Journal of Peptide Science
J. Peptide Sci. 2006; **12**: 258–266
Published online 2 September 2005 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/psc.716

JOURNAL OF PEPTIDE SCIENCE

Structural properties of orexins for activation of their receptors

MANJA LANG,[a] BERND BUFE,[b] SILVIA DE POL,[c] OLIVER REISER,[c] WOLFGANG MEYERHOF[b] and ANNETTE G. BECK-SICKINGER[a]*

[a] Institute of Biochemistry, University of Leipzig, 04103 Leipzig, Germany
[b] Department of Molecular Genetics, German Institute of Human Nutrition Potsdam-Rehbruecke, 14558 Nuthetal, Germany
[c] Institute of Organic Chemistry, University of Regensburg, 93040 Regensburg, Germany

Received 24 June 2005; Accepted 12 July 2005

Abstract: The closely related neuropeptides orexin A and orexin B mediate their actions, including the regulation of sleep and appetite, by the activation of the orexin 1 and 2 receptors. To elucidate the structural prerequisites for receptor activation and subtype selectivity, we performed multiple amino acid substitutions within the sequence of orexin A and human orexin B-(6-28)-peptide and analyzed their solution structures by CD spectroscopy and their activity at both receptors in Ca^{2+} mobilization assays. For orexin A, we showed that the basic amino acids within the segment of residues 6–14 were important for the activation of both receptors. Furthermore, we showed that the restriction via disulfide bonds is not required to maintain the active structure of orexin A. The kink region of h orexin B has been shown to be important for Ox_2R selectivity, which is not mediated by the restriction of the turn structure. Additionally, we showed that no particular secondary structure is required for receptor subtype selectivity. Copyright © 2005 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: orexin; hypocretin; structure-activity relationship; CD; β-aminocyclopropane carboxylic acids

INTRODUCTION

Neuropeptides regulate many physiological and behavioral functions by the interaction with their receptors. Mutations in receptors, changes in receptor expression or availabilities of peptide agonists may cause diseases. The disabling sleep disorder narcolepsy, characterized by daytime sleepiness, cataplexy, and stinging transitions from wakefulness into rapid-eye-movement (REM) sleep, is associated with orexin deficiency [1–3]. A phenotype similar to human narcolepsy was observed in orexin gene-targeted mice, suggesting that orexins have an important function in the regulation of sleep [4]. Further investigations in the autosomal recessive canine narcolepsy model identified mutations within the gene of the orexin 2 receptor (Ox_2R), which result in narcolepsy [5]. Mutations in the human Ox_2R gene were recently also found in narcolepsy patients [6]. Moreover, orexins have been implicated in several other physiological functions such as regulation of feeding behavior [7,8] and neuroendocrine function [9,10]. Thus, the orexins and their receptors are a promising target for the identification of new therapeutics.

The orexin system, consisting of the peptides orexin A and B (Figure 1) and the G-protein coupled receptors orexin 1 and orexin 2 receptor (Ox_1R and Ox_2R) [11], is distributed in the lateral hypothalamus, gastrointestinal tract and in the endocrine organs (for review see [12]). The orexin receptors are highly conserved across mammalian species, and human Ox_1R and Ox_2R show an overall sequence identity of 64%, with weak conservation of the extracellular *N*-terminal domain, the intracellular loop III and the *C*-terminal tail [11]. Activation of recombinantly expressed receptors and native neurons elevate the Ca^{2+} level via the G_q family of G-proteins [11,13].

The orexin peptides derive from a single mRNA encoding the 131 residue precursor prepro-orexin [11,14]. Posttranslational processing of the single precursor prepro-orexin generates both orexin A and orexin B, that share 46% sequence identity, with the *C*-terminus being well conserved [15]. The 33 amino acid peptide amide orexin A consists of a constrained structure at the *N*-terminus that is formed by two intramolecular disulfide bonds connecting residues 6 to 12 and 7 to 14 and activates both orexin receptors equally [11]. In contrast, the linear 28 residue peptide amide orexin B shows an approximately 10-fold decreased activity at the Ox_1R, but is equally potent at the Ox_2R [11]. However, the potency of both peptides is moderate compared to other neuropeptides that bind to GPCRs. The possible reason for the poor discrimination of both receptors between the orexin peptides was recently identified by the elucidation of the secondary structure by NMR spectroscopy. Both peptides consist of two related α-helices that are connected by a flexible bend. For orexin B, the α-helices range from residues Leu^7 to Ser^{18} and Ala^{22} to Met^{28} [16,17] and for orexin A from residues Cys^{14} to His^{21} and from Asn^{25} to Leu^{31} [18]. Additionally, orexin A consists of a rigid turn between residues Arg^8 and Thr^{11}.

Beside the structure, there are very few data available regarding the structure-activity relationship

*Correspondence to: A. G. Beck-Sickinger, Institute of Biochemistry, Bruederstraße 34, D-04103 Leipzig, Germany;
e-mail: beck-sickinger@uni-leipzig.de

Figure 1 Comparison of the amino acid sequences of human orexin A and B. Similarities are highlighted in grey.

of orexin peptides with their receptors. The minimal sequences required for receptor activation are *C*-terminal segments of at least 19 amino acids for orexin A [19,20] and 21 amino acids for orexin B [19], though the functional activity of these peptides is reduced. The same was observed for orexin A with replacements of the central residues 6–14 by linker moieties [19]. Preservation of full orexin A activity requires the complete amino acid sequence and only the disulfide bonds can be disconnected without loss of activity. Additionally, the replacement of each amino acid of orexin A-(15–33)-peptide by Ala showed that residues 16, 19, 20, 26 and 29–33 are important for the interaction with the Ox_1R [20,21]. Similar investigations of h orexin B [22,23] and h orexin B-(6–28)-peptide [19] showed that important residues for activity at both receptors are to be located in the *C*-terminal region as well as Leu^{11}, Leu^{14} and Leu^{15}. These investigations additionally led to the first Ox_2R preferring agonists by replacing central and *C*-terminal residues by Ala, Pro or D-amino acids [19,23]. Furthermore, there are few nonpeptidic Ox_1R and Ox_2R selective antagonists available [24–27] that may be used to elucidate the physiological roles of the orexin receptors. However, the conformation and residues involved in receptor subtype selectivity are not known.

