Structure−Activity Studies of Orexin A and Orexin B at the Human Orexin 1 and Orexin 2 Receptors Led to Orexin 2 Receptor Selective and Orexin 1 Receptor Preferring Ligands

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The neuropeptides orexin A and B (also known as hypocretins) play an important role in many physiological and behavioral activities. Orexins are ligands of two closely related G-protein-coupled receptors, that are the named orexin 1 and orexin 2 receptors. To clearly identify the minimal ligand sequences required for receptor activation, we synthesized and analyzed different centrally, C- and N-terminally truncated analogues of orexins A and B. Furthermore, we used the shortest active analogue to screen for important amino acid residues by L-alanine and L-proline replacement scans. For orexin A, only full-length peptides were able to show the same activity as orexin A, but interestingly, reduced orexin A and natural orexin A, which contains the two disulfide bonds, had the same activity. The shortest highly active orexin B analogue was orexin B 6−28. In addition, we identified orexin A 2−33 as the first analogue with orexin 1 receptor preference and orexin B 10−28, [A^{27}]orexin B 6−28, and [P^{11}]orexin B 6−28 as being highly potent orexin 2 receptor selective (>1000-fold) peptides.

Introduction

Orexins A and B (also known as hypocretins 1 and 2) were discovered in 1998 by two independent groups. By using directional tag PCR subtraction of hypothalamic rat mRNAs, a precursor was discovered encoding for two peptides, named hypocretin 1 and 2.[1] The approach included the isolation of two closely related peptides and their receptors by the search for endogenous peptide ligands for orphan GPCRs with a cell-based reporter system.[2] The HPLC fractions of rat brains that contain the new peptides were found in the lateral hypothalamus, which is described as the "feeding" center. Therefore, the new peptides were named orexins, which resembles the Greek word **orexis**, meaning appetite. Besides the regulation of appetite,[3−6] the orexins are important regulators of sleep−wake cycles[7−9] and neuroendocrine function.[10,11]

The orexin peptides derive from the same 131 residue precursor peptide, which is named prepro-orexin. The cleavage of prepro-orexin leads to the peptides orexins A and B (shown in Figure 1) after further modification.[12] Orexin A is a 33 amino acid peptide amide (MW 3562 Da) that contains two intramolecular disulfide bonds connecting positions 6 to 12 and 7 to 14. Orexin A is completely conserved in several mammalian and amphibian species, whereas rat and mouse orexin B contains two amino acid substitutions compared with the human peptide (proline instead of serine at position 2; asparagine instead of serine at position 18). Orexin B consists of 28 amino acids (MW 2937 Da) and an amidated C-terminus and shares, mainly in the C-terminal region, 46% sequence identity with orexin A. The three-dimensional solution structure of orexin B was analyzed by two-dimensional NMR spectroscopy and shows two α-helices that span the residues Leu^7 to Gly^{19} and Ala^{23} to Met^{28}. They are connected by a flexible loop at position 20−22.[13]

Figure 1. (A) Comparison of the amino acid sequence of human orexin A and B. Similarities of the sequences are highlighted in gray. (B) Comparison of the amino acid sequence of human, rat, mouse, and xenopus orexin A and B. Deviations from the human sequences are highlighted in gray. U = pyroglutamic acid.

The orexin peptides are ligands of two closely related G-protein coupled receptors, termed the orexin 1 and orexin 2 receptors (Ox_1R and Ox_2R). These receptors are highly conserved across mammalian species and share 64% sequence identity. The activity of orexin A at the Ox_1R is approximately 10-fold higher than that of orexin B, whereas the activity at the Ox_2R is in the same range for both peptides.[14] However, both orexin peptide−receptor systems show only moderate affinity compared to other neuropeptides that bind to GPCRs and only micromolar activity. Binding of orexins to their receptors causes an increase in intracellular Ca^{2+} in neurons as well as in recombinant receptor systems.[2,14] The Ox_1R

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is most abundant in the ventromedial hypothalamus, but it was found also in the tenia tecta, hippocampus, and locus coeruleus. The Ox_2R is expressed in the subthalamic and thalamic nuclei and several other regions in the brain.[15]

Until now there was little known about the structure−activity relationships of the orexin peptides with their receptors. Few investigations were done dealing with the relevance of the C-terminal sequence of orexin A at the Ox_1R[16] and the development of a human orexin B agonist at the Ox_2R.[17] The study of the C-terminal sequence of orexin A showed that orexin A 15−33-amide is the shortest analogue with affinity the Ox_1R; however, the functional activity is reduced. This analogue was used to identify important amino acid residues for activating the Ox_1R. By replacing each amino acid with L-alanine amino acid the positions 16, 19, 20, 26, and 29−33 were found to be important.[16] The activity of human orexin B at the Ox_2R was studied by L-alanine and D-amino acid replacements. A potent agonist with Ox_2R preference, [Ala[11],D-Leu[15]]orexin B, was identified.[17]

The object of the here presented study was to clearly identify the minimal sequences required for the activation of the two orexin receptors. Information about the primary structure for the activation of the Ox_1R and the Ox_2R, respectively, should be obtained by systematic modification of the orexin peptides. The contribution of the disulfide bounds of orexin A to the binding at the orexin receptors should be analyzed as well the exchanges between human and rodent orexin B. Furthermore, we intended to identify the shortest orexin A and B analogues that should be used to perform so-called scans, which helps to understand the contribution of each amino acid of a given sequence to the biological activity and the structural relevance.

Alanine scans contain systematic replacements of the natural amino acids with L-alanine. They are useful to identify the amino acid residues that are important for binding at the receptor. The side chain of alanine, a methyl group, maintains the secondary structure but has reduced possibilities to contribute to ligand−receptor interactions. Positions that are sensitive to conformational changes are identified by the proline scan, a replacement with L-proline, because proline is a conformationally rigid and turn-inducing amino acid.

