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Synthesis and Pharmacological Evaluation of 5-Pyrrolidinylquinoxalines as a Novel Class of Peripherally Restricted κ-Opioid Receptor Agonists

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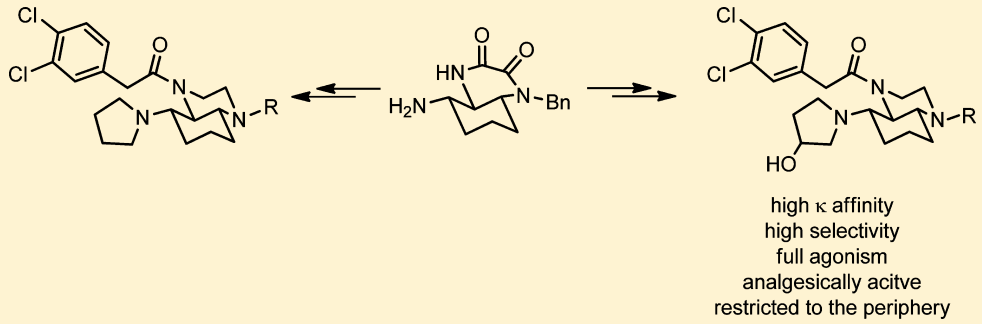
Supporting Information



ABSTRACT: 5-Pyrrolidinyl substituted perhydroquinoxalines were designed as conformationally restricted κ-opioid receptor agonists restricted to the periphery. The additional N atom of the quinoxaline system located outside the ethylenediamine κ pharmacophore allows the fine-tuning of the pharmacodynamic and pharmacokinetic properties. The perhydroquinoxalines were synthesized stereoselectively using the concept of late stage diversification of the central building blocks **14**. In addition to high κ-opioid receptor affinity they demonstrate high selectivity over μ, δ, σ_1, σ_2, and NMDA receptors. In the [^{35}S]GTPγS assay full agonism was observed. Because of their high polarity, the secondary amines **14a** (log $D_{7.4}$ = 0.26) and **14b** (log $D_{7.4}$ = 0.21) did not penetrate an artificial blood−brain barrier. **14b** was able to inhibit the spontaneous pain reaction after rectal mustard oil application to mice (ED_{50} = 2.35 mg/kg). This analgesic effect is attributed to activation of peripherally located κ receptors, since **14b** did not affect centrally mediated referred allodynia and hyperalgesia.

INTRODUCTION

In 1976 animal experiments with morphinoids led to a subclassification of the opioid receptor into three subtypes, which were termed according to their prototypical ligands μ-opioid (from morphine), σ-opioid (from SKF-10,047), and κ-opioid receptor (from ketocyclazocine).[1] Today the group of opioid receptors consists of four subtypes, μ (MOR), δ (DOR), κ (KOR), and ORL1 (NOR) receptor; the σ receptor has been removed from the class of opioid receptors.[2] The four opioid receptors belong to the peptide-binding γ subfamily of class A (rhodopsin-like) G-protein-coupled receptors (GPCRs), which are coupled predominantly to G_i/G_0 proteins. In the early 1990s the four opioid receptors were cloned. They reveal a sequence homology of more than 60%.[3]

The κ-opioid receptor consists of 380 amino acids and has a molecular weight of 42.7 kDa. Very recently the structure of a human κ-opioid receptor-T4 lysozyme construct in complex with the selective κ antagonist JDTic ((*R*)-7-hydroxy-*N*-{(*S*)-2-[(3*R*,4*R*)-4-(3-hydroxyphenyl)-1-isopropyl-3,4-dimethylpiperidin-1-yl]ethyl}-1,2,3,4-tetrahydroisoquinoline-3-carboxamide) was determined by X-ray crystal structure analysis giving insight into the binding pocket of the κ receptor. The polar interactions are formed by Asp138 (salt bridge with both protonated amino moieties) and Tyr312 (H-bond with carbonyl O-atom). Whereas conserved Asp138 plays a role in anchoring protonated κ ligands in the binding pocket, the

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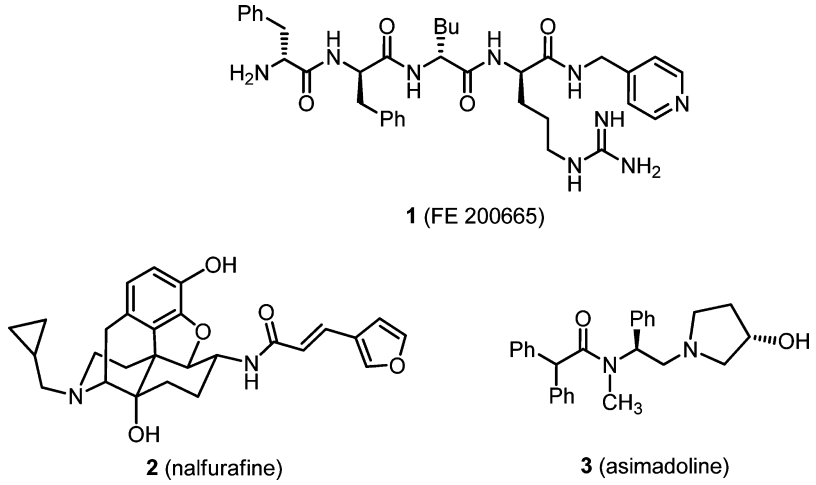

Figure 1. Peripherally acting κ receptor agonists.

amino acids Val108, Val118, Ile294, and Tyr312 are unique for the binding pocket of the κ receptor and are therefore postulated to contribute to the subtype selectivity of ligands.[4]

Activation of κ-opioid receptors leads to strong analgesia, which is not associated with the typical μ-mediated side effects, such as euphoria, constipation, respiratory depression, and development of dependency.[5] However, κ agonists induce different centrally mediated side effects including dysphoria, sedation, and strong diuresis.[6,7] Since the κ-opioid receptors are located not only in the central nervous system but also in the periphery, ligands activating predominantly peripherally located κ receptors would avoid these side effects. The therapeutic potential of κ agonists acting only in the periphery comprises treatment of visceral pain, inflammation, and inflammatory and pruritic skin diseases like atopic dermatitis and psoriasis.[8−10]

The known κ agonists belong to four structurally diverse compound classes: peptides derived from the endogenous agonist dynorphin A,[6,11] morphinoids including morphinans and benzomorphans,[6,11] the nonbasic natural product salvinorin A,[12,13] and ethylenediamines (or arylacetamides) with the first synthetic κ agonist U-50,488 (**4**)[14] (Figure 2). The pharmacophore of the fourth class of κ agonists is characterized by an ethylene moiety connecting a pyrrolidine ring and an arylacetamide moiety. On the basis of this key structure, several ligands with high κ affinity have been developed.[15,16]

In order to develop κ agonists restricted to the periphery, various approaches have been pursued (Figure 1). Tetrapeptides containing D-configured amino acids such as FE 200665 (**1**), also known as CR665, produce strong analgesia by activating selectively κ receptors in the periphery.[17−19] Nalfurafine (**2**) has been approved in Japan for the treatment of hemodialysis-related uremic pruritus. However, centrally mediated side effects were observed during the treatment of patients with **2**.[20−22] Introduction of polar functional groups such as hydroxy substituents of the pyrrolidine ring of ethylenediamine-based κ agonists led to the discovery of asimadoline (**3**), which has a preference for κ receptors in the periphery. **3** successfully passed a phase II clinical trial in irritable bowel syndrome (IBS). Recently, a phase III clinical trial for the treatment of patients with diarrhea-predominant IBS (D-IBS) was conducted by Tioga Pharmaceuticals, Inc.[23,24]

Herein we report on the synthesis and pharmacological evaluation of novel κ agonists, which should selectively activate κ receptors in the periphery. The ethylenediamine-based κ agonists **4** and **5** showing subnanomolar κ affinity (**4**, K_i = 0.34 nM; **5**, K_i = 0.31 nM[25]) were used as lead compounds (Figure 2). Their core structures should be combined in the perhydroquinoxaline ring system **6** while retaining the pharmacophoric ethylenediamine substructure. The designed perhydroquinoxalines **6a** result from connection of either the cyclohexane ring with the methyl moiety of **4** or the piperazine ring with the methyl moiety of **5** by a two-atom moiety (see arrows in Figure 2). The additional N-atom within the quinoxaline ring system of **6** will allow the fine-tuning of the pharmacodynamic and, moreover, the pharmacokinetic properties. In particular the introduction of polar substituents and/or ionizable functional groups should increase the polarity and inhibit the passage of the blood−brain barrier. The introduction of a polar OH-moiety into the pyrrolidine ring

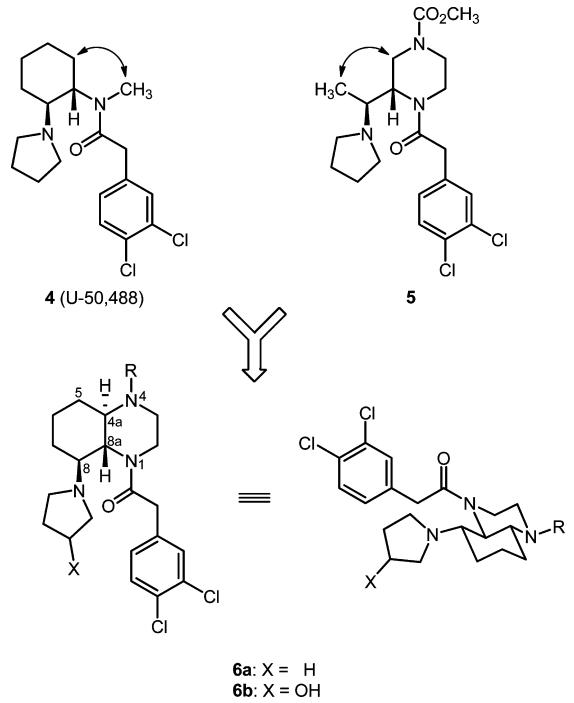

Figure 2. Development of a novel class of peripherally acting κ receptor agonists **6** based on the perhydroquinoxaline ring system.

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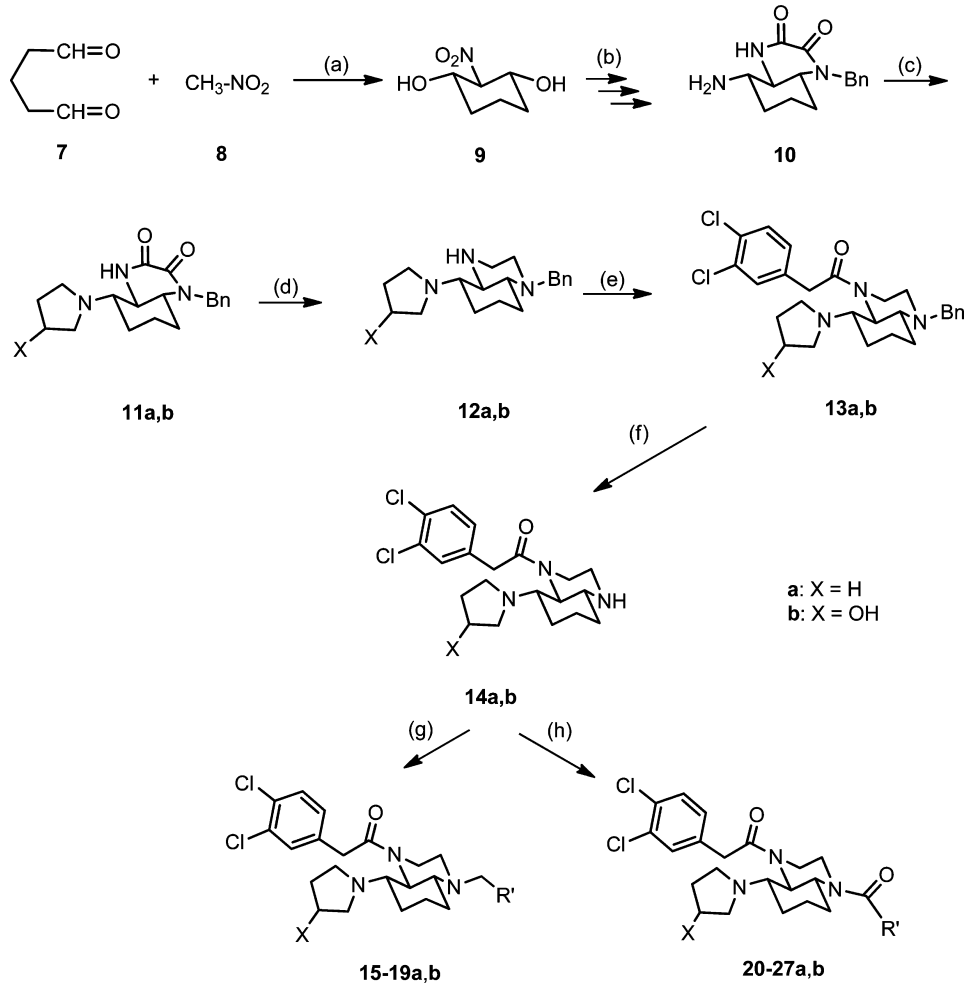
Scheme 1. Synthesis of κ Agonists with Perhydroquinoxaline Framework[a]



[a]Reagents and reaction conditions: (a) CH_3OH, NaOH, 80%;[26,27] (b) four steps (OH → NHBn exchange, NO_2 reduction, $(CO_2CH_3)_2$, NH_4HCO_2, Pd/C);[29] (c) 1,4-diiodobutane or 1,4-dibromobutan-2-ol, CH_3CN, $NaHCO_3$, 80 °C, 76% (**11a**), 79% (**11b**); (d) $AlCl_3/LiAlH_4$ 1:3, THF, 0 °C, then rt, 96% (**12a**), 77% (**12b**); (e) (3,4-dichlorophenyl)acetyl chloride, CH_2Cl_2, NaOH, rt, 86% (**13a**), 90% (**13b**); (f) H_2, 1 bar, Pd/C, THF, H_2O, HCl, rt, 30 min, 94% (**14a**), 89% (**14b**); (g) R′CH═O, $NaBH_3CN$ or $NaBH(OAc)_3$; for the definition of residues R (R = CH_2R') see Table 1; (h) R′C(═O)Cl or $(R'C═O)_2O$, CH_2Cl_2; for the definition of residues R (R = C═OR′) see Table 1.

as shown with **6b** should further increase the polarity of the κ agonists.