The work presented here was performed in order to identify the conformation and residues of orexin A required to obtain high activity at the Ox_1R and Ox_2R. Therefore, we replaced the rigid segment of residues 6–14 of orexin A by spacer building blocks and conformationally constrained β-aminocyclopropane carboxylic acids (β-ACC, Figure 2, [28]), respectively. Additionally, we exchanged all Cys by Abu to clearly demonstrate that the disulfide bonds are not required for full activity. Furthermore, we analyzed the prerequisites of Ox_2R selectivity by multiple amino acid replacements within the sequence of h orexin B-(6–28)-peptide and the predicted turn structure of h orexin B by incorporation of β-ACC moieties at various positions.

All analogs were analyzed for their ability to activate the Ox_1R and the Ox_2R, respectively, by recording changes of the intracellular Ca^{2+} levels in transfected cells using the fluorometric imaging technology. CD studies have been performed in order to correlate different activities with changes of the peptide conformation.

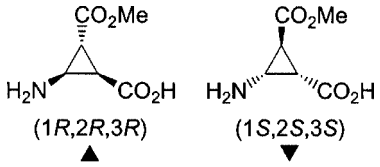

Figure 2 Structures of the substituted β-aminocyclopropane carboxylic acids employed.

EXPERIMENTAL SECTION

Materials

The N^{α}-Fmoc-protected amino acids, HOBt, TBTU and the 4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxy (Rink amide) resin were purchased from Novabiochem (Germany, Schwalbach). The side chain protecting groups were: Bu^t for Ser, Tyr, Thr, Asp and Glu; Trt for Asn, Gln, Cys and His; 2,2,4,6,7-pentamethyldihydrobenzofurane-5-sulfonyl (Pbf) for Arg and Boc for Lys. DIC and TFE were obtained from Sigma-Aldrich (Germany, Taufkirchen). TFA, 1-methyl-2-pyrrolidone, DIEA, thioanisole, *p*-thiocresol, 1,2-ethanedithiol, trimethylsilylbromide, *t*-butanol and piperidine were purchased from Fluka (Germany, Taufkirchen). MeCN for HPLC was obtained from Merck (Germany, Darmstadt). Diethyl ether, DCM and 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), PBS-EDTA, sodium pyruvate, nonessential amino acids, fetal calf serum (FCS) and glutamine were obtained from Invitrogen (Germany, Karlsruhe). BSA was obtained from Sigma-Aldrich. G418 was purchased from Biochrom (Germany, Berlin).

Peptide Synthesis

The peptides were synthesized by solid-phase technique on an automated multiple peptide synthesizer (Syro, MultiSynTech, Bochum, Germany) by using the Rink amide resin (30 mg, resin loading 0.6 mmol/g) as described recently [19]. The diastereomers ((▼) or (▲)-configured) of β-aminocyclopropane carboxylic acids (β-ACC) were synthesized with the following amino acid as diastereomer building blocks (Fmoc-Asp(*t*BuO)-β-ACC-OH, Fmoc-Gly-β-ACC-OH, Fmoc-Ala-β-ACC-OH) according to the general procedure described recently [29,30] and coupled manually as dipeptide building block after TBTU activation into the growing peptide chain as described by Koglin *et al.* [31].

After complete modification, all peptides were cleaved from the resin in one step by using TFA, precipitated with ice-cold diethyl ether, washed and finally lyophilized [19]. Partially oxidized Met were reduced after lyophilization by applying a mixture of TFA/ethanedithiol/trimethylbromosilane (97.2 : 1.6 : 1.2, v/v/v) for at least 20 min [32]. Subsequently, the peptides were recovered from ice-cold diethyl ether, washed and finally lyophilized.

Purification of the peptides was achieved by preparative HPLC on an RP C-18 column (Vydac, 250 × 25 mm, 10 μm) with a gradient of 20–60% B in A (A = 0.1% TFA in water; B = 0.08% TFA in MeCN) over 60 min and a flow of 10 mL/min (λ = 220 nm).

The peptides were analyzed by MALDI mass spectrometry on an Voyager-DE RP workstation (Applied Biosystems, Darmstadt, Germany) and by analytical reversed-phase HPLC on a

Vydac RP C-18 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 ($\lambda = 220$ nm). Found masses were in full agreement with calculated masses (Table 1) and purity of all peptides was >95% according to analytical HPLC (Data not shown).

CD Spectroscopy

CD spectra of 40 μM orexin peptides were measured in 10 mM phosphate buffer (pH 7.4) containing 30% TFE using a JASCO J-715 spectrapolarimeter. CD spectra were recorded from 250 to 190 nm at 20 °C in nitrogen atmosphere by a scanning rate of 20 nm/min with a time constant of 2 s. Each CD spectrum was obtained from an average of three recordings with a step resolution of 0.1 nm, a sensitivity range of 10 mdeg and a bandwidth of 2 nm in sample cells with a path length of 2 mm. High-frequency noise was reduced by means of a low-path Fourier-transform filter. The CD spectra of the pure solvent was subtracted from the peptide solution to eliminate interference from cell, solvent and optical equipment. The CD data are given as mean residue molar ellipticities $[\Theta]_R$ in deg cm^2 $dmol^{-1}$.