The contribution of the truncations and amino acid replacements to the activity of orexin A and B at the orexin receptors 1 and 2 was investigated by the measurement of the intracellular calcium level. This investigations led to the smallest known biologically active orexin B segment and to the first analogue with preference for Ox_1R, as well as to three highly potent Ox_2R selective peptides.

Results

Peptide Synthesis. The orexin A and B analogues (shown in Table 1) were synthesized on a Rink amide resin with an automated solid-phase peptide synthesizer by using the orthogonal Fmoc/*tert*-butyl strategy to directly obtain peptide amides after cleavage from the resin. The only peptide acid, orexin B 1−28-OH (**25**) was synthesized on Wang resin. The cleaved peptides were characterized by HPLC and matrix-assisted laser desorption ionization (MALDI) mass spectrometry. Peptides were purified by preparative HPLC to obtain products with >95% purity.

Functional Measurement. We have chosen a functional assay analysis because for functional activation of a receptor, binding to the receptor is prerequisite. The activity of the analogues was measured in HEK293 cells, stably transfected with the Ox_1R or Ox_2R, with a fluorimetric imaging plate reader assay (FLIPR) to investigate the transient mobilization of intracellular Ca^{2+}. All active compounds showed the same maximal response. Partial agonism or antagonism (effect of orexin A/B in the presence of testing compound) was not seen for any analogue (data not shown). The concentration of orexins A and B required to induce half-maximum response (EC_{50}) was in the same range as shown previously from Sakurai et al.[2]

Full Length Analogues. In the first set of peptides, full length orexin A analogues that contained no intramolecular disulfide bond were synthesized. Reduced orexin A (**2**) has the same peptide sequence as natural orexin A, but the four cysteine residues are not connected by disulfide bonds. This analogue exhibits the same activity at the Ox_1R (0.16 μM) and at the Ox_2R (0.09 μM) as natural orexin A (**1**). Replacement of the cysteines at positions 6 and 12 (**3**) or 7 and 14 (**4**) by α-amino butyric acid, an amino acid with similar properties as cysteine, results in an 10-fold decrease of activity at both receptors.

Full length analogues of human orexin B were synthesized to identify the relevance of amino acid exchanges that occurred during evolution. The synthesized r/m orexin B (**24**), which contains proline at position 2 instead of serine and asparagine at position 18 instead of serine, showed the same activity at both receptors as human orexin B (**23**). The EC_{50} values of human orexin B were 0.78 μM at the Ox_1R and 0.07 μM at the Ox_2R. The activity at the Ox_1R is nearly 10-fold decreased compared to orexin A and about the same at the Ox_2R. The C-terminal peptide acid of orexin B (**25**) led to a complete loss in activity at both receptors. While orexin A and B share 46% sequence identity mainly at the C-terminus and only the very C-terminal amino acid is different, we exchanged the C-terminal methionine of human orexin B with leucine (**26**). This exchange resulted in a 4-fold reduction of activity at the Ox_2R but did not alter activity at the Ox_1R.

Centrally Truncated and N-Terminal Analogues. To clearly identify the minimal receptor activating sequence of orexin A and B we performed a sequential shortening of the peptides at the C-terminus (**8**, **9**, **27**−**33**). All C-terminally truncated peptides displayed were inactive at both orexin receptors. Furthermore, centrally truncated compounds (compounds that lack the middle part of the peptide) were obtained by the replacement of disulfide bridges with the turn-inducing spacer Pro-Gly (**7**) or the flexible 6-aminohexanoic acid (Ahx) (**5**, **6**) spacer. Replacement of the orexin A segment 6−18 by Ahx (**6**) led to a complete loss in activity at the Ox_1R and the Ox_2R, whereas the replacement of the orexin A segment 6−14 (**5**, **7**) led to peptides with a reduced activity (55-fold at the Ox_1R and 10-fold at the Ox_2R).

C-Terminal Analogues. To get information on the relevance of the C-terminus, we synthesized stepwise

Table 1. Chemical and Biological Data of Synthesized Peptides[a]