SYNTHESIS

At first the cyclohexane ring of the perhydroquinoxaline system with three adjacent functional groups in trans-orientation was established by a double Henry reaction (nitro aldol reaction) of glutaraldehyde (**7**) and nitromethane (**8**).[26−28] The resulting achiral (2*r*)-configured nitrocyclohexanediol **9** was transformed via four reaction steps[29] (substitution of OH by BnNH, reduction of the nitro group, cyclization with dimethyl oxalate, hydrogenolytic removal of the first benzyl protective group) into racemic quinoxalinedione **10**, which represents the key intermediate of this project. (Scheme 1)

Alkylation of the primary amine **10** with 1,4-diiodobutane and 1,4-dibromobutan-2-ol provided the pyrrolidine derivatives **11a**[29] and **11b** in 76% and 79% yield, respectively. The reduction of the quinoxalinediones **11a,b** was performed with AlH_3, which was generated in situ by mixing $AlCl_3$ and $LiAlH_4$ in the ratio 1:3.[30] The resulting secondary amines **12a** and **12b** were subsequently acylated with 3,4-dichlorophenylacetyl chloride to afford the amides **13a**[29] and **13b** in 86% and 90% yield, respectively. The acylation of the hydroxypyrrolidine **12b** was performed with exactly 1 equiv of acid chloride to avoid the additional acylation of the OH moiety in the pyrrolidine ring.

To inhibit reductive dechlorination during the hydrogenolytic removal of the second benzyl protective group of **13a,b** with H_2 and Pd/C, HCl was added to the reaction mixture. The secondary amines **14a** and **14b** represent versatile building blocks, which allow the introduction of diverse alkyl, arylalkyl, acyl, and alkoxycarbonyl substituents at the 4-position of the perhydroqinoxaline ring system at the end of the synthesis according to the concept of late stage diversification. Alkyl and arylalkyl residues were attached by reductive alkylation using aldehydes and $NaBH_3CN$ or $NaBH(OAc)_3$. Acyl and alkoxycarbonyl residues were introduced by acylation with acid chlorides or anhydrides.

The N-residues were selected with the aim to achieve high κ affinity and receptor selectivity. Additionally, the residues should supply the compounds with high polarity to inhibit the penetration into the central nervous system. Therefore, the $clogD_{7.4}$ values of all designed compounds were calculated systematically prior to their synthesis.

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Since the hydroxypyrrolidine derivatives (**b**-series) contain an additional center of chirality, two diastereomers were formed during the synthesis of **11b** and its further transformations. Exemplarily the diastereomers of the secondary amine **14b** were separated by preparative HPLC. Since it was not possible to obtain crystals suitable for an X-ray crystal structure analysis, a precise assignment of the relative configuration of the diastereomeric derivatives **14bA** (shorter retention time) and **14bB** (longer retention time) was not possible.

PHARMACOLOGICAL EVALUATION

κ-Opioid Receptor Affinity. The κ receptor affinity of the quinoxalines **13**−**27** was determined in competitive receptor binding studies. Tritium labeled U-69,593 was employed as radioligand. Guinea pig brain preparations were used as receptor material, and the nonspecific binding was determined in the presence of 10 μM U-69,593.[31,32] The κ receptor affinity of the reference compounds **4**, **5**, U-69,593, and naloxone determined in this assay is included in Table 1.

The data in Table 1 indicate high κ affinity (i.e., K_i < 10 nM) for several compounds of this novel class of pyrrolidinylquinoxalines. Generally, the κ affinity of hydroxypyrrolidine derivatives (**b**-series) is slightly lower than the κ affinity of pyrrolidine derivatives (**a**-series) as demonstrated with the pair of secondary amines **14a** (K_i = 2.1 nM) and **14b** (K_i = 8.7 nM). Only the benzyl derivatives **13a**,**b** represent an exception of this general trend.

Small (Me, **15**) and medium alkyl groups (*n*-Bu, **16**) as well as benzyl moieties including the *p*-methoxybenzyl (**17**) and heteroarylmethyl substituents such as the 2-pyridylmethyl (**18**) and 5-imidazolylmethyl group (**19**) are well tolerated by the κ-opioid receptor. This result is remarkable, since the ligands **13**−**19** contain a second basic functional group within the quinoxaline ring in addition to the basic center of the pyrrolidine ring. These quinoxaline derivatives **13**−**19** represent another example of nonpeptidic κ agonists with two basic functional groups, in addition to our previously reported bicyclic κ agonists.[32] This observation broadens the possibilities of fine-tuning the pharmacodynamic and pharmacokinetic properties of this type of ligands.

In the group of *N*-acyl and *N*-alkoxycarbonyl substituted quinoxalines **20**−**27** the methoxycarbonyl derivatives **23a** and **23b** reveal the highest κ receptor affinity. The K_i values are 9.7 nM for the pyrrolidine derivative **23a** and 11 nM for the hydroxypyrrolidine derivative **23b**. Enlargement of the methoxycarbonyl moiety to an ethoxycarbonyl moiety (**24a**) and its replacement by acyl residues (**20**−**22**) led to reduced κ affinity. This observation correlates nicely with the high κ receptor affinity of monocyclic (e.g., **5**)[24] and bridged piperazines[31] bearing an *N*-methoxycarbonyl moiety.

The carboxylic acids **25** and **26** were designed and synthesized, since their high polarity ($clogD_{7.4}$ = −1.1 to +0.1) should not allow the passage of the blood−brain barrier. However, the κ affinity of the acids **25** and **26** is very low, and therefore, they are not suitable as peripherally restricted κ agonists.

The amides **27a** and **27b** bearing an ester moiety within the N-residue show promising κ receptor affinity of 11 and 16 nM. Topical application of the esters **27** could lead to activation of peripherally located κ receptors, e.g., in the skin. After systemic absorption, the esters **27** will be hydrolyzed by esterases present in the plasma. Since the hydrolysis products **25** show only low κ receptor affinity, systemic or centrally mediated side effects are not expected. Moreover, zwitterions usually do not readily pass the blood−brain barrier.

Exemplarily the diastereomers of the hydroxypyrrolidine **14b** were separated and tested. It was shown that **14bA**, the diastereomer with shorter retention time, has a considerably higher κ affinity (K_i = 2.8 nM) than its diastereomer **14bB** (K_i = 16 nM). Since in **3** (see Figure 1) and similar hydroxypyrrolidines (e.g., 2-(2-aminophenyl)-*N*-[2-(3-hydroxypyrrolidin-1-yl)-1-phenylethyl]-*N*-methylacetamide (EMD 60400)) the (*S*,*S*)-configured hydroxypyrrolidine enantiomers represent the eutomers,[33,34] it can be speculated that the more potent diastereomer **14bA** has the relative configuration (4a*RS*,8*SR*,8a*SR*-*SR*(pyrrolidine)).

The κ receptor affinity of the perhydroquinoxalines **13**−**27** was investigated in a cell-based assay. In this κ assay transfected HEK-293 cell lines expressing the human κ-opioid receptor served as receptor material and [^{3}H]Cl-977 was used as radioligand.[35,36] In Table 1 the data recorded with guinea pig brains/[^{3}H]U-69,593 and HEK-293 cells/[^{3}H]Cl-977 are compared.

The K_i values obtained with the transfected HEK-293 cells are generally 5- to 15-fold higher than the K_i values recorded with guinea pig brain preparations. However, both assay systems show the same tendencies; e.g., the K_i values of the methyl derivatives **15a** and **15b** are 2.7 and 5.4 nM in the guinea pig brain assay and 39 and 49 nM in the HEK-293 cell assay.

Discussion of the Dihedral Angle of the Ethylenediamine Substructure. It has been postulated that the relative orientation of the pyrrolidine ring and the dichlorophenylacetamide moiety is crucial for high κ receptor affinity. For the rather rigid cyclohexane derivative **4** (K_i = 0.34 nM) a dihedral angle N(pyrrolidine)−C−C−N(acyl) of 60° was postulated.[37] Recently we have shown that a dihedral angle of 70° represents the energetically most favored conformation of the piperazine **5** (K_i = 0.31 nM) with the flexible pyrrolidinylethyl side chain.[31]

Therefore, the dihedral angle of the ethylenediamine substructure (N(pyrrolidine)−C8−C8a−N(acyl)) of the pyrrolidinyl substituted perhydroquinoxalines was determined by AM1 calculations (Figure 3). Since the hybridization of the N atom in 4-position outside the ethylenediamine pharmacophore has an influence on the geometry of the perhydroquinoxaline system, the dihedral angles were calculated exemplarily for the secondary amine **14a** (tetraedric N atom, K_i = 2.1 nM) and the methoxycarbonyl derivative **23a** (trigonal N atom, K_i = 9.7 nM). The calculations were performed with (4a*R*,8*S*,8a*S*)-configured enantiomers, which correspond to the enantiomerically pure lead compounds **4** and **5**.

AM1 calculations provided two types of energetically favored conformers, respectively, which differ slightly in their dihedral angle. The energetically most favored conformers **14aI** and **23aI** show dihedral angles of 55° and 54°, respectively (Table 2). In the second type conformers **14aII** and **23aII** with slightly distorted geometry of the quinoxaline ring system resulting in slightly lower heat of formation, the dihedral angles are 71° and 69°, respectively. These results clearly indicate that the substituent at N-4 has only a small effect on the dihedral angle of the ethylenediamine pharmacophore.

Since the dihedral angles of both types of conformations are close to the dihedral angles reported for the lead compounds **4** and **5**, we assume that the geometry along the pharmacophoric ethylenediamine substructure of the conformationally con-

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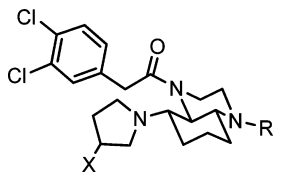
Table 1. Affinities of Perhydroquinoxalines toward κ-Opioid and Related Receptors



