pooledorganic phases were washed with water, dried over Na_2SO_4, filtered, and concentrated in vacuum. The oil obtained (6.8 g, 92%) was used in the next step without further purification.

5.1.6.4. Cis- and trans-3-methyl-4-(2-thienyl)tetrahydro-2H-thiopyran-4-amine

A solution of *cis*- and *trans*-3-methyl-4-(2-thienyl) tetrahydro-2*H*-thiopyran-4-azide (5.5 g, 23 mmol, 1 eq.) in THF (30 mL) was added dropwise at 0 °C to a solution of lithium aluminium hydride (0.87 g, 23 mmol, 4 eq.) in THF in a nitrogen atmosphere stirred for 24 h at room temperature. The strict minimum amount of NH_4OH necessary to destroy lithium aluminium hydride was added slowly, the precipitate filtered, washed with CH_2Cl_2 (300 mL), and the filtrate concentrated in vacuum. The brown oil obtained was diluted in ether, extracted with a 10% HCl solution (3 × 100 mL) and 20% NH_4OH added to the aqueous phase until neutrality. The aqueous phase was extracted with ether (3 × 100 mL) and the combined organic layers washed with water, dried over $MgSO_4$, filtered, and concentrated in vacuum. The crude diastereomeric oily mixture was purified by chromatography (SDS Chromagel 70–200 μ) in petroleum ether/ether (50:50) to yield 3.7 g (75%) of a *cis*/*trans* primary amines mixture as a colorless oil.

5.1.6.5. Cis- and trans-1-[3-methyl-4-(2-thienyl)tetrahydro-2H-thiopyran-4-yl]piperidine ***16, 17***

Potassium carbonate (5.8 g, 42 mmol, 2 eq.) and 1,5-dibromopentane (3.6 mL, 26.2 mmol, 1.25 eq.) was added in a nitrogen atmosphere to cis- and trans3-methyl-4-(2-thienyl)tetrahydro-2*H*-thiopyran-4-amine (4.5 g, 21 mmol, 1 eq.) dissolved in HMPT (50 mL) and the resulting mixture stirred at 60 °C for 48 h then cooled to room temperature. The mixture was poured onto water (200 mL) and extracted with ether (3 × 100 mL). The combined organic layers were extracted 3 times with 10% HCl and 20% NH_4OH was added to the aqueous phase until neutrality. The aqueous phase was extracted with ether and the final organic phase was washed with water, dried over $MgSO_4$, filtered, and concentrated in vacuum. The resulting yellow oil was chromatographied (SDS Chromagel 70–200 μ) in petroleum ether/ether (90:10) to yield the pure diastereomers as white solids: **17** (2 g, 34%) and **16** (0.7 g, 12%).

5.2. Biochemistry

5.2.1. Binding assays

[^{3}H]TCP (Amersham, 48 Ci/mmol) binding to the PCP receptor subtypes was measured as previously described [3]. Briefly, the rat (wistar) brain (minus the cerebellum) or the cerebellum was homogenized with an Ultraturax (Ika Werke, maximum setting) in a 50 mM Tris/HCl, pH 7.7 buffer for 20 s at 4 °C. The homogenate was then centrifuged at 49000 *g* for 20 min. The pellet was resuspended in the same buffer and the homogenization–centrifugation steps performed a second time. The final pellet was resuspended in 10 volumes of a 50 mM Tris/Hepes, pH 7.7 buffer and used without further purification.

The forebrain or cerebellum homogenate (0.5–0.8 mg protein/mL) was incubated with [^{3}H]TCP (1 nM or 2.5 nM respectively) in a 5 mM Tris/Hepes, pH 7.7 buffer (0.5 mL or 1 mL respectively) in the absence (total

binding) or in the presence of the competing drug for 30 min at 25 °C. The incubation was terminated by filtration over GF/B (Whatman) glass fibre presoaked in 0.05% polyethyleneimine (Aldrich) with an MR24 Brandel cell harvester. The filters were rinsed three times with 5 mL 50 mM NaCl, Tris HCl 10 mM, pH 7.7 buffer and the radioactivity retained was counted in 3.5 mL ACS (Amersham) with an Excel 1410 (LKB) liquid scintillation spectrophotometer. The non-specific binding was determined in parallel experiments in the presence of 100 μM unlabelled TCP.

5.2.2. Data analysis

In each experiment, values are the mean of three independent determinations. Each experiment was performed 3–5 times. The data from competition experiments were first analyzed by Hill's representation according to a single-site model, then by a non linear regression method (Marquardt–Levenberg algorithm) according to a two-site model using the Sigmaplot® 4 software (Jandel). The two-site interaction was represented by:

$$[LB] = (B1 + B2) - \{([I] \times B1/(IC_{50}1 + [I])) + ([I] \times B2/(IC_{50}2 + [I]))\}$$

where [LB] was the percentage of radioligand concentration specifically bound, [I] the competitor concentration, B1 and B2 the percentage of each binding site, $IC_{50}1$ and $IC_{50}2$ the concentrations of unlabeled competitor that inhibited 50% of specific [^{3}H]TCP binding on specified sites. Two constraints were fixed: (i) $IC_{50}1$, $IC_{50}2$, B1, B2 > 0; (ii) 95% < (B1 + B2) < 105% because of the uncertaincy on the total binding (10%). Experimental results were submitted to an ANOVA test followed by a Durbin–Watson test. The two-site model was preferred when it produced a significant reduction in the sum of squares ($P < 0.05$, Student's *t*-test) and when the Durbin–Watson coefficient was closer to 2 than in the single-site model and comprised between 1.5 and 2.5.

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