no.	peptide	sequence	mass (exp)	EC_{50} (μM) Ox_1 receptor	EC_{50} (μM) Ox_2 receptor	selectivity Ox_1/Ox_2
1	orexin A	UPLPDCCRQKTCSCRLYELLHGAGNHAAGILTL	3562.2	0.09 ± 0.05	0.06 ± 0.02	1.5
2	orexin A (reduced)	UPLPDCCRQKTCSCRLYELLHGAGNHAAGILTL	3567.0	0.16 ± 0.05	0.09 ± 0.01	1.8
3	[6.12 Abu]orexin A	UPLPD**B**CRQKT**B**SCRLYELLHGAGNHAAGILTL	3527.8	1.42 ± 0.03	0.91 ± 0.06	1.6
4	[7.14 Abu]orexin A	UPLPDC**B**RQKTCS**B**RLYELLHGAGNHAAGILTL	3528.6	1.34 ± 0.16	0.85 ± 0.18	1.6
5	orexin A 1−5-Ahx-15−33	UPLPD-**Ahx**-RLYELLHGAGNHAAGILTL	2663.4	5.52 ± 1.26	0.64 ± 0.25	8.6
6	orexin A 1−5-Ahx-19−33	UPLPD-**Ahx**-LLHGAGNHAAGILTL	2105.2	>1000	>1000	−
7	orexin A 1−5-Pro-Gly-15−33	UPLPD**PG**RLYELLHGAGNHAAGILTL	2603.3	4.52 ± 0.60	0.61 ± 0.33	7.4
8	orexin A 1−11	UPLPDCCRQKT	1269.5	>1000	>1000	−
9	orexin A 1−13 (reduced)	UPLPDCCRQKTCS	1458.7	>1000	>1000	−
10	orexin A 2−33 (reduced)	PLPDCCRQKTCSCRLYELLHGAGNHAAGILTL	3457.7	0.86 ± 0.53	2.51 ± 1.06	0.3
11	orexin A 4−33 (reduced)	PDCCRQKTCSCRLYELLHGAGNHAAGILTL	3239.8	0.64 ± 0.09	0.40 ± 0.37	1.6
12	orexin A 6−33 (reduced)	CCRQKTCSCRLYELLHGAGNHAAGILTL	3025.7	0.81 ± 0.29	0.79 ± 0.49	1.0
13	orexin A 8−33	RQKTCSCRLYELLHGAGNHAAGILTL	2822.8	0.68 ± 0.18	0.29 ± 0.14	2.3
14	orexin A 11−33	TCSCRLYELLHGAGNHAAGILTL	2414.4	1.21 ± 0.37	0.38 ± 0.14	3.2
15	orexin A 13−33	SCRLYELLHGAGNHAAGILTL	2210.2	1.03 ± 0.31	0.45 ± 0.26	2.3
16	orexin A 15−33	RLYELLHGAGNHAAGILTL	2020.0	1.79 ± 0.46	0.42 ± 0.08	4.3
17	orexin A 19−33	LLHGAGNHAAGILTL	1456.9	>1000	>1000	−
18	orexin A 23−33	AGNHAAGILTL	1036.6	>1000	>1000	−
19	orexin A 24−33	GNHAAGILTL	965.5	>1000	>1000	−
20	orexin A 25−33	NHAAGILTL	908.5	>1000	>1000	−
21	orexin A 26−33	HAAGILTL	794.5	>1000	>1000	−
22	S−S-[orexin A 13−33]$_2$	SCRLYELLHGAGNHAAGILTL | SCRLYELLHGAGNHAAGILTL	4420.4	0.69 ± 0.12	0.24 ± 0.05	2.9
23	h-orexin B	RSGPPGLQGRLQRLLQASGNHAAGILTM	2899.4	0.78 ± 0.07	0.07 ± 0.03	11.1
24	r/m-orexin B	R**P**GPPGLQGRLQRLLQANGNHAAGILTM	2937.5	0.93 ± 0.27	0.13 ± 0.06	7.2
25	h-orexin B 1−28-OH	RSGPPGLQGRLQRLLQASGNHAAGILTM-**OH**	2900.7	>1000	>1000	−
26	[L^{28}]h-orexin B	RSGPPGLQGRLQRLLQASGNHAAGILT**L**	2883.0	0.78 ± 0.22	0.25 ± 0.10	3.1
27	h-orexin B 1−27	RSGPPGLQGRLQRLLQASGNHAAGILT	2770.4	>1000	>1000	−
28	h-orexin B 1−25	RSGPPGLQGRLQRLLQASGNHAAGI	2555.2	>1000	>1000	−
29	h-orexin B 1−23	RSGPPGLQGRLQRLLQASGNHAA	2384.2	>1000	>1000	−
30	h-orexin B 1−21	RSGPPGLQGRLQRLLQASGNH	2243.6	>1000	>1000	−
31	h-orexin B 1−16	RSGPPGLQGRLQRLLQ	1775.2	>1000	>1000	−
32	h-orexin B 1−13	RSGPPGLQGRLQR	1420.9	>1000	>1000	−
33	h-orexin B 1−10	RSGPPGLQGR	1023.6	>1000	>1000	−
34	h-orexin B 6−28	GLQGRLQRLLQASGNHAAGILTM	2404.9	0.73 ± 0.04	0.13 ± 0.06	5.6
35	h orexin B 8−28	QGRLQRLLQASGNHAAGILTM	2235.6	22.23 ± 14.72	1.22 ± 0.80	18.2
36	h-orexin B 10−28	RLQRLLQASGNHAAGILTM	2049.5	>1000	0.57 ± 0.29	>1750
37	h-orexin B 14−28	LLQASGNHAAGILTM	1496.3	>1000	>1000	−
38	h-orexin B 18−28	SGNHAAGILTM	1070.2	>1000	>1000	−
39	[A^6]h-orexin B 6−28	**A**LQGRLQRLLQASGNHAAGILTM	2420.0	3.16 ± 1.76	0.57 ± 0.18	5.5
40	[A^7]h-orexin B 6−28	G**A**QGRLQRLLQASGNHAAGILTM	2364.6	7.41 ± 3.29	0.83 ± 0.61	8.9
41	[A^8]h-orexin B 6−28	GL**A**GRLQRLLQASGNHAAGILTM	2349.5	1.64 ± 0.57	0.24 ± 0.06	6.8
42	[A^9]h-orexin B 6−28	GLQ**A**RLQRLLQASGNHAAGILTM	2420.8	2.30 ± 1.39	0.21 ± 0.08	11.0
43	[A^{10}]h-orexin B 6−28	GLQG**A**LQRLLQASGNHAAGILTM	2321.8	6.95 ± 1.03	0.71 ± 0.21	9.8
44	[A^{11}]h-orexin B 6−28	GLQGR**A**QRLLQASGNHAAGILTM	2364.6	11.40 ± 1.41	0.51 ± 0.38	22.4
45	[A^{12}]h-orexin B 6−28	GLQGRL**A**RLLQASGNHAAGILTM	2349.1	1.30 ± 0.55	0.16 ± 0.09	8.1
46	[A^{13}]h-orexin B 6−28	GLQGRLQ**A**LLQASGNHAAGILTM	2320.8	4.34 ± 1.94	0.24 ± 0.11	18.1
47	[A^{14}]h-orexin B 6−28	GLQGRLQR**A**LQASGNHAAGILTM	2364.5	5.89 ± 0.85	2.28 ± 1.18	2.6
48	[A^{15}]h-orexin B 6−28	GLQGRLQRL**A**QASGNHAAGILTM	2364.5	>1000	2.33 ± 1.28	>430
49	[A^{16}]h-orexin B 6−28	GLQGRLQRLL**A**ASGNHAAGILTM	2347.1	1.64 ± 0.86	0.19 ± 0.15	8.6
50	[A^{18}]h-orexin B 6−28	GLQGRLQRLLQA**A**GNHAAGILTM	2389.3	1.46 ± 0.46	0.22 ± 0.08	6.6
51	[A^{19}]h-orexin B 6−28	GLQGRLQRLLQAS**A**NHAAGILTM	2420.0	2.21 ± 1.30	0.30 ± 0.27	7.4
52	[A^{20}]h-orexin B 6−28	GLQGRLQRLLQASG**A**HAAGILTM	2363.4	2.18 ± 0.81	0.32 ± 0.19	6.8
53	[A^{21}]h-orexin B 6−28	GLQGRLQRLLQASGN**A**AAGILTM	2339.7	1.14 ± 0.60	0.33 ± 0.40	3.5
54	[A^{24}]h-orexin B 6−28	GLQGRLQRLLQASGNHAA**A**ILTM	2419.9	>1000	3.15 ± 2.15	>320
55	[A^{25}]h-orexin B 6−28	GLQGRLQRLLQASGNHAAG**A**LTM	2363.4	>1000	>1000	−
56	[A^{26}]h-orexin B 6−28	GLQGRLQRLLQASGNHAAGI**A**TM	2362.7	>1000	15.63 ± 4.73	>65
57	[A^{27}]h-orexin B 6−28	GLQGRLQRLLQASGNHAAGIL**A**M	2375.0	>1000	0.76 ± 0.38	>1350
58	[A^{28}]h-orexin B 6−28	GLQGRLQRLLQASGNHAAGILT**A**	2345.6	4.84 ± 1.40	0.75 ± 0.21	6.5
59	[P^6]h-orexin B 6−28	**P**LQGRLQRLLQASGNHAAGILTM	2446.3	8.28 ± 3.06	0.49 ± 0.39	16.9
60	[P^7]h-orexin B 6−28	G**P**QGRLQRLLQASGNHAAGILTM	2390.7	1.70 ± 0.98	0.06 ± 0.04	28.3
61	[P^8]h-orexin B 6−28	GL**P**GRLQRLLQASGNHAAGILTM	2373.9	1.20 ± 0.63	0.16 ± 0.16	7.5
62	[P^9]h-orexin B 6−28	GLQ**P**RLQRLLQASGNHAAGILTM	2446.3	4.46 ± 2.33	0.19 ± 0.15	23.5
63	[P^{10}]h-orexin B 6−28	GLQG**P**LQRLLQASGNHAAGILTM	2346.8	5.41 ± 1.40	0.36 ± 0.17	15.0
64	[P^{11}]h-orexin B 6−28	GLQGR**P**QRLLQASGNHAAGILTM	2389.7	>1000	0.79 ± 0.47	>1265
65	[P^{12}]h-orexin B 6−28	GLQGRL**P**RLLQASGNHAAGILTM	2375.2	5.26 ± 0.97	0.28 ± 0.15	18.8
66	[P^{13}]h-orexin B 6−28	GLQGRLQ**P**LLQASGNHAAGILTM	2348.2	17.82 ± 2.36	0.68 ± 0.31	26.2
67	[P^{14}]h-orexin B 6−28	GLQGRLQR**P**LQASGNHAAGILTM	2389.7	>1000	1.79 ±1.59	>560
68	[P^{15}]h-orexin B 6−28	GLQGRLQRL**P**QASGNHAAGILTM	238 9.7	>1000	20.12 ± 9.85	>50
69	[P^{16}]h-orexin B 6−28	GLQGRLQRLL**P**ASGNHAAGILTM	237 4.7	9.49 ± 2.47	2.48 ± 1.47	3.8
70	[P^{17}]h-orexin B 6−28	GLQGRLQRLLQ**P**SGNHAAGILTM	243 2.2	8.37 ± 1.33	0.41 ± 0.28	20.4