			$K_i \pm$ SEM (nM)[a]						
compd	R	X	κ,[b] [^{3}H]U-69,593	κ,[c] [^{3}H]Cl-977	μ,[c] [^{3}H]naloxone	δ,[c] [^{3}H]deltorphine	σ_1, [^{3}H](+)-pentazocine	σ_2, [^{3}H]DTG	NMDA (PCP), [^{3}H]MK-801
13a (WMS-0610)	Bn	H	9.4 ± 1.6	25	3900	39%	1390	4340	29%
13b (WMS-0614)	Bn	OH	6.6 ± 1.4	38	4300	22%	765	24%	16%
14a (WMS-0611)	H	H	2.1 ± 0.4	28	2800	10%	3%	2340	nd
14b (WMS-0615)	H	OH	8.7 ± 1.1	19	4900	0%	4%	0%	18%
14bA	H	OH	2.8 ± 0.76						
14bB	H	OH	16 ± 2.6						
15a	CH_3	H	2.7 ± 0.6	39	5100	16%	3%	3%	0%
15b	CH_3	OH	5.4 ± 0.8	49	6000	27%	818	43%	nd
16a	*n*-Bu	H	3.1 ± 1.8	13	2300	32%	518	1590	20%
17a	*p*-H_3COBn	H	6.8 ± 2.0	47	740	26%	26%	0%	16%
18a	CH_2-2-pyridyl	H	4.2 ± 2.6	24	2260	4300	20%	0%	nd
19a	CH_2-imidazol-5-yl	H	4.3 ± 2.0	44	4690	27%	15%	6%	nd
19b	CH_2-imidazol-5-yl	OH	13%	49	4400	11%	21%	0%	21%
20a	C═OPh	H	15 ± 3.4	92	1300	15%	21%	24%	0%
20b	C═OPh	OH	22 ± 5.6	170	3620	22%	16%	3%	nd
21a	C═OCH_3	H	24 ± 2.8	200	2800	0%	24%	0%	0%
22a	C═OEt	H	26 ± 1.3	440	3335	10%	0%	0%	27%
23a	CO_2CH_3	H	9.7 ± 1.8	160	2355	28%	0%	0%	26%
23b	CO_2CH_3	OH	11 ± 2.8	104	4450	16%	15%	0%	nd
24a	CO_2Et	H	15 ± 3.0	180	1775	32%	0%	10%	0%
25a	C═OCH_2CO_2H	H	169 ± 63	41%	19%	7%	0%	4%	18%
25b	C═OCH_2CO_2H	OH	482 ± 113	870	35%	10%	17%	14%	12%
26a	C═$O(CH_2)_2CO_2H$	H	136 ± 31	45%	31%	21%	0%	7%	0%
27a	C═$OCH_2CO_2CH_3$	H	11 ± 5.6	100	2200	0%	9%	0%	0%
27b	C═$OCH_2CO_2CH_3$	OH	18 ± 2.2	180	6000	28%	19%	21%	nd
4			0.34 ± 0.07	1.5 ± 0.9					
5			0.31 ± 0.04						
U-69,593			0.97 ± 0.40						
naloxone			7.3 ± 0.40	15 ± 9.3	2.3 ± 1.1	103			
morphine					5.2 ± 1.6				
SNC80						1.2 ± 0.5			
(+)-pentazocine							5.4 ± 0.5		
haloperidol							6.6 ± 0.9	78 ± 2.3	
(+)-MK-801									2.9 ± 1.1
dexoxadrol									38 ± 9.9

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Table 1. continued

[a]A value in % reflects the inhibition of the radioligand binding at a test compound concentration of 10 μM. nd = not determined; because of low displacement of the radioligand, a K_i value or a precise inhibition could not be determined. [b]The number of experiments was 3 (n = 3). [c]The data represent the mean of two experiments (n = 2).

strained quinoxalines **13**−**27** is close to the bioactive conformation of the flexible piperazine derivative **5**.

Selectivity over μ- and δ-Opioid Receptors. The μ and δ receptor affinities of the perhydroquinoxalines **13**−**27** were investigated using transfected CHO-K_1 cell lines expressing the human μ- and δ-opioid receptors as receptor material. In the μ assay and the δ assay, [^{3}H]naloxone and [^{3}H]deltorphine served as radioligands, respectively.[35,36] The μ and δ affinity data of the quinoxalines **13**−**27** are summarized in Table 1.

The alkyl and arylalkyl substituted quinoxalines **13**−**19** show very low affinity toward μ and δ receptors resulting in high κ/μ and κ/δ selectivity of >100, respectively. With a κ/μ selectivity of only 16 the *p*-methoxybenzyl substituted derivative **17a** represents the only exception. The comparatively low κ/μ selectivity is due to a rather high submicromolar μ receptor affinity of 740 nM.

Compared with the alkyl and arylalkyl substituted quinoxalines **13**−**19**, the acyl and alkoxycarbonyl substituted quinoxalines **20**−**27** reveal reduced κ/μ and κ/δ selectivity values in the range of 10−40. The reduced selectivity of these compounds is explained with their lower κ receptor affinity.

It can be concluded that both types of compounds alkyl/arylalkyl and acyl/alkoxycarbonyl substituted quinoxalines show high selectivity for the κ-opioid over the related μ- and δ-opioid receptors.

Selectivity over σ and NNDA Receptors (PCP Binding Site). Although the structure of the κ receptor is not related to the structure of σ and NMDA receptors, it has been shown that small variations of κ agonists led to σ receptor ligands or NMDA antagonists. For example the cis-configured analogue of the trans-configured prototypical κ agonist **4** represents a high-affinity σ ligand.[38] Reduction of the phenylacetamide moiety of **4** to a phenylethylamino group increased the σ affinity and decreased the κ affinity.[39] In the class of benzomorphans the absolute configuration and the nature of the N-substituent control whether a ligand interacts preferably with κ-opioid, σ, or NMDA receptors.[40,41] Therefore, the affinity of this novel type of κ agonists toward σ_1, σ_2,[42,43] and NMDA receptors (PCP binding site)[44,45] was recorded in competitive radioligand binding assays. (Table 1).

The data in Table 1 clearly indicate that the quinoxaline-based κ agonists **13**−**27** do not interact considerably with σ_1, σ_2, and NMDA receptors. Generally, the test compounds did not compete with the radioligands up to a concentration of 1 μM, indicating IC_{50} values of at least greater than 1 μM. The *n*-butyl derivative **16a** possesses the highest σ_1 affinity (K_I = 518 nM) of these quinoxalines. Because of its high κ affinity (K_I = 3.1 nM) the κ/σ_1 selectivity of **16a** is still >100.

In conclusion, the new quinoxaline-based κ agonists show high selectivity against μ-opioid, δ-opioid, σ_1, σ_2, and NMDA (PCP binding site) receptors.

Functional Activity. The intrinsic activity of the substituted quinoxalines **13**−**27** was investigated with the [^{35}S]GTPγS ([^{35}S]guanosine 5′-3-*O*-(thio)triphosphate) binding assay using human HEK-293 (human embryonic kidney) cells as source of human κ-opioid receptors.[46,47]

In Table 3 the EC_{50} values of the quinoxaline-based κ agonists are summarized and compared with the EC_{50} value of the prototypical full agonist U-69,593. Promising EC_{50} values below 40 nM were obtained for the benzylamine **13a** (EC_{50} = 29 nM), the secondary amines **14a** (EC_{50} = 33 nM) and **14b** (EC_{50} = 35 nM), the methylamine **15a** (EC_{50} = 20 nM), the butylamine **16a** (EC_{50} = 12 nM), and the *p*-methoxybenzyl-

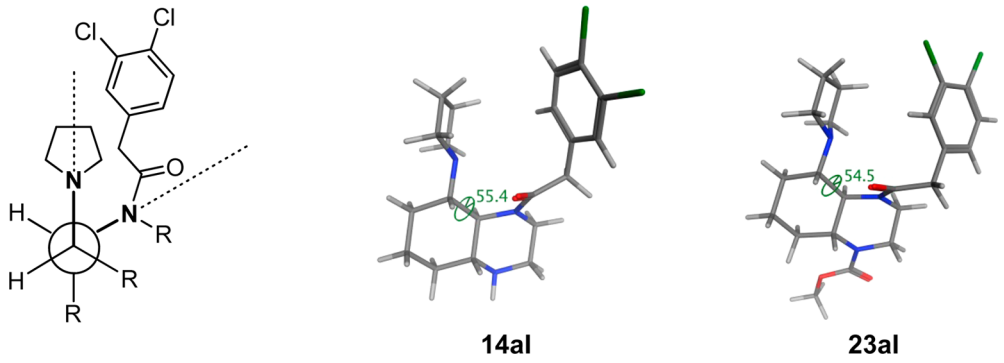

Figure 3. Left: Schematic view of the dihedral angle N(pyrrolidine)−C8−C8a−N(acyl). Middle: Energetically most favored conformation of **14a** with a dihedral angle of 55°. Right: Energetically most favored conformation of **23a** with a dihedral angle of 54°.

Table 2. Correlation of the Dihedral Angle N(yrrolidine)−C−C−N(acyl) of the Pyrrolidinyl Substituted Perhydroquinoxalines 14a and 23a and the Lead Compounds 4 and 5 with Their κ Receptor Affinity

compd	K_i (nM) (κ receptor)	conformer	dihedral angle[a] (deg)	ΔH^0 [b] (kcal/mol)
14a	2.1	I	55	0.0
		II	71	3.7
23a	9.7	I	54	0.0
		II	69	2.5
4	0.34		60	
5	0.31		70[c]	

[a]Dihedral angle defined by N(pyrrolidine)−C−C−N(acyl). [b]ΔH^0 = H^0(conformer II) − H^0(conformer I); H^0 = heat of formation calculated with AM1 (MOE). [c]Dihedral angle of the energetically most favored conformation of the flexible κ agonist **5**.

amine **17a** (EC_{50} = 26 nM). These EC_{50} values are close to the EC_{50} value of the prototypical κ agonist U-69,593 (EC_{50} = 12 nM). Obviously, an additional basic functional group (N-4 of the perhydroquinoxaline ring) is not detrimental for the κ agonistic activity of these ligands. However, elimination of the basicity the N-4-atom of the quinoxaline ring by acylation or alkoxycarbonylation (compounds **20**−**27**) resulted in considerably reduced agonistic activity with EC_{50} values higher than 85 nM. Compounds **25a** and **25b** bearing an acyl moiety with carboxylic acid substituent did not show a significant activation of the κ opioid receptor at concentrations up to 1 μM. However, these compounds also exhibited a reduced affinity toward κ receptor in the binding assay.

In Figure 4 the binding curves of the secondary amines **14a** and **14b**, the butylamine **16a**, and the methyl carbamate **23a** are compared with the binding curve of the prototypical full agonist U-69,593, respectively. All compounds behave as full agonists reaching the same maximal effect as the full κ agonist U-69,593. Even the methyl carbamate **23a** with a rather high EC_{50} value of 110 nM is a full κ receptor agonist. The binding curve of the butyl derivative **16a** is remarkable, since it is almost identical with the binding curve of U-69,593 resulting in the same EC_{50} value of 12 nM.

In conclusion, all perhydroquinoxaline derivatives but the compounds bearing a carboxylic acid substituted acyl group in 4-position behave as full κ-opioid receptor agonists in the [^{35}S]GTPγS binding assay independently of the substituent at N-4 (H, alkyl, arylalkyl, acyl, alkoxycarbonyl). However, the compounds with an additional basic structural element in 4-position reveal higher agonistic activity than the N-4 acylated and N-4 alkoxycarbonylated derivatives.

log *D* Value. Lipophilicity in terms of log *P* value is an important physicochemical parameter indicating the ability of a compound to penetrate lipophilic membranes. However, the log *P* value is a descriptor for neutral compounds and therefore does not take into account the ionization of compounds under physiological conditions. The pH dependent log *D* value (coefficient of distribution) describes the *n*-octanol/water distribution of ionizable species at a given pH value. Therefore, the calculation and measurement of the log *D* value at a physiologically relevant pH value (e.g., pH 7.4) provide a more realistic description of the lipophilicity properties of bioactive compounds.[48,49]

In order to obtain κ agonists, which are not able to pass the blood−brain barrier and stay in the periphery, we are interested in rather polar compounds. Therefore, the log $D_{7.4}$ value (log *D* value at pH 7.4) was determined experimentally for the benzylamines **13a** and **13b**, the secondary amines **14a** and **14b**, and the methyl carbamates **23a** and **23b**. According to a standardized protocol in our lab, the compounds were partitioned between an aqueous buffer (3-morpholinopropanesulfonic acid (MOPS), pH 7.4) and *n*-octanol. The amount of the compound in the aqueous layer was determined quantitatively by mass spectrometry. The results are summarized in Table 4.

In addition to the experimentally determined log $D_{7.4}$ values, the log *P* and log *D* values were calculated using different methods (see Table 4). The log *P* values calculated for neutral compounds (ClogP and milogP values) do not correlate with the experimentally determined log $D_{7,4}$ values. However, calculations taking the positive charge of the quinoxalines into account, either with completely protonated species (milogP(H^+)) or at physiological pH value ($clogD_{7.4}$), lead to values close to the experimentally determined log $D_{7,4}$ values. Since the distribution between two layers at pH 7.4 characterizes better the behavior of compounds under physiological conditions, the $clogD_{7,4}$ values of all test compounds are included in Table 3.

According to all different calculations, the log *P* values of the pyrrolidine derivatives (**a**-series) are higher by 0.7−1.3 than the log *P* values of the analogous hydroxypyrrolidine derivatives (**b**-series). However, this difference in the polarity could not be reproduced by the experiments. The experimentally determined log *D* values of the pyrrolidine/hydroxypyrrolidine pairs **13a**/**b**, **14a**/**b**, and **23a**/**b** do not differ, respectively.