Cell Culture and Transfection

The human orexin receptor cDNAs (Ox_1R, Ox_2R) in pcDNA3 were kindly provided by Dr F. M. Dautzenberg, Axovan Ltd. Innovation Center. To obtain stable cell lines, HEK293 Flip-in cells (Invitrogen) were transfected with linearized vector. Clones were selected using 800 ng/μL G418. For each receptor, about 15 single cell foci were collected and separately grown to obtain individual cell lines. The best responding cell line was selected by monitoring the calcium response of the cells to orexin. The selected cell lines were maintained in DMEM supplemented with 10% fetal calf serum, 2 mM glutamine and 200 ng/μL G418 at 37 °C in a humidified atmosphere (95%) and 5% CO_2.

Calcium Imaging

The agonistic potency of orexin analogs was examined by calcium imaging experiments using an automated fluorometric imaging plate reader (FLIPR-Tetra, Molecular Devices, Germany, Munich). HEK293 cells stably expressing the human Ox_1R and Ox_2R, respectively, were seeded at ~30% confluency in 96-well black-wall, clear bottom microtiter plates (Molecular Devices) coated with poly-D-lysine (10 μg/mL). After 24–48 h, the cells were loaded for 1 h with 1 mM calcium-sensitive dye Fluo4-AM (Molecular Probes, Germany, Karlsruhe) in serum-free DMEM. Prior to recording, cells were washed three times with an automated plate washer (Denley Cellwash, Labsystems) using C1 buffer (10 mM HEPES, 130 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 10 mM glucose, pH 7.4). Ligands were appropriately diluted (10^{-4}–10^{-10} M) in C1 before measuring. Calcium signals were recorded as described previously [33]. Each experiment was performed at least three times independently in duplicate. Data analysis was performed with ScreenWorks (Molecular Devices). Data were fitted by nonlinear regression using the Prism program (GraphPad software Inc., USA, San Diego) to obtain EC_{50} values. The resulting values are given in Table 1 as mean ± s.e.mean.

RESULTS

Peptide synthesis. All analogs (Table 1) were prepared by automated solid-phase peptide synthesis on a Rink amide resin using the orthogonal Fmoc/Bu^t strategy with *in situ* activation by DIC/HOBt, except that β-ACC residues were introduced manually as dipeptide building blocks into the growing peptide chain of analogs nos. 9, 10, 25–30. All peptides were identified by MALDI mass spectrometry (Table 1) and analyzed by HPLC. An additional purification step was included to obtain products with >95% purity. Partially oxidized Met was successfully reduced by applying trimethylbromosilane in the presence of ethanedithiol and TFA.

Activity of orexin A analogs. The ability of orexin analogs to activate both receptors was measured by observing the transient mobilization of intracellular Ca^{2+} in HEK293 cells, stably transfected with Ox_1R and Ox_2R, respectively. The concentrations of orexin A (no. 1) and h orexin B (no. 11) required to induce half-maximal responses (EC_{50}) were in the same range as shown previously [11,19].

Substitutions within the segment of residues 6–14 of orexin A were investigated in order to identify prerequisites for orexin receptor activity (nos. 3–10). Previously, we suggested that activity of orexin A at both receptors does not require the *N*-terminal disulfide bonds. However, our conclusions were restricted because of the fact that we analyzed only reduced 33mer orexin A, which consists of unmodified Cys-residues, and exchanged only either $Cys^{6,12}$ or $Cys^{7,14}$ by the isosteric Abu residue [19]. In order to clearly demonstrate that neither any disulfide bond nor a Cys-residue is required for activity of orexin A at both receptors, we exchanged all Cys by the isosteric Abu residue (no. 3) in the present study. The activity of peptide no. 3 was approximately decreased fivefold at both receptors compared to native orexin A that showed at the Ox_1R an EC_{50} of 0.24 μM and at the Ox_2R of 0.06 μM. But it was comparable to that of reduced orexin A (no. 2) at the Ox_1R and threefold more potent at the Ox_2R. This indicates that the Cys-residues are not required for receptor activity and suggests that a rigid structure in this segment seems to be necessary to maintain high activity. Therefore, we replaced the Cys-containing segment of residues 6–14 by turn-inducing residues. Previously, we have shown that both the applied spacer building blocks, Pro-Gly and 6-aminohexanoic acid (Ahx), result in about 55-fold decreased activity at the Ox_1R and tenfold decreased activity at the Ox_2R [19]. To improve the activity, we now elongated the turn-inducing spacer Pro-Gly by each of the originally occurring residues in the natural segment: Arg, Gln, Lys and Thr, respectively (nos. 4–7). All modifications resulted in increased activity at the Ox_1R and almost in the same activity at the Ox_2R compared to the Pro-Gly spacer, but the activity was