Table 1 (Continued)

no.	peptide	Sequence	mass (exp)	EC_{50} (μM) Ox_1 receptor	EC_{50} (μM) Ox_2 receptor	selectivity Ox_1/Ox_2
71	[P^{18}]h-orexin B 6−28	GLQGRLQRLLQA**P**GNHAAGILTM	241 6.3	2.69 ± 1.38	0.69 ± 0.24	3.9
72	[P^{19}]h-orexin B 6−28	GLQGRLQRLLQAS**P**NHAAGILTM	244 5.4	1.90 ± 1.56	1.03 ± 0.93	1.8
73	[P^{20}]h-orexin B 6−28	GLQGRLQRLLQASG**P**HAAGILTM	238 9.8	12.66 ± 0.09	0.59 ± 0.24	21.5
74	[P^{21}]h-orexin B 6−28	GLQGRLQRLLQASGN**P**AAGILTM	236 5.9	1.89 ± 1.25	0.84 ± 0.64	2.3
75	[P^{22}]h-orexin B 6−28	GLQGRLQRLLQASGNH**P**AGILTM	243 2.5	>1000	4.40 ± 2.00	>230
76	[P^{23}]h-orexin B 6−28	GLQGRLQRLLQASGNHA**P**GILTM	243 2.3	>1000	3.60 ± 0.55	>280
77	[P^{24}]h-orexin B 6−28	GLQGRLQRLLQASGNHAA**P**ILTM	244 6.7	>1000	9.43 ± 4.27	>105
78	[P^{25}]h-orexin B 6−28	GLQGRLQRLLQASGNHAAG**P**LTM	239 0.7	>1000	>1000	−
79	[P^{26}]h-orexin B 6−28	GLQGRLQRLLQASGNHAAGI**P**TM	239 0.7	8.77 ± 2.12	0.63 ± 0.31	13.9
80	[P^{27}]h-orexin B 6−28	GLQGRLQRLLQASGNHAAGIL**P**M	240 2.7	>1000	2.67 ± 1.26	>375
81	[P^{28}]h-orexin B 6−28	GLQGRLQRLLQASGNHAAGILT**P**	237 2.6	>1000	3.63 ± 2.44	>275
82	h-orexin B Ac-6−28	**Ac**-GLQGRLQRLLQASGNHAAGILTM	244 7.6	2.09 ± 1.26	0.59 ± 0.29	3.5

[a] Ac = acetyl; Ahx = 6-amino hexanoic acid; B = α-aminobutyric acid; U = pyroglutamic acid.