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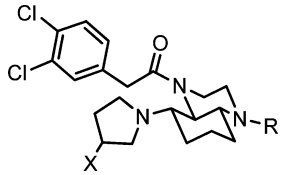
Table 3. Agonistic Activity (EC_{50}) in the [^{35}S]GTPγS Assay and clog$D_{7.4}$ of Quinoxaline-Based κ Agonists



compd	R	X	κ affinity, K_i (nM)	κ agonistic activity, EC_{50} (nM)	clog$D_{7.4}$ [a]
13a	Bn	H	9.4	29	3.2
13b	Bn	OH	6.6	64	2.5
14a	H	H	2.1	33	1.1
14b	H	OH	8.7	35	0.4
15a	CH_3	H	2.7	20	1.4
15b	CH_3	OH	5.4	79	0.7
16a	*n*-Bu	H	3.1	12	2.7
17a	*p*-H_3COBn	H	6.8	26	3.0
18a	CH_2-2-pyridyl	H	4.2	41	2.2
19a	CH_2-imidazol-5-yl	H	4.3	56	1.1
19b	CH_2-imidazol-5-yl	OH	13%	nd[b]	0.5
20a	C═OPh	H	15	260	3.2
20b	C═OPh	OH	22	470	2.5
21a	C═OCH_3	H	24	330	1.3
22a	C═OEt	H	26	600	2.0
23a	CO_2CH_3	H	9.7	110	2.0
23b	CO_2CH_3	OH	11	85	1.3
24a	CO_2Et	H	15	150	2.4
25a	C═OCH_2CO_2H	H	169	na[c]	0.1
25b	C═OCH_2CO_2H	OH	482	na[c]	−1.1
26a	C═O$(CH_2)_2CO_2$H	H	136	nd[b]	0.1
27a	C═O$CH_2CO_2CH_3$	H	11	220	1.3
27b	C═O$CH_2CO_2CH_3$	OH	18	170	0.7
U-69,593			0.97	12	

[a] clog$D_{7.4}$ = calculated log *D* value at pH 7.4 (MarvinSketch 6.2.3, ChemAxon). [b] nd = not determined. [c] na = not active; i.e., at a test compound concentration of 1 μM the agonistic activity was lower than 50%.

High polarity was a criterion for the selection of the substituents at the additional N-atom in 4-position of the quinoxaline ring system outside the κ pharmacophore. Low clog$D_{7.4}$ values were calculated for the secondary amines **14**, the methylamines **15**, the pyridylmethyl and imidazolylmethyl substituted derivatives **18** and **19**, the methyl carbamates **23** as well as the compounds **25** and **26** with a carboxy moiety in the side chain.

For the further tests the secondary amines **14a** and **14b** were selected because of their high κ receptor affinity (K_i = 2.1 and 8.7 nM), high selectivity over related receptors, full κ agonistic activity (EC_{50} = 33 and 35 nM) and high polarity (log $D_{7.4}$ = 0.26 and 0.21).

Passage of an Artificial Blood–Brain Barrier. A model of the blood–brain barrier (Figure 5) was used to determine the penetration of the potent and polar κ agonists **14a** and **14b** into the brain. For the purpose of comparison the potent but more lipophilic benzylamine **13a** was selected for being tested in this model as well. In this model pig cerebral capillary endothelial cells were grown on a filter forming a tight monolayer. The polarity of the monolayer is improved by coating the filter with collagen. After addition of a compound solution to the apical compartment, which corresponds to the blood side of the blood–brain barrier, its penetration into the basolateral compartment corresponding to the brain side was measured. Since active transporters for the compounds under consideration are shown to be not present in this blood–brain barrier model, only passive diffusion of compounds had to be considered.[50−52]

Prior to the penetration test, the potential toxicity of the compounds toward the capillary endothelial cells has to be determined. For this purpose various concentrations of the test compounds (1, 100, 500, 1000 μM) were added to the apical compartment of the model together with ^{14}C-labeled sucrose. The amount of [^{14}C]sucrose reaching the basolateral compartment was determined by a β counter over an incubation period of 180 min. Since sucrose cannot penetrate an intact endothelial monolayer, the amount of [^{14}C]sucrose in the basolateral compartment indicates a damage of the integrity of the endothelial monolayer.

Concentrations of 100 μM and higher of the benzylamine **13a** increased the permeability of the endothelial monolayer considerably, indicating nontolerable toxicity. A concentration of 1 μM was tolerated by the endothelial cells, but this concentration is too low to quantify **13a** in the apical and basolateral compartments. Therefore, this compound was no longer considered for this assay.

The secondary amines **14a** and **14b** were well tolerated by the endothelial cells up to a concentration of 500 μM. Only a concentration of 1000 μM led to increased permeability of [^{14}C]sucrose. Therefore, the permeability test was performed with 500 μM solutions of the secondary amines **14a** and **14b**.

After incubation of the apical compartment with 500 μM solutions of **14a** and **14b** for 3 h, the amounts of **14a** and **14b**

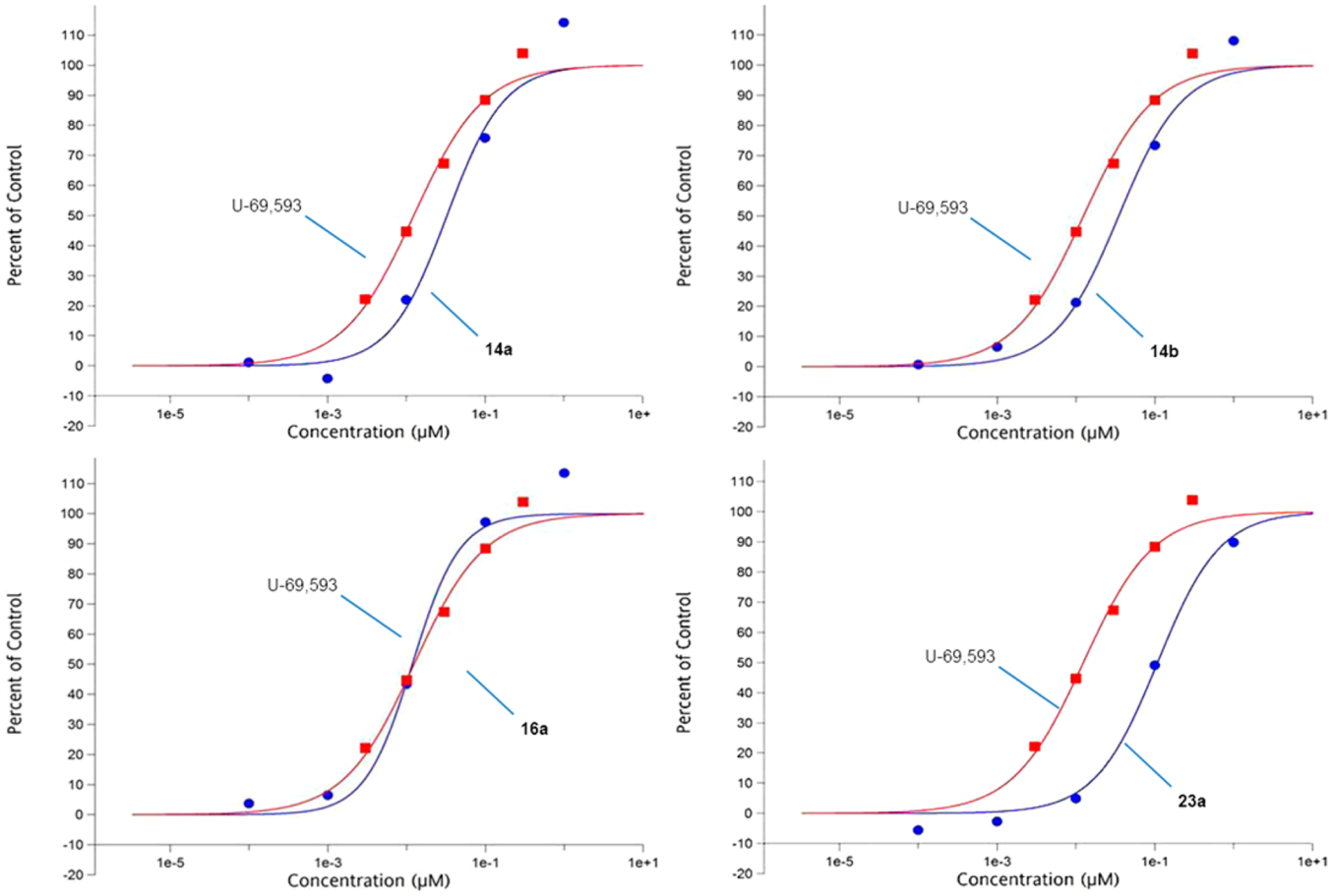


Figure 4. Binding curves of the secondary amines **14a** (top left) and **14b** (top right), the butylamine **16a** (bottom left), and the methyl carbamate **23a** (bottom right) at human κ receptors in the [^{35}S]GTPγS assay. The binding curves of the test compounds are compared with the binding curves of the prototypical κ agonist U-69,593, respectively.

Table 4. Correlation of Variously Calculated log *P*/log *D* and Experimentally Determined log *D* Values of Selected Perhydroquinoxalines

compd	K_i (nM) (κ receptor)	ClogP [a]	milogP [b]	milogP(H^+) [c]	clogD$_{7.4}$ [d]	log $D_{7.4}$, [e] exptl
13a	9.4	6.4	5.3	2.2	3.2	2.38 ± 0.09
13b	6.6	5.1	4.4	1.3	2.5	2.51 ± 0.09
14a	2.1	4.1	3.7	0.5	1.1	0.26 ± 0.10
14b	8.7	2.8	2.8	−0.4	0.4	0.21 ± 0.09
23a	9.7	4.9	3.8	0.6	2.0	1.29 ± 0.08
23b	11	3.7	2.9	−0.3	1.3	1.34 ± 0.08

[a]ClogP = calculated log *P* value (ChemDraw). [b]milogP = calculated log *P* value (www.molinspiration.com). [c]milogP(H^+) = calculated log *P* value of the monoprotonated species (www.molinspiration.com). [d]clogD$_{7.4}$ = calculated log *D* value at pH 7.4 (MarvinSketch 6.2.3, ChemAxon) (www.chemaxon.com). [e]log $D_{7.4}$ = experimentally determined log *D* value at pH 7.4, $n \geq 8$.

in the basolateral compartment were recorded by RP-HPLC analysis. Table 5 shows that 2.1%/cm^2 and 2.2%/cm^2 of the secondary amines **14a** and **14b** passed the cerebral capillary endothelial monolayer, respectively. The very low penetration of the endothelial monolayer by the secondary amines **14a** and **14b**, which is only slightly higher than the penetration of sucrose, can be attributed to their low log $D_{7.4}$ values determined experimentally.

Analgesic Activity in Animal Models of Visceral Pain. The secondary amines **14a** and **14b** demonstrate high κ-opioid agonistic activity and low permeability of the endothelial monolayer as artificial model of the blood−brain barrier. Therefore, these compounds were selected to investigate their analgesic activity in animal models of visceral pain. For this purpose the phenylquinone writhing assay and the mustard oil assay in mice were performed.

The phenylquinone writhing assay is an unspecific pain test comprising both visceral and nonvisceral afferent structures.[53] Various doses of the test compounds **14a** and **14b** were iv administered to male mice. After 10 min, an aqueous solution of 2-phenyl-1,4-benzoquinone (phenylquinone) was given intraperitoneally and the resulting pain-induced writhing reactions, such as stretching, twisting a hind leg inward, or contraction of abdomen, were counted for 15 min (from 5 to 20 min after phenylquinone administration).

Whereas doses of 10 and 21.5 mg/kg body weight of the pyrrolidine **14a** did not lead to a decreased number of writhing reactions, a dose of 46.4 mg/kg induced lethality. In contrast,

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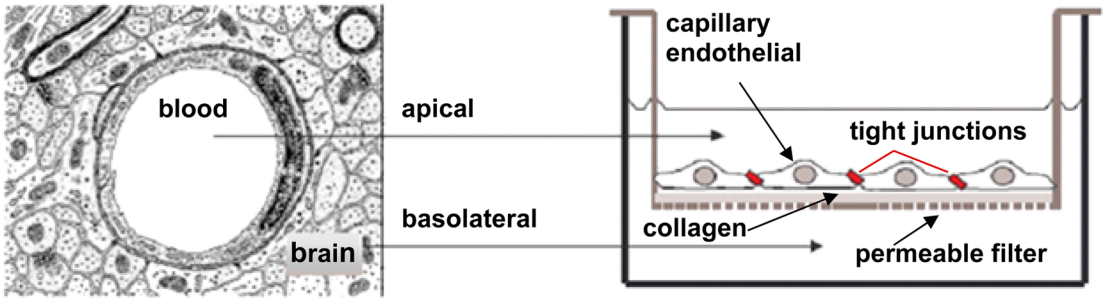

Figure 5. Cell culture model of the blood–brain barrier. A monolayer of pig cerebral capillary endothelial cells is grown on top of a permeable collagen coated filter. According to the polarity of the cells on the filter, the medium in the upper (apical) compartment of the cell monolayer corresponds to the blood side of the capillary; the compartment below the filter (basolateral) corresponds to the extravasal (brain) part.