Table 1 Chemical and biological data of synthesized peptides

No.	Peptide	Sequence	Mass		EC_{50} [μM]	
			calc.	exp.	Ox_1 receptor	Ox_2 receptor
1	Orexin A	Glp-PLPDCCRQKTCSCRLYELLHGAGNHAAGILTL	3562.2	3562.2	0.24 ± 0.02	0.06 ± 0.03
2	Orexin A (reduced)	Glp-PLPDCCRQKTCSCRLYELLHGAGNHAAGILTL	3566.2	3567.0	0.91 ± 0.29	1.72 ± 0.85
3	[$Abu^{6,7,12,14}$]-orexin A	Glp-PLPD-**Abu**-**Abu**-RQKT-**Abu**-S-**Abu**-RLYELLHGAGNHAAGILTL	3495.1	3494.1	0.98 ± 0.21	0.47 ± 0.44
4	Orexin A-(1–5)-Pro-Arg-Gly-(15–33)-peptide	Glp-PLPD**PRG**RLYELLHGAGNHAAGILTL	2863.2	2863.2	2.02 ± 0.70	1.70 ± 1.57
5	Orexin A-(1–5)-Pro-Gln-Gly-(15–33)-peptide	Glp-PLPD**PQG**RLYELLHGAGNHAAGILTL	2835.3	2838.3	3.41 ± 1.71	1.55 ± 0.95
6	Orexin A-(1–5)-Pro-Lys-Gly-(15–33)-peptide	Glp-PLPD**PKG**RLYELLHGAGNHAAGILTL	2835.3	2835.3	1.40 ± 0.81	0.50 ± 0.21
7	Orexin A-(1–5)-Pro-Thr-Gly-(15–33)-peptide	Glp-PLPD**PTG**RLYELLHGAGNHAAGILTL	2808.2	2808.3	5.62 ± 1.57	2.13 ± 1.88
8	Orexin A-(1–5)-Ala-Aib-(15–33)-peptide	Glp-PLPD**A-Aib-**RLYELLHGAGNHAAGILTL	2710.0	2710.1	13.78 ± 7.02	3.63 ± 3.45
9	Orexin A-(1–5)-▲-(15–33)-peptide	Glp-PLPD▲RLYELLHGAGNHAAGILTL	2694.3	2693.1	>100	>100
10	Orexin A-(1–5)-▼-(15–33)-peptide	Glp-PLPD▼RLYELLHGAGNHAAGILTL	2694.3	2693.5	4.63 ± 3.40	1.84 ± 1.59
11	h orexin B	RSGPPGLQGRLQRLLQASGNHAAGILTM	2897.6	2899.4	0.80 ± 0.17	0.13 ± 0.09
12	h orexin B-(6–28)-peptide	GLQGRLQRLLQASGNHAAGILTM	2404.8	2404.9	0.34[a]	0.13[a]
13	[S^{11},A^{27}]-h orexin B-(6–28)-peptide	GLQGR**S**QRLLQASGNHAAGIL**A**M	2348.7	2348.6	>100	>100
14	[P^{11},A^{27}]-h orexin B-(6–28)-peptide	GLQGR**P**QRLLQASGNHAAGIL**A**M	2358.8	2349.0	>100	>100
15	[P^{11},A^{27},L^{28}]-h orexin B-(6–28)-peptide	GLQGR**P**QRLLQASGNHAAGIL**AL**	2340.8	2340.4	>100	>100
16	[P^{11},A^{15},A^{27}]-h orexin B-(6–28)-peptide	GLQGR**P**QRL**A**QASGNHAAGIL**A**M	2316.7	2317.2	>100	>100
17	[P^{11},A^{15},P^{27}]-h orexin B-(6–28)-peptide	GLQGR**P**QRL**A**QASGNHAAGIL**P**M	2342.7	2343.0	>100	>100
18	[P^{11},I^{15},A^{27}]-h orexin B-(6–28)-peptide	GLQGR**P**QRL**I**QASGNHAAGIL**A**M	2358.8	2358.9	>100	13.94 ± 8.03
19	[P^{11},P^{27}]-h orexin B-(6–28)-peptide	GLQGR**P**QRLLQASGNHAAGIL**P**M	2384.8	2385.0	>100	43.74 ± 25.35
20	[P^{14},A^{27}]-h orexin B-(6–28)-peptide	GLQGRLQR**P**LQASGNHAAGIL**A**M	2358.8	2359.1	>100	10.59 ± 8.68
21	[P^{7},A^{27}]-h orexin B-(6–28)-peptide	G**P**QGRLQRLLQASGNHAAGIL**P**M	2384.8	2384.2	43.49 ± 26.12	2.26 ± 1.56
22	[D^{21},P^{23}]-h orexin B-(6–28)-peptide	GLQGRLQRLLQASGN**D**A**P**GILTM	2408.8	2409.2	>100	1.65 ± 0.94
23	[L^{21},P^{23}]-h orexin B-(6–28)-peptide	GLQGRLQRLLQASGN**L**A**P**GILTM	2406.9	2408.3	>100	3.63 ± 1.88
24	[▲^{20}]-h orexin B-(6–28)-peptide	GLQGRLQRLLQASG▲HAAGILTM	2408.8	2408.7	15.45 ± 6.81	0.86 ± 0.28
25	[▼^{20}]-h orexin B-(6–28)-peptide	GLQGRLQRLLQASG▼HAAGILTM	2408.8	2408.7	>100	1.24 ± 0.55
26	[A^{20},▲^{21}]-h orexin B-(6–28)-peptide	GLQGRLQRLLQASG**A**▲AAGILTM	2365.8	2366.0	>100	1.87 ± 1.20
27	[A^{20},▼^{21}]-h orexin B-(6–28)-peptide	GLQGRLQRLLQASG**A**▼AAGILTM	2365.8	2367.9	>100	2.29 ± 0.52
28	[A^{21},▲^{22}]-h orexin B-(6–28)-peptide	GLQGRLQRLLQASGN**A**▲AGILTM	2431.8	2431.7	>100	26.28 ± 11.14
29	[A^{21},▼^{22}]-h orexin B-(6–28)-peptide	GLQGRLQRLLQASGN**A**▼AGILTM	2431.8	2431.9	>100	8.96 ± 3.73
30	[H^{16},G^{17},A^{18}]-h orexin B-(6–28)-peptide	GLQGRLQRLL**HGA**GNHAAGILTM	2383.8	2384.4	1.09 ± 0.58	0.23 ± 0.08

[a] In accordance with previous results [19]; ▼ = (1*S*, 2*S*, 3*S*) and ▲ = (1*R*, 2*R*, 3*R*) substituted *β*-aminocyclopropane carboxylic acids, see Figure 2 for structures.