N-terminally shorted segments. Orexin A as well as reduced orexin A showed similar activity at the orexin receptors. Thus, we decided to use the reduced segment for further SAR studies. Removal of the first amino acid of orexin A (**10**) led to a Ox_1R preferring analogue with a 10-fold decrease in activity at the Ox_1R and a 40-fold reduced activity at the Ox_2R. Surprisingly, further shortening of orexin A up to orexin A 15−33 (**11**−**16**) led to a loss of Ox_1R preference. The activity of all truncated analogues up to orexin A 15−33 showed a 10-fold reduced activity at both orexin receptors. Shorter analogues (**16**−**21**) of orexin A led to a total loss of receptor interaction at both receptors. Dimers of hormones can bind with significantly higher affinities at receptors then the corresponding monomers.[18−20] Therefore, we synthesized a cysteine dimer (**22**) of orexin A 13−33, which showed a slightly better activity at the orexin receptors than orexin A 13−33. In a similar manner we got information on the relevance of the C-terminus of human orexin B (**34**−**38**). The most remarkable truncated peptide was orexin B 6−28 (**34**), because of its high activity at the orexin receptors, which was in the same range as full length orexin B. Further truncation of orexin B to generate orexin B 10−28 (**36**) created a highly selective agonist with a loss of activity at the Ox_1R but retaining high activity at the Ox_2R. With a selectivity of >1750 for Ox_2R over Ox_1R, this is the most selective agonist reported so far. In contrast, orexin B 8−28 (**35**) exhibited a 30-fold decreased activity at the Ox_1R and a 10-fold reduced activity at the Ox_2R. C-Terminal orexin B analogues shorter than 15 amino acids (**37**, **38**) are inactive at both orexin receptors.

L-Alanine Scan of Human Orexin B 6−28. The relevance of single amino acid residues at the orexin receptors was investigated by an L-alanine scan (shown as hatched bars in Figures 2 and 3). Therefore, we used orexin B 6−28 as the shortest orexin B segment with high activity at the Ox_1R and the Ox_2R. The replacement of single amino acids by L-alanine (**39**−**58**) resulted in a loss of activity at the Ox_1R for the amino acids Leu^{15}, Gly^{24}, Ile^{25}, Leu^{26}, and Thr^{27}, which are mainly located at the C-terminus. The only peptide that was not able to activate both orexin receptors was [Ala^{25}]-orexin B 6−28, which means that isoleucine at position 25 is highly important for binding or activating the orexin receptors. Surprisingly, the replacement of Thr^{27} with L-alanine, which is located at the conserved C-terminus, led to a nearly identical behavior at the Ox_2R and a loss of activity at the Ox_1R.

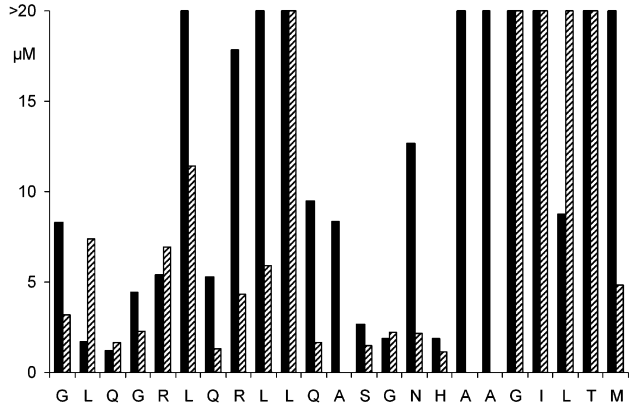

Figure 2. Activity of the human orexin B 6−28 analogues at the Ox_1R. Single amino acids of the original sequence (given at the *x*-axis) were replaced by L-alanine (hatched bars) or L-proline (black bars).

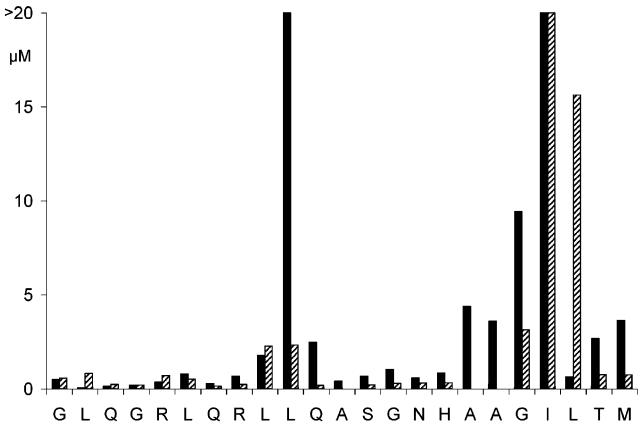

Figure 3. Activity of the human orexin B 6−28 analogues at the Ox_2R. Single amino acids of the original sequence (given at the *x*-axis) were replaced by L-alanine (hatched bars) or L-proline (black bars).

Taken together, the activity of the analogues was not strongly affected when the polar and uncharged amino acids were replaced with L-alanine, whereas exchanges of some of hydrophobic residues (Leu^{15}, Ile^{25}, Leu^{26}) had a strong impact on the activity.