Table 5. Penetration of the Secondary Amines 14a and 14b via the Endothelial Monolayer[a]

compd	permeability[b] (%/cm^2)	log $D_{7,4}$/log P [c]
14a	2.1 ± 0.9	0.26 ± 0.10
14b	2.2 ± 0.5	0.21 ± 0.09
diazepam	57	2.84
sucrose	0.63	−3.18

[a]The permeability is correlated with log $D_{7,4}$/log P values. [b]The permeability was standardized taking the size of the filter (1.13 cm^2) into account. Values are the mean of three independent experiments (n = 3). [c]log $D_{7,4}$ values of **14a** and **14b** were experimentally determined. log P values of the reference compounds diazepam and sucrose were calculated with ChemDraw.

the hydroxypyrrolidine **14b** showed a dose dependent inhibition of the writhing reactions indicating analgesic activity (Table 6). However, increasing occurrence of side effects (decreased locomotion, flat posture, ptosis) was observed when increasing doses were administered.

Table 6. Analgesic Activity of the Hydroxypyrrolidine 14b in the Phenylquinone Writhing Assay

dose (mg/kg)	no. of writhing reactions	inhibition of writhing reactions (%)
0 (control)	98	0
3.16	93	5.1
10.0	59	40
21.5	7	93

In the second assay visceral pain was induced in mice by rectal administration of mustard oil.[54,55] The pain induced by a chemical stimulation of a defined visceral organ, i.e., the colon, was quantified in three parameters. Spontaneous visceral pain behavior (e.g., abdominal licking, stretching, squashing, contraction of flank muscles) occurs during the first minutes after mustard oil administration. This period of spontaneous pain is followed by referred allodynia and referred hyperalgesia measured by assessing the animals reaction toward mechanical stimulation (von Frey filaments) at the abdominal wall, a dermatom corresponding to the colon. Allodynia is characterized by pain induced by a non-nociceptive stimulus, which can be measured using a 1 mN von Frey filament (non-nociceptive). Hyperalgesia is defined as increased pain reaction induced by a nociceptive stimulus. In this model hyperalgesia was evoked by a 16 mN von Frey filament.

Intravenous application of a dose of 21.5 mg/kg of the pyrrolidine **14a** led to complete inhibition of visceral pain behavior but did not affect referred allodynia and referred hyperalgesia. Since only one dose was investigated, ED_{50} values were not determined for the analgesic activity of **14a**.

In Figure 6 the effect of the hydroxypyrrolidine **14b** is displayed. The spontaneous pain reaction of mice was inhibited dose-dependently leading to an ED_{50} value of 2.35 (1.87–2.87) mg/kg. Obviously, the hydroxypyrrolidine **14b** represents a potent analgesic for the treatment of visceral pain. The efficacy of **14b** for the treatment of referred allodynia and hyperalgesia was recorded 20–40 min after mustard oil application using von Frey filaments of 1 and 16 mN, respectively. As shown in Figure 6B and Figure 6C, **14b** did not have any effect on these readouts. According to the low passage of the artificial blood–brain barrier, a low passage of the real blood barrier is expected, which might explain the lack of efficacy of **14b** in these centrally mediated readouts.

These findings were further substantiated by intracerebroventricular (icv) administration of the hydroxypyrrolidine **14b** in the same experimental models. After icv administration of **14b** spontaneous pain response, referred allodynia, and referred hyperalgesia were reduced dose-dependently with corresponding ED_{50} values of 2.72 (1.01–5.76) μg/animal, 3.31 (2.53–4.39) μg/animal, and 2.16 (1.63–2.73) μg/animal, respectively.

These results led to the conclusion that the hydroxypyrrolidine **14b** is a highly potent peripherally acting analgesic. Systemic (iv) application of **14b** only reduced the spontaneous pain reactions but did not affect the centrally mediated allodynia and hyperalgesia. However, after icv application these readouts were also inhibited with similar potency.

■ CONCLUSION

Following the concept of late stage diversification a large set of differently substituted perhydroquinoxalines has been prepared. The κ receptor affinity, κ-agonistic activity, receptor selectivity, and polarity of the κ agonists were modulated by the additional substituent at N-4 outside the ethylenediamine κ pharmacophore. The very polar secondary amine **14b** (log $D_{pH7,4}$ = 0.21) did not penetrate an artificial blood–brain barrier but was efficacious (ED_{50} = 2.35 mg/kg) in mustard oil induced spontaneous visceral pain measures. Differential efficacy on centrally mediated referred allodynia and hyperalgesia depending on peripheral/systemic (iv) or central (icv) administration supports the concept of peripherally acting κ-opioid analgesics.

■ EXPERIMENTAL SECTION

Chemistry. General. For flash chromatography (fc), silica gel 60, 40–64 μm (Merck) was used. Data presented in parentheses include diameter of the column, length of the column, eluent, fraction size, and

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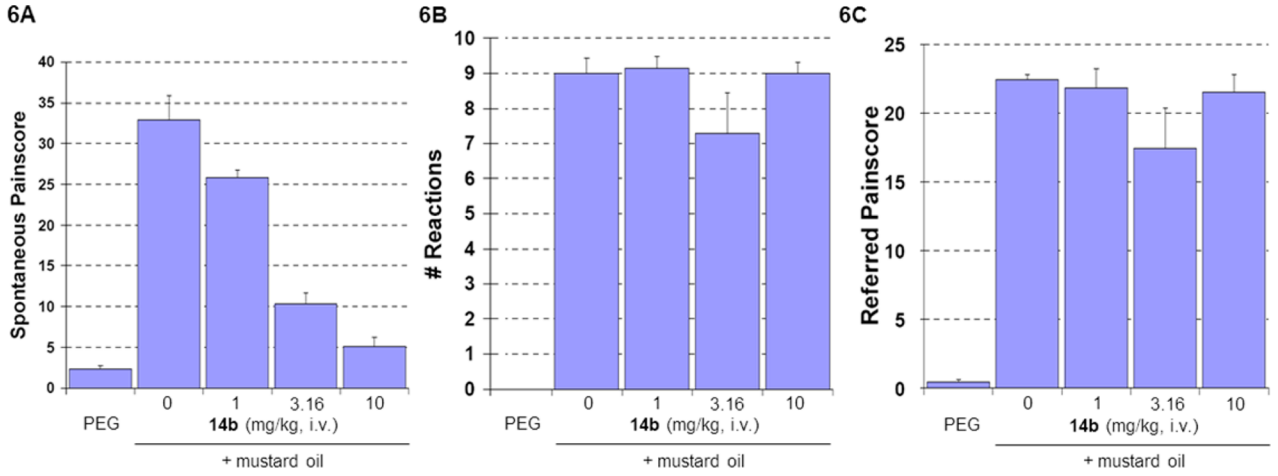

Figure 6. Analgesic activity after iv administration of various doses of hydroxypyrrolidine **14b** in the mustard oil assay: (A) spontaneous pain behavior; (B) referred allodynia; (C) referred hyperalgesia.

R_f value. Melting points were determined with an SMP 3 (Stuart Scientific) melting point apparatus and are uncorrected.

^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) data were obtained using a Mercury 400BB spectrometer (Varian). δ in ppm is referenced to tetramethylsilane, and coupling constants are given with 0.5 Hz resolution.

According to HPLC methods A and B, the purity of all test compounds was greater than 95%. For details of the methods, see Supporting Information.

Synthetic Procedures. (4a*RS*,5*SR*,8a*RS*)-1-Benzyl-5-(3-hydroxypyrrolidin-1-yl)perhydroquinoxaline-2,3-dione (11b). A mixture of primary amine **10** (144 mg, 0.53 mmol), racemic 1,4-dibromobutan-2-ol (purity 85%, 1.15 g, 4.20 mmol, 0.57 mL), $NaHCO_3$ (300 mg, 3.57 mmol), and CH_3CN (16 mL) was heated to reflux for 24 h. $NaHCO_3$ was filtered off, and the solvent was removed in vacuo. The residue was dissolved in CH_2Cl_2, and the solution was extracted with 2 M HCl (3×). Then 2 M NaOH was added to the combined aqueous layers until pH 8 was obtained and the alkaline solution was extracted with CH_2Cl_2 (3×). The combined organic layer was dried (Na_2SO_4), filtered, concentrated in vacuo, and the residue was purified by fc (2 cm, 17 cm, acetone/MeOH/Et_2NH 9.5:0.5:0.1, 5 mL, R_f = 0.63 (CH_3OH)). Pale yellow solid, mp 151 °C, yield 143 mg (79%). $C_{19}H_{25}N_3O_3$ (343.4). MS (EI): m/z (%) = 343 (M^+, 18), 325 ($(M - H_2O)^+$, 52), 258 ($(M - C_4H_7NO)^+$, 19), 252 ($(M - C_7H_7)^+$, 21), 234 ($(M - H_2O - C_7H_7)^+$, 100), 167 ($(M - C_7H_7 - C_4H_7NO)^+$, 33), 91 ($C_7H_7^+$, 36). IR: $\tilde{\nu}$ (cm^{-1}) = 3458 (m, ν (O–H)), 3202 (m, ν (N–H)), 1695 (s, ν (C═O), tert amide), 1667 (s, ν (C═O), sec amide), 1327 (w, δ (OH)), 1142 (w, ν (C–OH)). ^{1}H NMR ($CDCl_3$): δ (ppm) = 1.18–1.40 (m, 3 H, 6-H_a, 7-H_a, 8-H_a), 1.66–1.88 (m, 3 H, N(CH_2CH_2), 6-H_e, 7-H_e), 2.05–2.16 (m, 2 H, N(CH_2CH_2), 8-H_e), 2.50 (q, ^3J = 8.1 Hz, 1 H, N(CH_2CH_2)), 2.57–2.78 (m, 3 H, 5-H, N(CH_2CHOH), N(CH_2CH_2)), 2.80–2.88 (m, 1 H, N(CH_2CHOH)), 3.42/3.43 (2 t, ^3J = 10.8 Hz, 1 H, 4a-H), 3.54–3.64 (m, 1 H, 8a-H), 4.24–4.32 (m, 1 H, N(CH_2CHOH)), 4.59 (d, ^2J = 15.7 Hz, 1 H, Ph-CH_2), 5.06 (d, ^2J = 15.7 Hz, 1 H, Ph-CH_2), 7.22–7.28 (m, 3 H, ortho-Ph-H, para-Ph-H), 7.30–7.37 (m, 2 H, meta-Ph-H). Signals for the NH and OH protons are not visible in the spectrum. ^{13}C NMR ($CDCl_3$): δ (ppm) = 19.9/20.2 (C-6), 21.8/21.9 (C-7), 27.9 (C-8), 33.7/33.9 (N(CH_2CH_2)), 43.5 (N(CH_2CH_2)), 45.6 (Ph-CH_2), 55.9/56.2 (C-4a), 57.1 (N(CH_2CHOH)), 58.9 (C-8a), 59.0/59.1 (C-5), 70.3/70.6 (N(CH_2CHOH)), 126.8 (2 C, Ph-C), 127.2 (Ph-C), 128.8 (2 C, Ph-C), 137.1 (quart. Ph-C), 158.2/158.3 (C-3), 160.2/160.3 (C-2). HPLC (method A): purity 97.7%, t_R = 12.8 min. HPLC (method B): purity 97.0%, t_R = 11.3 min.

(4a*RS*,5*RS*,8a*SR*)-1-Benyzl-5-[(3*SR*)- and (3*RS*)-3-hydroxypyrrolidin-1-yl]perhydroquinoxaline (12b). Under N_2, dry $AlCl_3$ (940 mg, 6.8 mmol) was dissolved in absolute THF (52 mL), and the solution was cooled to 0 °C. A solution of $LiAlH_4$ (1.0 M in THF, 21 mL, 21 mmol) was added dropwise. The suspension was warmed to rt and stirred for 20 min. A solution of dilactam **11b** (1.29 g, 3.8 mmol) in THF (65 mL) was added to the freshly prepared solution of AlH_3 at 0 °C. The mixture was stirred at 0 °C for 45 min and at rt for 20 min. Then 2 M NaOH (13 mL) was added dropwise under cooling and the mixture was extracted with CH_2Cl_2 (5 × 50 mL). The organic layer was dried (Na_2SO_4), filtered, and concentrated in vacuo. R_f = 0.26 (CH_2Cl_2/MeOH/NH_3 = 9:1:0.1), R_f = 0.19 (CH_3OH). Pale yellow solid, mp 142 °C, yield 908 mg (77%). $C_{19}H_{29}N_3O$ (315.5). MS (ESI): m/z (%) = 316 (MH^+, 100). MS (EI): m/z (%) = 315 (M^+, 8), 297 ($(M - H_2O)^+$, 100), 91 ($C_7H_7^+$, 93). IR: $\tilde{\nu}$ (cm^{-1}) = 3400–3100 (m, ν (O–H)), 3276 (m, ν (N–H)), 1336 (w, δ (OH)), 1132 (w, ν (C–OH)). ^{1}H NMR ($CDCl_3$): δ (ppm) = 1.08–1.35 (m, 3 H, 6-H_a, 7-H_a, 8-H_a), 1.62–1.81 (m, 2 H, 6-H_e, N(CH_2CH_2)), 1.81–1.90 (m, 1 H, 7-H_e), 1.96–2.09 (m, 2 H, 8a-H, N(CH_2CH_2)), 2.14 (td, ^2J = ^3J = 11.3 Hz, ^3J = 3.2 Hz, 1 H, 2-H_a), 2.18–2.24 (m, 1 H, 8-H_e), 2.40 (t, ^3J = 10.6 Hz, 1 H, 4a-H), 2.58–2.95 (m, 8 H, 2-H, 3-H (2 H), 5-H, N(CH_2CHOH) (2 H), N(CH_2CH_2) (2 H)), 3.13 (d, ^3J = 13.3 Hz, 1 H, Ph-CH_2), 4.13 (d, ^3J = 13.2 Hz, 1 H, Ph-CH_2), 4.24–4.31 (m, 1 H, N(CH_2CHOH)), 7.24–7.32 (m, 5 H, Ph-H). Signals for the NH and OH protons are not visible in the spectrum. ^{13}C NMR ($CDCl_3$): δ (ppm) = 21.9/22.2 (C-6), 22.8/22.9 (C-7), 28.9 (C-8), 34.8/34.9 (N(CH_2CH_2)), 46.2 (C-3), 47.1 (N(CH_2CH_2)), 53.2 (C-2), 55.7 (N(CH_2CHOH)), 58.1 (Ph-CH_2), 60.5/60.7 (C-5), 62.5/62.6 (C-4a), 66.0/66.1 (C-8a), 71.2/71.3 (N(CH_2CHOH)), 127.0 (Ph-C), 128.4 (2 C, Ph-C), 129.4 (2 C, Ph-C), 139.3 (quart. Ph-C). HPLC (method A): purity 95.4%, t_R = 7.1 min.