still decreased compared to orexin A (no. 1). The most potent spacer contained the basic amino acid Lys (no. 6) with only sixfold decreased activity at the Ox_1R and eightfold at the Ox_2R, followed by Arg (no. 4, eightfold at the Ox_1R and 28-fold at the Ox_2R), Gln (no. 5, 14-fold at the Ox_1R and 26-fold at the Ox_2R) and Thr (no. 7, 23-fold at the Ox_1R and 35-fold at the Ox_2R). To increase the turn-formatting tendency, we replaced the segment 6–14 of orexin A by the Ala–Aib dipeptide (no. 8). This analog displayed the same activity at both receptors as previous substitutions with Ahx and Pro-Gly spacers, with a 50-fold decrease. Furthermore, we investigated the conformational requirements within this segment by the conformationally constrained β-ACC moieties, which have been recently shown to stabilize peptide structures [31,34]. Testing of the analogs orexin A-(1–5)-▲-(15–33)-peptide (no. 9) and orexin A-(1–5)-▼-(15–33)-peptide (no. 10) revealed that the configuration of the β-ACC residue had a strong effect on activity. Only the analog with the (▼)-configured residue was active at both receptors, with 20-fold decreased activity at the Ox_1R and 30-fold decreased activity at the Ox_2R. In contrast, the analog with the (▲)-configured β-ACC residue was devoid of any detectable orexin receptor activity (EC_{50} >100 μM).

Receptor activity of h orexin B-(6–28)-peptide analogs. Recently, it was shown that substitutions of central and *C*-terminal residues within h orexin B led to Ox_2R selectivity [19,23]. To get further information about the differences in activating orexin receptors, we performed multiple amino acid exchanges within the sequence of the high-affinity analog h orexin B-(6–28)-peptide (nos. 13–21). The combined substitutions of Thr^{27} by Ala and Leu^{11} by Ser (no. 13) or Pro (no. 14) led to a complete loss of activity at both receptors. The same was observed for analogs with an additional substitution of position 28 by Leu (no. 15) and position 15 by Ala (no. 16). Also the analog [P^{11},A^{15},P^{27}]-h orexin B-(6–28)-peptide (no. 17) was devoid of any activity at both receptors up to the tested concentration. In contrast, the analogs [P^{11},I^{15},A^{27}]-h orexin B-(6–28)-peptide (no. 18), [P^{11},A^{27}]-h orexin B-(6–28)-peptide (no. 19) and [P^{14},A^{27}]-h orexin B-(6–28)-peptide (no. 20) showed Ox_2R selectivity, however, the activity was above 80-fold decreased. Owing to the poor Ox_2R selectivity of multiple amino acid exchanges in the central and *C*-terminal region of h orexin B, we suggested that a substitution within the *N*-terminal region (this was shown to lead to high active analogs at the Ox_2R and poor at the Ox_1R [19,23]) in combination with an amino acid exchange at the *C*-terminus would result in highly active Ox_2R selective peptides. But surprisingly, the analog [P^{7},A^{27}]-h orexin B-(6–28)-peptide (no. 21) displayed activity at both receptors (43.49 μM at the Ox_1R and 2.26 μM at the Ox_2R).

Frequently binding to and activation of GPCRs is obtained by turn regions of the peptide ligands [35]. Orexin B possesses a turn that spans residues 20–22 [17]. This turn may be involved in the discrimination between the orexin receptors by direct interaction or stabilization of a distinct secondary structure. To examine the contribution of the turn to receptor activity, we replaced amino acids in the kink region (nos. 22–29). The peptides [D^{21},P^{23}]-h orexin B-(6–28)-peptide (no. 22) and [L^{21},P^{23}]-h orexin B-(6–28)-peptide (no. 23) showed selectivity for the Ox_2R with 10- to 30-fold decreased potency compared to the natural peptide. In order to rigidify the peptide backbone and to induce or stabilize the secondary structure within this region, we applied β-ACC building blocks. The absolute configuration of the β-ACC derivative had only minor influence on Ox_2R activity at all investigated positions (nos. 24–29). However, the position of the β-ACC residue determined the level of activity. Position 20 was the most potent one with a tenfold decrease of activity, followed by position 21 with 15-fold decreased activity. Position 22 was more sensitive to exchange by β-ACC building blocks with a 70-fold decrease for the (▲)-configured residue and 200-fold decrease for the (▼)-configured residue. At the Ox_1R only [$▲^{20}$]-h orexin B-(6–28)-peptide (no. 28) was active (15.45 μM), while all other analogs showed no activity at concentrations up to 100 μM.

Furthermore, we investigated the role of residues 16–18 of h orexin B for receptor selectivity. These three amino acids are different between orexin A and h orexin B, although this segment belongs to the conserved region. By the exchange of all three residues of h orexin B-(6–28)-peptide by the corresponding amino acids of orexin A (no. 30), the activity was still similar to h orexin B at both receptors.