Proline Scan of Human Orexin B 6−28. We also used the short orexin B analogue for studying the structural role of specific amino acids by replacements

with L-proline (**59**−**81**, shown as gray bars in Figures 2 and 3). Most of the peptides were active at the Ox_2R. Only the exchange of Ile^{25} resulted in a complete loss of activity at both receptors. Other amino acids that resulted in reduced activity at the Ox_1R after the replacement with L-proline were Leu^{11}, Leu^{14}, Leu^{15}, Ala^{22}, Ala^{23}, Gly^{24}, Ile^{25}, Thr^{27}, and Met^{28}. Most of these amino acids are located at the C-terminus, which means that the secondary structure element, an α-helix,[13] at this position seems to be essential for activating the Ox_1R. A further interesting replacement was found at Leu^7 (**60**), [P^7]orexin B 6−28 resulted in slightly better activation of the Ox_2R and a slightly reduced activity at the Ox_1R compared with the parent peptide. The analogues with replacement of Leu^{11} or Leu^{14} (**64**, **67**) showed a high activity at the Ox_2R and were inactive at the Ox_1R. Further replacements (Leu^{15}, Ala^{22}, Ala^{23}, Pro^{27}, and Met^{28}) resulted in a loss of activity at Ox_1R and a reduced activity at the Ox_2R.

In addition, we wanted to characterize the importance of the N-terminal amino group by N-terminal acetylation of human orexin B 6−28 (**82**). The activity to the Ox_1R and the Ox_2R was not significantly reduced, suggesting that a modification of the N-terminus does not influence binding at the orexin receptors.

Discussion

Orexin A is completely conserved in different species and displays the highest activity at the Ox_1R. To identify structures or amino acid side chains of orexin A that are necessary to activate the orexin receptors we investigated at first the relevance of the disulfide bonds. Because the reduced and the native orexin A showed the same activity, we suggest that the disulfide bonds are not required for binding to the orexin receptors and subsequent stimulation of intracellular calcium release. This is in contrast to previously reported results.[21] However, Okumura et al. replaced cysteines by L-alanines at different positions to achieve orexin A analogues with one or none disulfide bridge that showed reduced intracellular calcium mobilization and a very low gastric acid output. On the basis of this result they concluded that the disulfide bridges are necessary for a full activity. However, it remained open whether the L-alanine replacement of the disulfide bridge is responsible for the loss of activity. In contrast, we used analogues with reduced cysteine residues with the more bulky aminobutyric acid (Abu) at the relevant positions. The size of aminobutyric acid is similar to cysteine and Abu is frequently used to replace cysteine. Although we cannot completely exclude that <5% of the cysteine might be reoxidized under assay conditions, this cannot account for the full activity of the reduced orexin peptide. As the Abu analogue shows only slightly reduced activity, we conclude that the size or the electronic properties of the cysteine residues are essential for activating orexin receptors, but a connection between them is not necessary. This is further supported by the data obtained with centrally truncated peptides, which show reduced activity at the Ox_1R and the Ox_2R. Additionally, we observed that the length of the truncation influences activity. Whereas orexin A 1−5-Ahx-19−33 was inactive, orexin A 1−5-Ahx-15−33 maintained activation properties at both receptors. To further prove this theory, we removed stepwise the N-terminal amino acids of orexin A. Thereby we found the minimal sequence 15−33, which is necessary to activate the receptors. This nonadecapeptide is identical with the conserved C-terminus of orexins A and B and was first reported by Darker et al.[16] Most of the short analogues that activate orexin receptors displayed a drop in activity at both receptors and confirm previous results.[16] Surprisingly, the removal of the first amino acid of orexin A led to an increased activation of the Ox_1R compared to the Ox_2R. All other peptides preferred the Ox_2R. It was reported that dimerization of peptides led to significant increase in the affinity to receptors in the neuropeptide Y field.[18] The dimerized orexin A 13−33 activated the orexin receptors better than the monomer, but the effect was less pronounced than observed for other peptide receptor families.[19,20]

All orexin A analogues that miss the C-terminus were inactive at both receptors, confirming our previous data.[22]

Orexin B is characterized by a high degree of sequence homology across various species. Surprisingly, the replacement of two amino acids within the sequence of human and rodent orexin B did not resulte in a different activation behavior at the human orexin receptors. Furthermore, the C-terminal region of human orexin A and B is highly conserved. However, the substitution of the C-terminal amino acid of human orexin B with the C-terminal amino acid of orexin A resulted in a slight decrease in the activity at the Ox_2R, which indicates that the C-terminal amino acid is h ighly important for activating the Ox_2R. We could also show that the C-terminal amidation of orexin B is necessary for functional activity at both orexin receptors, because the peptide acid of human orexin B (**25**) showed no activity at the receptors. Additionally, we could demonstrate that the C-terminus is required for activating the orexin receptors, because all fragments only covering the N-terminal parts of the orexin B peptide turned out to be inactive at both receptors, whereas C-terminal fragments longer than 19 amino acids were active. An interesting short analogue was human orexin B 10−28 which was only active at the Ox_2R. Therefore, we suggest that only the segment orexin B 10−28 is in contact with the Ox_2R. Furthermore, we identified orexin B 6−28 to be the shortest analogue with high activity at both orexin receptors.