1-{(4a*RS*,8*SR*,8a*SR*)-4-Benzyl-8-[(3*SR*)- and (3*RS*)-3-hydroxypyrrolidin-1-yl]perhydroquinoxalin-1-yl}-2-(3,4-dichlorophenyl)ethan-1-one (13b). 2-(3,4-Dichlorophenyl)acetyl chloride (1.8 g, 8.1 mmol) was added dropwise to a solution of **12b** (2.6 g, 8.1 mmol) in absolute CH_2Cl_2 (200 mL), and the mixture was stirred at rt. After 30 min, 2 M NaOH (200 mL) was added, and the mixture was stirred overnight. The aqueous layer was separated, and the organic layer was extracted with 1 M HCl (3×). The pH value of the aqueous layer was adjusted to pH 8 by addition of 2 M NaOH. The alkaline aqueous layer was extracted with CH_2Cl_2 (3×). The combined organic layer was dried (Na_2SO_4), filtered, and concentrated in vacuo. R_f = 0.14 (CH_3OH); R_f = 0.55 and 0.62 (CH_2Cl_2/MeOH/NH_3 = 9:1:0.1). Pale yellow solid, mp 78 °C, yield 3.7 g (90%). $C_{27}H_{33}Cl_2N_3O_2$ (502.5) MS (ESI): m/z (%) = 502 (MH^+, 2 × ^{35}Cl, 100), 504 (MH^+, ^{35}Cl/^{37}Cl, 65), 506 (MH^+, 2 × ^{37}Cl, 12). IR: $\tilde{\nu}$ (cm^{-1}) = 3100–3500 (m, ν (O–H)), 1636 (s, ν (C═O)), 875 (w, out-of-plane (Ar–H)), 814 (w, out-of-plane (Ar–H)). ^{1}H NMR (toluene-d_8, 100 °C): δ (ppm) = 0.86–1.01 (m, 2 H, 5-H_a, 7-H_a), 1.01–1.12 (m, 1 H, 6-H_a), 1.45–1.65 (m, 3 H, 6-H_e, 5-H_e, N(CH_2CH_2)), 1.67–1.86 (m, 2 H, 7-H_e, N(CH_2CH_2)), 1.86–1.92 (m, 1 H, 3-H), 2.23–2.32 (m, 1 H, 8-H), 2.36–2.52 (m, 3 H, 2-H, 3-

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H, N(CH_2CHOH)), 2.60−2.66 (m, 1 H, N(CH_2CHOH)), 2.67−2.76 (m, 2 H, N(CH_2CH_2)), 2.87/2.96 (2 d, ^2J = 13.7 Hz, 1 H, Ph-CH_2-N), 2.93−3.01 (m, 1 H, 2-H), 3.20−3.29 (m, 3 H, Ph-CH_2-C=O (2 H), 8a-H), 3.49−3.58 (m, 1 H, 4a-H), 3.62/3.70 (2 d, ^2J = 13.4 Hz, 1 H, Ph-CH_2-N), 3.96−4.02 (m, 1 H, N(CH_2CHOH)), 6.85−6.89 (m, 2 H, Ph-H), 7.02−7.08 (3 H, Ph-H), 7.09−7.13 (m, 2 H, Ph-H), 7.21−7.23 (m, 1 H, Ph-H). A signal for the OH proton is not visible in the spectrum. ^{13}C NMR (toluene-d_8, 100 °C): δ (ppm) = 23.4/23.7 (C-6), 25.7 (C-5), 31.0/31.2 (C-7), 35.3/35.5 (N(CH_2CH_2)), 41.6/41.7 (Ph-CH_2-C=O), 46.4 (N(CH_2CH_2), 48.1 (C-2), 53.2/53.4 (N-(CH_2CHOH)), 56.9 (C-3), 57.1/57.4 (Ph-CH_2-N), 58.2 (C-4a), 62.1/62.3 (C-8), 67.1 (C-8a), 71.4/71.5 (N(CH_2CHOH)), 127.3 (2 C, Ph-C), 128.4 (Ph-C), 128.7 (2 C, Ph-C), 129.0 (Ph-C), 129.3 (Ph-C), 128.9 (Ph-C), 130.7 (Ph-C), 131.5/131.6 (Ph-C), 137.1/137.3 (quart. C), 140.3 (quart. C), 170.4/170.7 (C=O). HPLC (method A): purity 98.0%, t_R = 16.6 min. HPLC (method B): purity 96.6%, t_R = 13.7 min.

2-(3,4-Dichlorophenyl)-1-{(4a*RS*,8*SR*,8a*SR*)-8-[(3*SR*)- and (3*RS*)-3-hydroxypyrrolidin-1-yl]perhydroquinoxalin-1-yl}-ethan-1-one (14b). A mixture of **13b** (373 mg, 0.74 mmol), conc HCl (7.4 mL), Pd/C (158 mg), and THF/H_2O (1:1, 74 mL) was stirred at rt under H_2 (1 bar) for 30 min. The suspension was filtered, and the organic solvent was removed in vacuo. The pH value of the aqueous layer was adjusted to pH 8 by addition of 2 M NaOH. The aqueous layer was extracted with CH_2Cl_2 (5×). The combined organic layer was dried (Na_2SO_4), filtered, concentrated in vacuo, and the residue was purified by fc (3 cm, 18 cm, CH_2Cl_2/MeOH/NH_3 = 9:1:0.1, 10 mL, R_f = 0.25 and 0.29 (CH_2Cl_2/MeOH/NH_3 = 9:1:0.1), R_f = 0.04 (CH_3OH)). Pale yellow resin, yield 272 mg (89%). $C_{20}H_{27}Cl_2N_3O_2$ (412.4 g). MS (ESI): m/z (%) = 412 (MH^+, 2 × ^{35}Cl, 100), 414 (MH^+, ^{35}Cl/^{37}Cl, 62), 416 (MH^+, 2 × ^{37}Cl, 10). IR: $\tilde{\nu}$ (cm^{-1}) = 3100−3500 (m, ν (O−H, N−H)), 1633 (s, ν (C=O)), 875 (w, out-of-plane (Ar−H)), 828 (w, out-of-plane (Ar−H)). ^{1}H NMR (DMSO-d_6, 100 °C): δ (ppm) = 1.11−1.33 (m, 3 H, 7-H_a, 5-H_a, 6-H_a), 1.45−1.55 (m, 1 H, N(CH_2CH_2)), 1.71−1.80 (m, 2 H, 6-H_e, 7-H_e), 1.81−1.91 (m, 2 H, 5-H_e, N(CH_2CH_2)), 2.50−2.57 (m, 2 H, 3-H, 8-H), 2.61−2.85 (m, 5 H, 2-H (2 H), N(CH_2CHOH), N(CH_2CH_2) (2 H)), 2.86−2.93 (m, 2 H, 3-H, N(CH_2CHOH),), 3.18−3.27 (m, 1 H, 8a-H), 3.31−3.42 (m, 1 H, 4a-H), 3.72−3.79 (d broad, ^2J = 16.2 Hz, 1 H, Ph-CH_2-C=O), 3.87/3.89 (2 d, ^2J = 15.5 Hz, 1 H, Ph-CH_2-C=O), 4.07−4.15 (m, 1 H, N(CH_2CHOH)), 7.23−7.28 (m, 1 H, Ph-6-H), 7.45−7.49 (m, 2 H, Ph-5-H, Ph-2-H). Signals for the NH and OH protons are not visible in the spectrum. HPLC (method A): purity 97.3%, t_R = 13.8 min. HPLC (method B): purity 96.6%, t_R = 11.2 min.

2-(3,4-Dichlorophenyl)-1-[(4a*RS*,8*SR*,8a*RS*)-4-methyl-8-(pyrrolidin-1-yl)perhydroquinoxalin-1-yl]ethan-1-one (15a). Formalin (37%, 223 mg, 2.7 mmol) was dissolved in MeOH (5 mL), and $NaBH_3CN$ (17.2 mg, 0.27 mmol) was added. The pH value of the mixture was adjusted with conc acetic acid to pH 5. Then a solution of **14a** (109 mg, 0.27 mmol) in MeOH (15 mL) was added and the mixture was stirred at rt for 1.5 h. A saturated Na_2CO_3 solution (12 mL) was added, and the mixture was stirred at rt for 15 min. The precipitate was filtered off, and MeOH was evaporated in vacuo. The remaining aqueous layer was extracted with CH_2Cl_2 (5×). The combined organic layer was dried (Na_2SO_4), filtered, concentrated in vacuo, and the residue was purified by fc (2 cm, 16 cm, CH_2Cl_2/MeOH/NH_3 = 9.5:0.5:0.05, 5 mL, R_f = 0.48 (CH_2Cl_2/MeOH/NH_3 = 9:1:0.1), R_f = 0.04 (MeOH)). Pale yellow resin, yield 34 mg (31%). $C_{21}H_{29}Cl_2N_3O$ (410.4). MS (ESI): m/z (%) = 410 (MH^+, 2 × ^{35}Cl, 100), 412 (MH^+, ^{35}Cl/^{37}Cl, 62), 414 (MH^+, 2 × ^{37}Cl, 10). IR: $\tilde{\nu}$ (cm^{-1}) = 1639 (s, ν (C=O)), 873 (w, out-of-plane (Ar−H)), 789 (w, out-of-plane (Ar−H)). ^{1}H NMR (toluene-d_8, 100 °C): δ (ppm) = 0.84 (qd, ^2J = ^3J = 12.7 Hz, ^3J = 3.9 Hz, 1 H, 7-H_a), 0.94 (qd, ^2J = ^3J = 12.3 Hz, ^3J = 3.2 Hz, 1 H, 5-H_a), 1.07 (qt, ^2J = ^3J = 13.3 Hz, ^3J = 3.1 Hz, 1 H, 6-H_a), 1.47−1.52 (m, 5 H, 6-H_e, N(CH_2CH_2)$_2$), 1.61−1.67 (m, 1 H, 5-H), 1.71−1.78 (m, 1 H, 7-H_e), 1.81−1.88 (m, 1 H, 3-H), 1.94 (s, 3 H, CH_3), 1.99 (td, ^3J = 10.0 Hz, ^3J = 4.3 Hz, 1 H, 8-H), 2.41−2.48 (m, 3 H, 3-H, N(CH_2CH_2)$_2$), 2.48−2.54 (m, 2 H, N(CH_2CH_2)$_2$), 2.84 (t, ^3J = 10.5 Hz, 1 H, 8a-H), 3.01−3.13 (m, 2 H, 2-H (2 H)), 3.27 (d, ^2J = 15.6 Hz, 1 H, Ph-CH_2-C=O), 3.35 (d, ^2J = 15.5 Hz, 1 H, Ph-CH_2-C=O), 3.62−3.71 (m, 1 H, 4a-H), 6.87 (d broad, ^3J = 8.1 Hz, 1 H, Ph-6-H), 7.02 (d, ^3J = 8.4 Hz, 1 H, Ph-5-H), 7.24 (d, ^4J = 1.9 Hz, 1 H, Ph-2-H). HPLC (method A): purity 97.4%, t_R = 15.0 min, HPLC (method B): purity 97.7%, t_R = 12.0 min.