Structure analysis. The solution structure of orexin analogs was investigated by CD spectroscopy in phosphate buffer at neutral pH supplemented with 30% of the membrane mimicking solvent TFE. The CD spectra of all orexin A analogs displayed a strong positive band at 192 nm, a negative band at 205 or rather 207 nm and a shoulder at 220 nm (Figure 3). According to the intensity of the bands and the wavelength of the negative band, we classified the analogs into two groups. The first group consists of natural orexin A (no. 1) and all centrally truncated analogs (nos. 4–10), which display a maximal positive Cotton effect of about 17 000 deg cm^2 $dmol^{-1}$ and a negative cotton effect at 205 nm of about 12 500 deg cm^2 $dmol^{-1}$ (Figure 3a). The second group includes the full-length analogs without any disulfide bond (nos. 2, 3) and is different from the first group by a 1.5-fold higher Cotton effect along with a red shift of the negative band to 207 nm (Figure 3b). Analysis of the spectra by a secondary structure estimating program (JASCO, J-700 for Windows) based on the method of Yang *et al.* [36] confirmed this classification, except for analog no. 9. The first group is characterized by a lower content of α-helical (23%) and higher

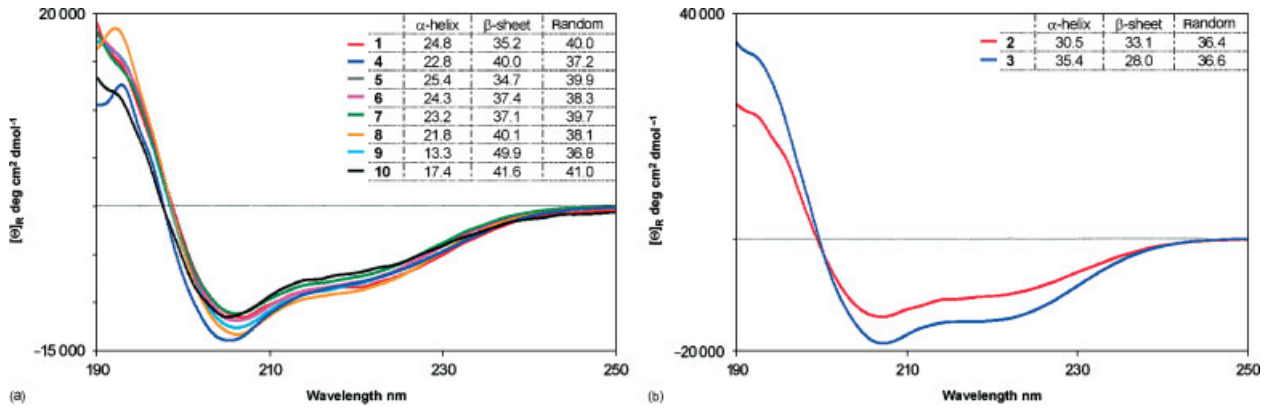

Figure 3 CD spectra and the percentage distribution of secondary structure elements of orexin A analogs according to the calculation of Yang *et al.* [36]. (a) orexin A and centrally truncated orexin A analogs. (b) full-length orexin A analogs without disulfide bonds.

β-sheet structure (38%) compared to the second group with 33% α-helix and 31% β-sheet (Figure 3). Additionally, we identified that the inactive analog orexin A-(1–5)-▲-(15–33)-peptide (no. 9) consists of a totally different pattern of secondary structure (13% α-helix, 50% β-sheet, 37% random structure).

The h orexin B analogs adopted a distinct secondary structure in correlation with their biological activity (Figure 4). All analogs with activity at both receptors (nos. 11, 12, 21, 24, 30) and the Ox_2R selective analog [▲^{20}]-h orexin B-(6–28)-peptide (no. 25) displayed CD spectra similar to the second group of orexin A analogs (Figure 4a). A similar shape of CD spectra, but approximately 1.6-fold decreased molar ellipticity, was also recorded for analogs with amino acid exchanges in the kink region that showed Ox_2R selectivity with activity below 10 μM (nos. 22, 23, 27, 29, Figure 4b). Particularly [A^{20},▲^{21}]-h orexin B-(6–28)-peptide (no. 26)