This short analogue was used to identify important positions for activating the orexin receptors by single amino acid replacement (shown in Figures 2 and 3). Most important the replacement of Ile^{25} with L-alanine and L-proline led to inactive analogues of orexin B 6−28 at both orexin receptors, as was previously reported for the full length orexin B.[17] Ile^{25} is located in the middle of the C-terminal α-helix,[13] which is proposed to play a key role for recognition and activation of orexin receptors. Therefore, it is possible that Ile^{25} is the anchor amino acid for maintaining the helical structure, binding, or activating the orexin receptors. The loss of activity at the Ox_1R during replacement of the amino acids Leu^{15}, Ala^{22}, Ala^{23}, Gly^{24}, and Thr^{27} with L-alanine and/or L-proline supports the suggestion of the impor-

tance of the C-terminal α-helix.[13] Similar result have been reported for the replacement of the C-terminal amino acids with the corresponding D-amino acids.[17] Furthermore, we observed a reduced activation of the Ox_2R in the C-terminal region after the exchange with L-proline and to a lesser extent with L-alanine. The results from the exchange with L-proline were not surprising, because L-proline induces turn structures and destroys helices. So we conclude that the C-terminal helix is necessary for binding or activating both receptors. We observed also a reduced activity at the Ox_1R by replacement of amino acids located in the first helix with L-proline. The replacement with L-alanine showed that mostly the polar and uncharged amino acids are not involved in activating both receptors. This might be of high importance for the rational design of small molecule ligands as peptidomimetic. In any case, hydrophobic interactions have to be strongly conserved. Additionally, we observed that the exchange of the N-terminal Leu^7 with L-proline (**60**) is tolerated but not with L-alanine (**40**). This might be due to the stabilization of a turn structure by L-proline or due to the reduced size of L-alanine that does not allow formation of the required hydrophobic ligand−receptor interaction. The exchange of amino acids within the segment 16−23 with L-alanine led to analogues without any loss of activity at the orexin receptors. This region contains the flexible linker of the two α-helixes as well as the end of the first α-helix and suggests that the backbone of this region is structurally important for functional activity, whereas the amino acid side chains are not important. Only the exchange of Asn^{20} with L-proline led to a highly reduced activity at the Ox_1R but not at the Ox_2R. The replacement of L-alanine at this position had no influence on the activation of the orexin receptors. This indicates that a turn structure within segment 20−23 might be favorable for the activation of the Ox_1R. We also could show that the N-terminal amino acids were not involved in binding and activating of the receptors. We also observed in general the Ox_1R to be more sensitive to amino acid replacements than the Ox_2R. Accordingly, the native conformation and amino acid side chains are important for the Ox_1R activation, and the ligand−receptor contact site seems to be larger.

Until now there was little known about the physiological role of the two orexin receptors because of the lack of selective ligands. Only orexin A, which prefers the Ox_1R with 10-fold selectivity, was used to discover the role of this receptor so far. Up to now only Ox_2R-preferring ligands (selectivity around 400-fold) were published.[17] We now identified analogues that activate the Ox_2R more than 1000-fold as efficient as the Ox_1R. Three of these analogues, orexin B 10−28, [A^{27}]orexin B 6−28, and [P^{11}]orexin B 6−28, showed only a slight decrease in activity at the Ox_2R, whereas replacement of Leu^{15}, Gly^{24}, and Leu^{26} with L-alanine as well as the exchange of Leu^{14}, Leu^{15}, Ala^{22}, Ala^{23}, Thr^{27}, and Met^{28} with L-proline led to a decrease in the activity at the Ox_2R as well. All of them were inactive at the Ox_1R up to 1 mM concentrations. The new selective ligands represented here can be used as novel tools for the investigation of the function and physiological role of each receptor subtype. Using labeled analogues it will be possible to match receptor expression on the protein level and finally assign the different activities to the different receptor subtypes.

Conclusion

To understand more about the interaction of orexins and orexin receptors we performed guided structure−activity relationship studies. We could confirm previous investigations that binding at orexin receptors requires a C-terminal sequence of at least 19 amino acids for orexin A and 15 amino acids for orexin B. High activity at the orexin receptors was reached for orexin A only with full length peptides. However, we found that reduced orexin A and orexin A with two disulfide bonds displayed the same activity. Moreover, we identified reduced orexin A 2−33 to be the first Ox_1R-preferring peptide. We found the shortest segment with high activity at the Ox_1R and the Ox_2R to be human orexin B 8−28. This analogue was used to screen for important amino acid residues and their structural role. Important amino acid residues for binding at the orexin receptors were placed in the C-terminal region as well as glycine, leucine, and arginine in the N-terminal region. The structural role of the amino acids was studied by replacements with L-proline, which induces turns. The analogues with replacements in the C-terminal region were inactive at the Ox_1R, whereas at the Ox_2R replacement led to a reduced activity. In the N-terminal region no difference was found for activating the Ox_1R and Ox_2R. On the basis of these studies, the first analogue with Ox_1R preference (orexin A 2−33) and three highly potent Ox_2R-selective peptides (orexin B 10−28, [A^{27}]-orexin B 6−28; [P^{11}]orexin B 6−28; selectivity for Ox_2R over Ox_1R >1000-fold) were identified and can be used for further investigations to study the physiological role of orexin receptor subtypes.

Experimental Section

Materials. For peptide synthesis the following were used: N^{α}-Fmoc-protected amino acids, 1-hydroxybenzotriazole (HOBt), Wang resin, and the 4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)phenoxy (Rink amide) resin purchased from Novabiochem (Germany, Schwalbach). The side chain protecting groups for the amino acids were *tert*-butyl (tBu) for Ser, Thr and Tyr; *tert*-butoxy (tBuO) for Asp and Glu; trityl (Trt) for Asn, Cys, Gln, and His; 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf) for Arg; and *tert*-butoxycarbonyl (Boc) for Lys. *N*,*N*′-diisopropylcarbodiimide (DIC) was obtained from Sigma-Aldrich (Germany, Taufkirchen). Trifluoroacetic acid (TFA), thioanisole, *p*-thiocresol, 1,2-ethanedithiol, hydrazine hydrate solution, trimethylsilyl bromide, *tert*-butyl alcohol, and piperidine were purchased from Fluka (Germany, Taufkirchen). Acetonitrile (ACN, for HPLC) was obtained from Merck (Germany, Darmstadt). Diethyl ether, dichloromethane, and *N*,*N*′-dimethylformamide (DMF, peptide synthesis grade) were obtained from Biosolve (Netherlands, Valkenswaard).