2-(3,4-Dichlorophenyl)-1-{(4a*RS*,8*SR*,8a*SR*)-4-methyl-8-[(3*SR*)- and (3*RS*)-3-hydroxypyrrolidin-1-yl]-perhydroquinoxalin-1-yl}ethan-1-one (15b). Formalin (37%, 170 mg, 2.1 mmol) was dissolved in MeOH (5 mL), and $NaBH_3CN$ (132 mg, 2.1 mmol) was added. The pH value of the mixture was adjusted with conc acetic acid to pH 5. Then a solution of **14b** (86 mg, 0.21 mmol) in MeOH (15 mL) was added and the mixture was stirred at rt for 15 min. A saturated Na_2CO_3 solution (15 mL) was added, and the mixture was stirred at rt for 2 h. The precipitate was filtered off, and MeOH was evaporated in vacuo. The remaining aqueous layer was extracted with CH_2Cl_2 (5×). The combined organic layer was dried (Na_2SO_4), filtered, concentrated in vacuo, and the residue was purified by fc (2 cm, 16 cm, CH_2Cl_2/MeOH/NH_3 = 9:1:0.1, 5 mL, R_f = 0.34 and 0.36 (CH_2Cl_2/MeOH/NH_3 = 9:1:0.1), R_f = 0.05 (CH_3OH)). Pale yellow resin, yield 54 mg (60%). $C_{21}H_{29}Cl_2N_3O_2$ (426.4). MS (ESI): m/z (%) = 426 (MH^+, 2 × ^{35}Cl, 100), 428 (MH^+, ^{35}Cl/^{37}Cl, 77), 430 (MH^+, 2 × ^{37}Cl, 12). IR: $\tilde{\nu}$ (cm^{-1}) = 3600−3000 (m, ν (O−H)), 1636 (s, ν (C=O)), 874 (w, out-of-plane (Ar−H)), 812 (w, out-of-plane (Ar−H)). ^{1}H NMR (toluene-d_8, 100 °C): δ (ppm) = 0.82 (q broad, ^2J = ^3J = 12.8 Hz, 1 H, 7-H_a), 0.90 (qd, ^2J = ^3J = 12.3 Hz, ^3J = 3.1 Hz, 1 H, 5-H_a), 0.98−1.10 (m, 1 H, 6-H_a), 1.44−1.63 (m, 4 H, N(CH_2CH_2) (2 H), 6-H_e, 5-H_e), 1.65−1.77 (m, 1 H, 7-H_e), 1.79−1.88 (m, 1 H, 3-H), 1.91/1.92 (s, 3 H, CH_3), 1.93−1.99 (m, 1 H, 8-H), 2.32−2.45 (m, 1 H, N(CH_2CHOH)), 2.45−2.52 (m, 1 H, 3-H), 2.52−2.60 (m, 1 H, N(CH_2CHOH)), 2.62−2.68 (m, 1 H, N(CH_2CH_2)), 2.68−2.74 (m, 1 H, N(CH_2CH_2)), 2.80 (t, ^3J = 10.3 Hz, 1 H, H-8a), 2.95−3.11 (m, 2 H, 2-H (2 H)), 3.22/3.25 (d, ^2J = 15.4 Hz, 1 H, Ph-CH_2-C=O), 3.32 (d, ^2J = 15.3 Hz, 1 H, Ph-CH_2-C=O), 3.61−3.70 (m, 1 H, 4a-H), 3.94−4.00 (m, 1 H, N(CH_2CHOH)), 6.87 (d broad, ^3J = 8.6 Hz, 1 H, Ph-6-H), 7.06 (d, ^3J = 8.2 Hz, 1 H, Ph-5-H), 7.22 (d, ^4J = 1.9 Hz, 1 H, Ph-2-H). A signal for the OH proton is not visible in the spectrum. HPLC (method A): purity 98.8%, t_R = 14.1 min. HPLC (method B): purity 98.2%, t_R = 11.4 min.

1-[(4a*RS*,8*SR*,8a*RS*)-4-Butyl-8-(pyrrolidin-1-yl)-perhydroquinoxalin-1-yl]-2-(3,4-dichlorophenyl)ethan-1-one (16a). Butyraldehyde (93 mg, 1.3 mmol) was dissolved in MeOH (5 mL), and $NaBH_3CN$ (82 mg, 1.3 mmol) was added. The pH value of the mixture was adjusted with conc acetic acid to pH 5. Then a solution of **14a** (101 mg, 0.25 mmol) in MeOH (15 mL) was added and the mixture was stirred at rt overnight. A saturated Na_2CO_3 solution (15 mL) was added, and the mixture was stirred at rt for 15 min. The precipitate was filtered off, and MeOH was evaporated in vacuo. The remaining aqueous layer was extracted with CH_2Cl_2 (3×). The combined organic layer was dried (Na_2SO_4), filtered, concentrated in vacuo, and the residue was purified by fc (2 cm, 15 cm, CH_2Cl_2/MeOH/NH_3 = 9.5:0.5:0.05, 5 mL, R_f = 0.67 (CH_2Cl_2/MeOH/NH_3 = 9:1:0.1), R_f = 0.09 (MeOH)). Colorless resin, yield 45 mg (40%). $C_{24}H_{35}Cl_2N_3O$ (452.5). MS (ESI): m/z (%) = 452 (MH^+, 2 × ^{35}Cl, 100), 454 (MH^+, ^{35}Cl/^{37}Cl, 61), 456 (MH^+, 2 × ^{37}Cl, 11). IR: $\tilde{\nu}$ (cm^{-1}) = 1641 (s, ν (C=O)), 876 (w, out-of-plane (Ar−H)), 790 (w, out-of-plane (Ar−H)). ^{1}H NMR (toluene-d_8, 100 °C): δ (ppm) = 077−0.82 (t, ^3J = 8.0 Hz, 3 H, N($CH_2CH_2CH_2CH_3$)), 0.83−0.92 (m, 1 H, 7-H_a), 0.98 (qd, ^2J = ^3J = 12.1 Hz, ^3J = 3.0 Hz, 1 H, 5-H_a), 1.07 (qt, ^2J = ^3J = 13.3 Hz, ^3J = 3.3 Hz, 1 H, 6-H_a), 1.10−1.22 (m, 4 H, N($CH_2CH_2CH_2CH_3$)), 1.47−1.55 (m, 5 H, 6-H_e, N-(CH_2CH_2)$_2$), 1.62−1.68 (m, 1 H, 5-H_e), 1.72−1.78 (m, 1 H, 7-H_e), 1.94−2.00 (m, 1 H, 3-H), 2.20 (td, ^3J = 9.9 Hz, ^3J = 4.1 Hz, 1 H, 8-H), 2.38−2.52 (m, 5 H, 3-H, N(CH_2CH_2)$_2$ (4 H)), 2.61 (m, 1 H, 2-H), 2.97 (t, ^3J = 8.7 Hz, 3 H, 8a-H, N($CH_2CH_2CH_2CH_3$) (2 H)), 3.18−3.27 (m, 1 H, 4a-H), 3.32−3.39 (m, 3 H, Ph-CH_2-C=O (2 H), 2-H), 6.89−6.92 (m, 1 H, Ph-6-H), 7.03 (d, ^3J = 8.2 Hz, 1 H, Ph-5-H), 7.27 (d, ^4J = 1.9 Hz, 1 H, Ph-2-H). HPLC (method A): purity 97.5%, t_R = 17.5 min.

Methyl (4a*RS*,8*SR*,8a*RS*)-1-[2-(3,4-Dichlorophenyl)acetyl]-8-(pyrrolidin-1-yl)perhydroquinoxaline-4-carboxylate (23a). Under N_2, **14a** (100.9 mg, 0.25 mmol) was dissolved in CH_2Cl_2 (13

mL) and methyl chloroformate (28.9 mg, 0.31 mmol) was added. The mixture was stirred at rt for 2 h. The solvent was removed in vacuo, and the residue was purified by fc (2 cm, 16 cm, CH_2Cl_2/MeOH/NH_3 = 9.5:0.5:0.05, 5 mL, R_f = 0.76 (CH_2Cl_2/MeOH/NH_3 = 9:1:0.1), R_f = 0.17 (CH_3OH)). Pale yellow resin, yield 67 mg (59%). $C_{22}H_{29}Cl_2N_3O_3$ (454.4). MS (ESI): m/z (%) = 454 (MH^+, 2 × ^{35}Cl, 100), 456 (MH^+, $^{35}Cl/^{37}Cl$, 66), 458 (MH^+, 2 × ^{37}Cl, 10). IR: $\tilde{\nu}$ (cm^{-1}) = 1700 (s, ν (N-C=O-O)), 1646 (s, ν (N-C=O)), 873 (w, out-of-plane (Ar−H)). ^1H NMR (CD_2Cl_2, −40 °C): δ (ppm) = 0.74−0.85 (m, 1 H, 5-H_a), 1.10−1.40 (m, 4 H, 5-H_e, 6-H_a, 7-H_a, 7-H_e), 1.55−1.62 (m, 1 H, 6-H_e), 1.63−1.75 (m, 4 H, N(CH_2CH_2)$_2$), 1.82−1.92 (m, 2 H, 2-H, 3-H), 2.00−2.07 (m, 1 H, 8a-H), 2.56−2.71 (m, 4 H, N(CH_2CH_2)$_2$), 3.00−3.07 (m, 1 H, 2-H), 3.44−3.54 (m, 3 H, $COOCH_3$), 3.55−3.61 (m, 2 H, Ph-CH_2-C=O), 3.76−3.82 (m, 1 H, 8-H), 3.89−3.97 (m, 1 H, 4a-H), 4.00−4.12 (m, 1 H, 3-H), 6.98−7.03 (m, 1 H, Ph-6-H), 7.28−7.32 (m, 1 H, Ph-2-H), 7.33−7.38 (m, 1 H, Ph-5-H). HPLC (method A): purity 98.4%, t_R = 18.6 min. HPLC (method B): purity 97.3%, t_R = 15.4 min.

Methyl (4a*RS*,8*SR*,8a*RS*)-1-[2-(3,4-Dichlorophenyl)acetyl]-8-[(3*SR*)- and (3*RS*)-3-hydroxypyrrolidin-1-yl]-perhydroquinoxaline-4-carboxylate (23b). Under N_2, **14b** (132 mg, 0.32 mmol) was dissolved in CH_2Cl_2 (20 mL) and methyl chloroformate (30 mg, 0.32 mmol) was added dropwise. The mixture was stirred at rt for 3 h. Then it was concentrated in vacuo, and the residue was purified by fc (2 cm, 15 cm, CH_2Cl_2/MeOH/NH_3 9:1:0.05, 5 mL, R_f = 0.36 and 0.41 (CH_2Cl_2/MeOH/NH_3 = 9:1:0.1), R_f = 0.22 (MeOH)). The solvent was removed in vacuo. The solid residue was dissolved in CH_2Cl_2, and the solution was extracted with 1 M HCl (3×). The pH value of the aqueous layer was adjusted with 2 M NaOH to pH 8, and the solution was extracted with CH_2Cl_2 (3×). The combined organic layer was dried (Na_2SO_4), filtered, concentrated in vacuo, and the residue was purified once more by fc (2 cm, 15 cm, CH_2Cl_2/MeOH/NH_3 = 9.5:0.5:0.05, 5 mL). The solvent was removed in vacuo, and the residue was purified by preparative HPLC method C (eluent, MeOH/H_2O/Et_2NH 70:30:0.1). The organic solvent was removed in vacuo, and the aqueous layer was extracted with CH_2Cl_2 (3×). The combined organic layer was dried (Na_2SO_4), filtered, and concentrated in vacuo. Pale yellow resin, yield 93 mg (62%). $C_{22}H_{29}Cl_2N_3O_4$ (470.4). MS (ESI): m/z (%) = 470 (MH^+, 2 × ^{35}Cl, 100), 472 (MH^+, $^{35}Cl/^{37}Cl$, 61), 474 (MH^+, 2 × ^{37}Cl, 11). IR: $\tilde{\nu}$ (cm^{-1}) = 3600−3100 (m, ν (O−H)), 1693 (s, ν (MeO-C=O)), 1642 (s, ν (N-C=O)), 875 (w, out-of-plane (Ar−H)), 792 (w, out-of-plane (Ar−H)). ^1H NMR (CD_2Cl_2, −50 °C): δ (ppm) = 1.05−1.19 (m, 3 H, 5-H_a, 6-H_a, 7-H_a), 1.23−1.35 (m, 2 H, 5-H_e, 7-H_e), 1.57−1.74 (m, 2 H, 6-He, N(CH_2CH_2)), 1.76−1.93 (m, 3 H, 2-H, 3-H, N(CH_2CH_2)), 1.97−2.06 (m, 1 H, 8a-H), 2.56−2.68 (m, 2 H, N(CH_2CH_2)), 2.71−2.87 (m, 2 H, N(CH_2CHOH)), 3.00−3.08 (m, 1 H, 2-H), 3.49−3.54 (m, 1 H, Ph-CH_2-C=O), 3.58 (s, 3 H, $COOCH_3$), 3.65 (d, ^2J = 15.9 Hz, 1 H, Ph-CH_2-C=O), 3.73−3.80 (m, 1 H, 8-H), 3.85−3.98 (m, 1 H, 4a-H), 4.01−4.06 (m, 1 H, 3-H), 4.14−4.19 (m, 1 H, N(CH_2CHOH)), 7.04−7.08 (m, 1 H, Ph-6-H), 7.26−7.30 (m, 1 H, Ph-2-H), 7.32−7.39 (m, 1 H, Ph-5-H). A signal for the OH proton is not visible in the spectrum. HPLC (method A): purity 99.9%, t_R = 17.4 min. HPLC (method B): purity 99.3%, t_R = 14.8 min.