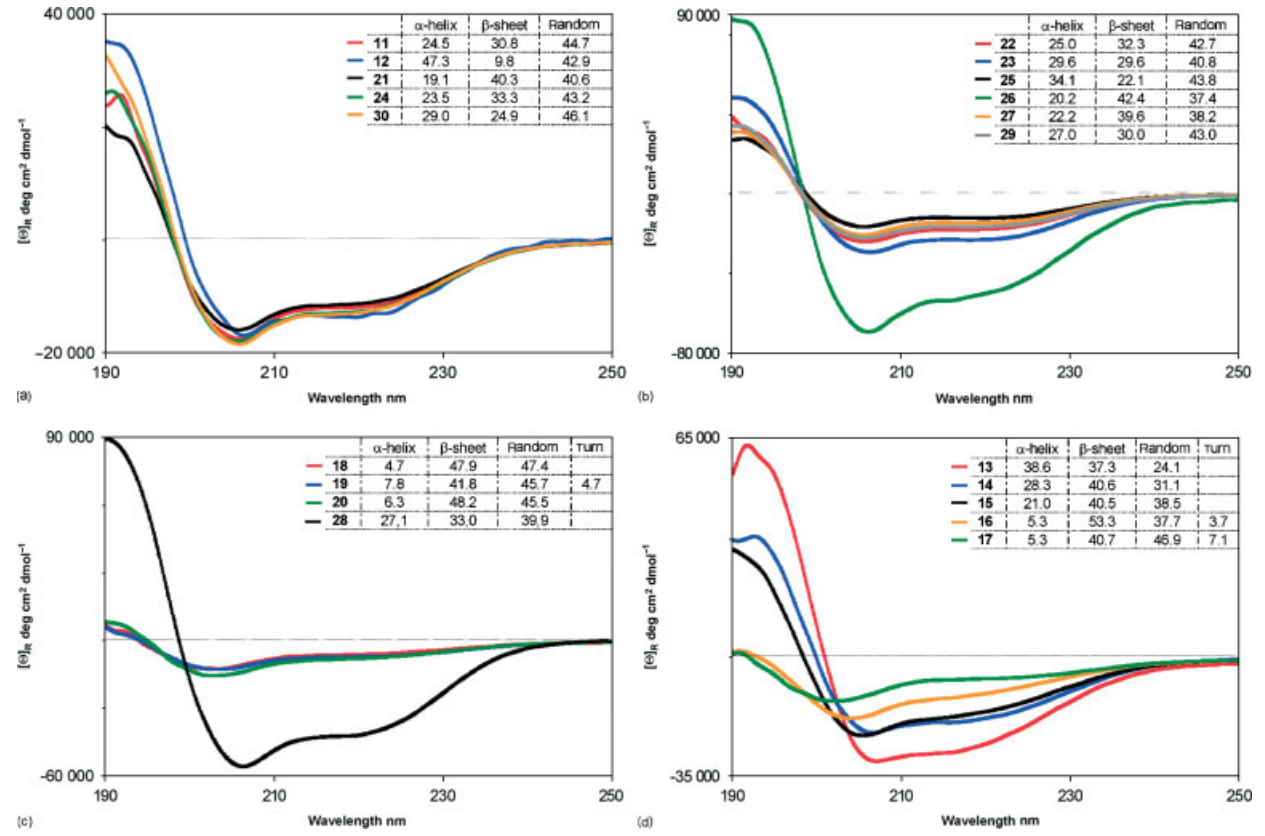

Figure 4 CD spectra and the percentage distribution of secondary structure elements according to the calculation of Yang *et al.* [36] of orexin B analogs. (a) human orexin B analogs with activity at both orexin receptors. (b) Ox_2R selective h orexin B analogs with activity <10 μM (c) Ox_2R selective h orexin B analogs with activity >10 μM (d) completely inactive h orexin B analogs.

displayed bands with high molar ellipticity (80 000 deg cm^2 $dmol^{-1}$). Totally different CD spectra were recorded for most of the Ox_2R selective analogs with low activity (>10 μM, nos. 18, 19, 20). Only a single strong negative band at 202 nm was observed for those analogs with combination of amino acid replacements at positions 11, 15 and 27 as well as positions 14 and 27 (no. 18–20) (Figure 4c). A different CD spectrum showed [A^{21},▲^{22}]-h orexin B-(6–28)-peptide (no. 28), which was similar to those recorded for analog no. 26. In Figure 4(d), all completely inactive h orexin B-(6–28)-peptide analogs are combined. They show a large variety of CD spectra without any tendency to preclude special secondary structures that were adopted by active analogs. [P^{11},A^{15},A^{27}]-h orexin B-(6–28)-peptide (no. 16) and [P^{11},A^{15},P^{27}]-h orexin B-(6–28)-peptide (no. 17) showed spectra similar to those of Ox_2R selective analogs with activity above 10 μM, however, the intensity of the band at 202 nm was increased about 1.4-fold. The CD spectra of [S^{11},A^{27}]-h orexin B-(6–28)-peptide (no. 13), [P^{11},A^{27}]-h orexin B-(6–28)-peptide (no. 14) and [P^{11},A^{27},L^{28}]-h orexin B-(6–28)-peptide (no. 15) showed strong positive (192 nm) and negative (206 nm, 220 nm) bands that were similarly seen for h orexin B analogs with activity at both receptors (Figure 4a). The estimation of secondary structure of h orexin B analogs by using the JASCO software (results displayed in Figure 4) led to the assumption that active analogs exist highly unstructured in solution (>40%). The differentiation of analogs with activity at both receptors, with Ox_2R selectivity and inactive analogs is not supported by the percentage distribution of secondary structure elements. Only the analogs with Ox_2R activity below 10 μM and some of the inactive compounds (nos. 16–20) adopt α-helical structure <10%, whereas all other analogs consist of about 30% α-helix.

DISCUSSION

The orexin peptides are highly homologous to each other in their primary and secondary structure, hence they show similar activity at the Ox_2R and only at the Ox_1R, orexin A has a tenfold higher potency compared to orexin B. To identify residues that contribute to this receptor selectivity, we investigated several orexin A and orexin B analogs for their ability to activate Ox_1R and Ox_2R. First, we examined the role of the disulfide bonds of orexin A by replacing each Cys by the isosteric Abu to obtain orexin A that lacks both the electronic properties of Cys and the rigid structure. Since the activity of this analog was decreased to a lesser extent than by the absence of the disulfide bonds with Abu at position 6 and 12 or rather 7 and 14 [19], we confirmed the previous assumption that neither the Cys-residues nor the constriction of the structure is required for maintaining activity and thereby disproved the hypothesis of Okumura *et al.*, suggesting that the two disulfide bonds play a major role in receptor activation [37]. Furthermore, the unimportance of the disulfide bonds was also shown previously by active orexin A analogs that consists of turn-inducing and linker residues instead of residues 6–14, respectively. However, the activity of these analogs was decreased at both receptors [19]. In the study presented here, we obtained analogs with activities at both receptors close to that of native orexin A by the modification of the turn-inducing spacer Pro-Gly to the type Pro-Xaa-Gly, with Xaa being residues that occur naturally in the replaced segment. Thereby, the alkaline property and length of the side chain of Xaa correlated with activity. This may by due to an electrostatic interaction of this amino acid with the receptor or to the flexibility of the long and narrow side chains, particularly of the Lys residue. Furthermore, the elongation of the spacer may be essential to bring the *C*- and *N*-terminal segment of orexin A in the right position for the interaction with the receptor. This was shown to be not the case by the investigation of the flexible Ala-Aib linker that displayed the same activity as shown previously for the flexible Ahx linker [19]. On the other hand, the disulfide bonds within this segment may constrain the overall structure of the peptide, and accordingly, may provide the right orientation of *C*- and *N*-terminal residues to obtain high activity. With constrained β-ACC building blocks that adopt opposite conformations, we observed that the orientation of the sequence is important to obtain biologically active analogs. We confirm that β-ACC building blocks are optimal tools to stabilize bioactive conformations in peptide analogs as it was recently shown for neuropeptide Y analogs [31]. Investigation of the secondary structure of orexin A analogs by CD spectroscopy elucidated that at least 17% α-helicity is required for activity at both receptors. However, an overall correlation of the relationship between secondary structure and affinity could not be found. This again indicates that besides the conformational properties the amino acid side chains of orexin A also play an important role in receptor activity. Similar results were obtained by the investigation of several truncated orexin A and B analogs [38].