For cell culturing, the following media and supplements were used: Dulbecco's modified eagle medium (DMEM), phosphate-buffered saline (PBS), phosphate-buffered saline−ethylenediaminetetraacetic acid (PBS−EDTA), Hanks buffered salt solution (HBSS), fetal calf serum (FCS), glutamine, and Geneticin, obtained from Gibco Life Technologies (Germany, Karlsruhe). Bacitracin and bovine serum albumin were obtained from Sigma-Aldrich.

Fluorimetric imaging plate reader assay (FLIPR) was done with 384-well, poly-D-lysine-coated black-wall microtiter plates from TPP (Trassadingen, Switzerland). HEPES was purchased from Roth (Karsruhe, Germany). Probenecid and dimethyl sulfoxide (DMSO) were purchased from Sigma (Germany,

Taufkirchen). Fluo-4-AM and pluronic acid were from Molecular Probes (Leiden, Nederland).

Peptide Synthesis. The orexin A and B analogues were synthesized by automated multiple solid-phase peptide synthesis (Syro, MultiSynTech, Bochum, Germany) using the Wang resin to obtain a peptide acid or the Rink amide resin, to obtain a peptide amide (30 mg, resin loading 0.6 mmol/g). The Fmoc-amino acids (10-fold excess) were introduced by double coupling procedures (2 × 36 min) using in situ activation with DIC and HOBt. The Fmoc removal was carried out with 40% piperidine in DMF for 3 min, 20% piperidine for 7 min and finally 40% piperidine for 5 min. Peptides which containing methionine or cysteine were cleaved from the resin by adding a mixture of trifluoroacetic acid/thioanisol/ethandithiol (90:7:3, v/v) for 3 h. All other peptides were cleaved by using a mixture of trifluoroacetic acid/thioanisol/thiocresol (90:5:5, v/v) for 3 h. The peptides were precipitated from ice-cold diethyl ether, collected by centrifugation and washed four times. Then they were dissolved in *tert*-butyl alcohol/water (1:3, w/w) and lyophilized. Partially oxidized methionines were reduced after lyophilization by dissolving the peptide in 1 mL trifluoroacetic acid, followed by addition of 15.7 μL ethanedithiol and 13 μL trimethylsilylbromide. The solution was shaken for at least 20 min.[23] The reduced peptides were precipitated from ice-cold ether, washed as described above and finally lyophilized.

Purification of the peptides was achieved by preparative HPLC on a RP C-18 column (Waters, 300 × 25 mm, 5 μm) with a gradient of 20−60% B in A (A = 0.1% trifluoroacetic acid in water; B = 0.08% trifluoroacetic acid in acetonitrile) over 45 min and a flow of 15 mL/min. The synthesis of the oxidized orexin A analogues was performed as described previously.[24] Homogeneity of all peptides was >95% accordingly to analytical HPLC. Retention times are given in the Supporting Information.

Dimerization of orexin A 13−33 and simultaneous cleavage of potential trifluoroacetic acid esters was achieved by dissolving 8 mg (3.6 μmol) of the peptide in 1 mL of methanol/piperidine (9:1, v/v). After 1 h of shaking at room temperature, the peptide was precipitated from diethyl ether, dissolved in 50 μL of trifluoroacetic acid, precipitated from diethyl ether again, dissolved in water, and lyophilized. Purification of the dimerized peptide was carried out as described above.

The peptides were analyzed by matrix-assisted laser desorption ionization (MALDI) mass spectrometry on an Voyager-DE RP workstation (Applied Biosystems, Darmstadt, Germany), by analytical reversed-phase HPLC on a Vydac RP18-column (4.6 × 250 mm; 5 μm, 300 Å) using linear gradients of 10−60% B in A over 30 min and a flow rate of 0.6 mL/min and on a Phase Sep RP8-column (4.6 × 250 mm; 5 μm, 300 Å) using linear gradients of 30−80% B in A over 30 min and a flow rate of 0.6 mL/min. Found masses were in full agreement with calculated masses and are listed in Table 1.

Cell Culture. HEK293 cells stably transfected with human Ox_1R and Ox_2R were grown in DMEM supplemented with 10% fetal calf serum, 1% glutamine, and 250 mg Geneticin G418 in a 5% CO_2, humified atmosphere (95%) at 37 °C.

Calcium Mobilization Assays. HEK293 cells stably expressing human Ox_1R and Ox_2R were washed once with phosphate-buffered saline and detached with PBS−EDTA buffer. Afterward, cells were washed once with assay buffer [Hanks Balanced Salts Solution (HBSS), 10 mM HEPES, 200 mM Ca^{2+}, 0.1% BSA, 2.5 mM probenecid] and then batch-loaded for 1 h with 1 mM Fluo-4-AM fluorescent indicator dye in assay buffer. Finally, cells were seeded (50 000 cells/well) into poly-D-lysine-coated 384-well black-well, clear-bottom microtiter plates and then returned to the incubator for 10 min before assay on a T-channel fluorometric imaging plate reader (FLIPR, Molecular Devices, Wokingham, U.K.). Ligands, appropriately diluted in assay buffer, were added and the maximum change in fluorescence over baseline was used to determine agonist response.

Each trial was performed as three independent experiments in quadruplicate. Data analysis and curve fitting were conducted using the Xlfit program (IDBS, Guilford, UK). The resulting EC_{50} ± SD (standard deviation) are shown in Table 1.

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Supporting Information Available: Table of chemical data of synthesized peptides. This material is available free of charge via the Internet at http://pubs.acs.org.

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