Molecular Modeling. The conformational analysis was performed with force field MMFF94x of the molecular modeling program MOE (molecular operating environment), version 2013.08 (Chemical Computing Group AG).

Receptor Affinity Using Animal Sources as Receptor Material. *Materials.* The guinea pig brains and rat liver for the κ, σ_1, and σ_2 receptor binding assays were commercially available (Harlan-Winkelmann, Borchen, Germany). Pig brains for the NMDA assay were obtained from the local slaughterhouse. The following pieces of equipment were used. Homogenizer: Elvehjem Potter (B. Braun Biotech International, Melsungen, Germany). Cooling centrifuge model Rotina 35R (Hettich, Tuttlingen, Germany) and high-speed cooling centrifuge model Sorvall RC-5C Plus (Thermo Fisher Scientific, Langenselbold, Germany). Multiplates: standard 96-well multiplates (Diagonal, Muenster, Germany). Shaker: self-made device with adjustable temperature and tumbling speed (scientific workshop of the institute). Vortex: Vortex Genie 2 (Thermo Fisher Scientific, Langenselbold, Germany). Harvester: MicroBeta FilterMate-96 harvester. Filter: Printed Filtermat types A and B. Scintillator: Meltilex (type A or B) solid state scintillator. Scintillation analyzer: MicroBeta Trilux (all PerkinElmer LAS, Rodgau-Jügesheim, Germany). Chemicals and reagents were purchased from different commercial sources and of analytical grade.

Preparation of Membrane Homogenates from Guinea Pig Brain.[31,32] Five guinea pig brains were homogenized with the potter (500−800 rpm, 10 up-and-down strokes) in 6 volumes of cold 0.32 M sucrose. The suspension was centrifuged at 1200*g* for 10 min at 4 °C. The supernatant was separated and centrifuged at 23 500*g* for 20 min at 4 °C. The pellet was resuspended in 5−6 volumes of buffer (50 mM Tris, pH 7.4) and centrifuged again at 23 500*g* (20 min, 4 °C). This procedure was repeated twice. The final pellet was resuspended in 5−6 volumes of buffer and frozen (−80 °C) in 1.5 mL portions containing about 1.5 mg protein/mL.

Protein Determination. The protein concentration was determined by the method of Bradford,[56] modified by Stoscheck.[57] The Bradford solution was prepared by dissolving 5 mg of Coomassie Brilliant Blue G 250 in 2.5 mL of EtOH (95%, v/v). Then 10 mL of deionized H_2O and 5 mL of phosphoric acid (85%, m/v) were added to this solution. The mixture was stirred and filled to a total volume of 50.0 mL with deionized water. The calibration was carried out using bovine serum albumin as a standard in nine concentrations (0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 1.5, 2.0, and 4.0 mg/mL). In a 96-well standard multiplate, an amount of 10 μL of the calibration solution or an amount of 10 μL of the membrane receptor preparation was mixed with 190 μL of the Bradford solution, respectively. After 5 min, the UV absorption of the protein−dye complex at λ = 595 nm was measured with a plate reader (Tecan Genios, Tecan, Crailsheim, Germany).

General Protocol for the Binding Assays. The test compound solutions were prepared by dissolving approximately 10 μmol (usually 2−4 mg) of test compound in DMSO so that a 10 mM stock solution was obtained. To obtain the required test solutions for the assay, the DMSO stock solution was diluted with the respective assay buffer. The Filtermats were presoaked in 0.5% aqueous polyethylenimine solution for 2 h at room temperature before use. All binding experiments were carried out in duplicate in 96-well multiplates. The concentrations given are the final concentrations in the assay. Generally, the assays were performed by addition of 50 μL of the respective assay buffer, 50 μL of test compound solution in various concentrations (10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, and 10^{-10} mol/L), 50 μL of corresponding radioligand solution, and 50 μL of the respective receptor preparation into each well of the multiplate (total volume 200 μL). The receptor preparation was always added last. During the incubation, the multiplates were shaken at a speed of 500−600 rpm at the specified temperature. Unless otherwise noted, the assays were terminated after 120 min by rapid filtration using the harvester. During the filtration each well was washed five times with 300 μL of water. Subsequently, the Filtermats were dried at 95 °C. The solid scintillator was melted on the dried Filtermats at a temperature of 95 °C for 5 min. After solidification of the scintillator at room temperature, the trapped radioactivity in the Filtermats was measured with the scintillation analyzer. Each position on the Filtermat corresponding to one well of the multiplate was measured for 5 min with the ^3H-counting protocol. The overall counting efficiency was 20%. The IC_{50} values were calculated with the program GraphPad Prism 3.0 (GraphPad Software, San Diego, CA, USA) by nonlinear regression analysis. Subsequently, the IC_{50} values were transformed into K_i values using the equation of Cheng and Prusoff.[58] The K_i values are given as mean value ± SEM from three independent experiments.

Protocol of the κ Receptor Binding Assay (Guinea Pig Brain Homogenates). The assay was performed with the radioligand [^3H]U-69,593 (55 Ci/mmol, Amersham, Little Chalfont, U.K.). The thawed guinea pig brain membrane preparation (about 100 μg of the protein) was incubated with various concentrations of test compounds, 1 nM [^3H]U-69,593, and Tris-$MgCl_2$ buffer (50 mM, 8 mM $MgCl_2$, pH 7.4)

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at 37 °C. The nonspecific binding was determined with 10 μM unlabeled U-69,593. The K_d value of U-69,593 is 0.69 nM.

[^{35}S]GTPγS Binding Assay. Agonistic Activity at the κ-Opioid Receptor. The [^{35}S]guanosine 5′-3-*O*-(thio)triphosphate (GTPγS) assay was carried out as described in ref 45. The receptor material was obtained from human HEK 293 (human embryonic kidney) cells. Parameters were the following. Vehicle, 1.00% DMSO. Incubation time, 30 min. Incubation temperature, 30 °C. Incubation buffer, 20 mM HEPES, pH 7.4, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, 1 mM EDTA. Quantification of bound [^{35}S]GTPγS. Significance criterion for agonists: >50% increase of bound [^{35}S]GTPγS relative to U-69,593 response. The EC_{50} values were determined by a nonlinear, least-squares regression analysis using MathIQTM (ID Business Solutions Ltd., U.K.). Reference standards were run as an integral part of each assay to ensure the validity of the results obtained.

Passage of an Artificial Blood−Brain Barrier. *Blood−Brain Barrier Cell Culture Model.* Pig brain cortex endothelial cells (BCECs) were gently thawed and seeded (250 000/cm^2) on rat tail collagen-coated (0.54 mg/mL) Transwell microporous polycarbonate filter inserts (Corning, Wiesbaden, Germany, 1.12 cm^2 growth area, 0.4 μm pore size) on DIV2 in plating medium (medium 199 Earle supplemented with 10% newborn calf serum, 0.7 mM L-glutamine, 100 μg/mL gentamycin, 100 U/mL penicillin, 100 μg/mL streptomycin (all Biochrom, Berlin, Germany)) in the apical compartment at 37 °C with 5% CO_2 and 100% humidity. After PBCECs reached confluence (DIV4), the plating medium was replaced by serum-free culture medium (Dulbecco's modified Eagle medium/Ham's F 12 (1:1) containing 4.1 mM L-glutamine, 100 μg/mL gentamycin, 100 U/mL penicillin, 100 μg/mL streptomycin (all Biochrom, Berlin, Germany)) and 550 nM hydrocortisone (Sigma-Aldrich, Deisenhofen, Germany) in order to induce differentiation. Permeability ([^{14}C]sucrose, test compounds) and barrier integrity studies were started on day DIV6. For quantification of the barrier integrity, the transendothelial electrical resistance (TER) device, a cellZscope (nanoAnalytics, Muenster, Germany), was used. Only wells with TER values of >600 Ω·cm^2 and capacitance values between 0.45 and 0.6 μF/cm^2 on day DIV6 indicate a confluent PBCEC monolayer with sufficient barrier properties and were thus used for the respective experiments.

Transfer over the Barrier Model. To study transfer across the in vitro barrier, the model was exposed to the test compounds on the apical side. Thereby test compounds were applied by replacing 10% of the apical volume to reach finally concentration (500 μM). After 3 h the amount of the test compound in the basolateral compartment was recorded by RP-HPLC analysis.

Analgesic Activity in Animal Models of Visceral Pain. *Phenylquinone Writhing Assay.*[53] Male NMRI mice with a weight of 25−35 g were used. Ten animals were used per compound dose. Ten minutes after intravenous injection of 0.3 mL/mouse of a solution ot the test compound, an aqueous solution of phenylquinone (2-phenyl-1,4-benzoquinone, 0.02% in water with 5% ethanol, 0.35 mL) was injected intraperitoneally. The phenylquinone solution was kept in a water bath of 45 °C until use. Each animal was put into a single cage and observed. The pain-induced writhing reactions (=stretching, twisting a hind leg inward, contraction of abdomen) were counted for 15 min (5−20 min after ip injection of phenylquinone). Animals treated with vehicle (iv) and animals treated with phenylquinone (ip) served as negative and positive controls, respectively. Test compounds were used in the standard dose of 10 mg/kg. If necessary, higher or lower doses were employed.

Mustard Oil Assay.[54,55] Male NMRI mice (20−35 g) were habituated on a lattice in a cage of plexiglas (14.5 cm × 14.5 cm × 10 cm) for 30 min (test conditions). The mice were stimulated with von Frey filaments onto the abdominal wall. Ten stimulations with von Frey filaments of 1, 4, 8, 16, 32 mN were applied in ascending order (i.e., 10 × 1 mN, 10 × 4 mN, etc.). Animals with more than 25 positive reactions during this phase were excluded. Vaseline was applied in the perianal area to avoid the stimulation of somatic areas with the irritant chemical. Colitis was induced by rectal administration of 50 μL of mustard oil (3.5% in PEG200). Control animals were treated with vehicle (50 μL of PEG200). Compound or vehicle was given iv or icv 5 min before mustard oil. Seven animals were tested per group.

At 2−12 min after mustard oil administration the spontaneous pain score was determined by counting and scoring of visceral pain behaviors (score 1 = licking of abdominal wall; score 2 = stretching, squashing, mounting, backward movement, or contraction of the flank muscles.

At 20−40 min after mustard oil administration referred allodynia and hyperalgesia were determined by tactile stimulations with von Frey filaments. In the referred allodynia test the number of withdrawal reactions against 10 stimulations with a 1 mN von Frey filament was determined. In the hyperalgesia assay the withdrawal reactions against 10 stimulations with a 16 mN von Frey filament were counted and scored (score 1 = lifting of abdomen, licking, movement; score 2 = extrusion of flinching of hind paws, slight jumping, strong licking; score 3 = strong jumping, vocalization).

Statistical Analysis. Data were analyzed by means of two-factor analysis of variance (ANOVA) with repeated measures. Significance of effects was analyzed by means of Wilks' Lambda statistics. In the case of a significant treatment effect, pairwise comparison was performed at every test time point on raw data by Fischer's least significant difference test. Results were considered statistically significant if $p < 0.5$. ED_{50} values and 95% confidence intervals were calculated by linear regression.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Physical, spectroscopic, and purity data of all compounds; synthetic methods; calculation and determination of log *P* and log $D_{7.4}$ values; details of the receptor binding assays and the [^{35}S]GTPγS binding assay; passage of an artificial blood−brain barrier; experimental details of the phenylquinone writhing assay and the mustard oil assay. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

GPCR, G-protein-coupled receptor; JDTic, (*R*)-7-hydroxy-*N*-{(*S*)-2-[(3*R*,4*R*)-4-(3-hydroxyphenyl)-1-isopropyl-3,4-dimethylpiperidin-1-yl]ethyl}-1,2,3,4-tetrahydroisoquinoline-3-carboxamide; DTG, di(*o*-tolyl)guanidine; MOE, molecular operating environment; HEK, human embryonic kidney; GTPγS, guanosine 5′-3-*O*-(thio)triphosphate; APCI, atmospheric pressure chemical ionization; DTG, di-*o*-tolylguanidine; SEM, standard error of the mean; MOPS, 3-morpholinopropanesulfonic acid

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