Receptor selective ligands are important tools for the investigation of receptor subtype specific functions. We identified the kink region of h orexin B-(6–28)-peptide, residues 20–22, and the combination of exchanges at position 11 and 27 to be required for Ox_2R selectivity. The amino acid side chains appeared to have even a higher influence on receptor activation than the overall conformation of the peptide, because the data from the secondary structure estimation did not correlate with biological activity. This is surprising with respect to the applied β-ACC building blocks that have been shown to stabilize peptide structures depending on their conformation [31,34]. However, the secondary

structure was investigated in solution in 30% TFE and may not represent the biological active conformation that is adopted in proximity of the receptor, indicated by the high content (>40%) of unordered structure.

Multiple amino acid substitutions in orexin B have been shown to contribute differently to Ox_2R selectivity. For position 21, we found no direct interaction with the receptors because of the exchangeability of His by acidic and hydrophobic amino acids. This is in agreement with the results of previous investigations [19,22,23]. In contrast, direct interactions with the receptor of residues 11, 14, 15 and 27 of the peptide are conceivable because the substitution of at least two of them led to a complete loss in activity or rather strongly dropped Ox_2R selectivity. We agree that residue 11 activate the Ox_2R by hydrophobic interactions [22], since the exchange with hydrophilic Ser was devoid of any activity. However, Pro is as well a hydrophobic amino acid, but has no bias toward helix conformation and the h orexin B consists from residue 7–19 of a α-helix [16,17]. Therefore, the poor activity of the Pro^{11} as well as Pro^{14} containing analogs might result in the interruption of the helix. This is supported by the CD spectra of analogs no. 16–20. Amino acids in position 15 may directly interact with the receptor, since all analogs with Ala^{15} are devoid of any receptor activity and analogs with Ile^{15} display only poor Ox_2R selectivity. This is in agreement with the information obtained by the investigation of [D-Leu^{15}]-h orexin B [23]. The role of position 27 remains unclear because exchanges at this position in combination with other amino acid replacements resulted in complete inactive, Ox_2R selective peptides or peptides active at both receptors, although the single exchange by Ala resulted in Ox_2R selectivity [19]. The Pro-residue of the active analog [P^7,A^{27}]-h orexin B-(6–28)-peptide might be able to stabilize the *N*-terminal structure that is suggested to directly interact with the receptor [19,22].

The amino acid sequence at the end of the *C*-terminal helix differs in three residues between orexin A and B. Because the replacement of these three amino acids in h orexin B-(6–28)-peptide by those of orexin A did not alter the activity of h orexin B, we suggest that this region stabilizes the *N*-terminal structure of orexin A, which might be responsible for the high Ox_1R activity [39].

CONCLUSION

Orexin A and B analogs have been tested for their activity at the Ox_1R and Ox_2R in order to identify structural properties for high activity and subtype selectivity. For orexin A, we have shown that activation of both receptors requires a distinct conformation of the segment between residues 6–14, but constriction by the two disulfide bonds is not essential. Additionally, an ionic interaction between the basic amino acids within this segment and the receptors seems to be reasonable. Furthermore, we have localized the Ox_2R selectivity of orexin B to be situated in the kink region between the two helices. Especially, the conformation at position 20 is a determinant of Ox_2R selectivity. On the basis of these findings, the identification of the physiological function of the different orexin receptors through application of selective compounds may be enforced.

Acknowledgement

We would like to thank Doris Haines for the technical assistance in peptide synthesis, Regina Reppich for MALDI mass measurements and Ellen Schöley-Pohl for assistance with some calcium mobilization experiments. The financial contribution of DFG (BE1264-3/2, RE948-4/2 and GRK760) and the Fonds der Chemischen Industrie is kindly acknowledged.

REFERENCES

1. Peyron C, Faraco J, Rogers W, Ripley B, Overeem S, Charnay Y, Nevsimalova S, Aldrich M, Reynolds D, Albin R, Li R, Hungs M, Pedrazzoli M, Padigaru M, Kucherlapati M, Fan J, Maki R, Lammers GJ, Bouras C, Kucherlapati R, Nishino S, Mignot E. A mutation in a case of early onset narcolepsy and a generalized absence of hypocretin peptides in human narcoleptic brains. *Nat. Med.* 2000; **6**: 991–997.
2. Chabas D, Taheri S, Renier C, Mignot E. The genetics of narcolepsy. *Annu. Rev. Genomics Hum. Genet.* 2003; **4**: 459–483.
3. Wieczorek S, Jagiello P, Arning L, Dahmen N, Epplen JT. Screening for candidate gene regions in narcolepsy using a microsatellite based approach and pooled DNA. *J. Mol. Med.* 2004; **82**: 696–705.
4. Chemelli RM, Willie JT, Sinton CM, Elmquist JK, Scammell T, Lee C, Richardson JA, Williams SC, Xiong Y, Kisanuki Y, Fitch TE, Nakazato M, Hammer RE, Saper CB, Yanagisawa M. Narcolepsy in orexin knockout mice: molecular genetics of sleep regulation. *Cell* 1999; **98**: 437–451.
5. Lin L, Faraco J, Li R, Kadotani H, Rogers W, Lin X, Qiu X, de Jong PJ, Nishino S, Mignot E. The sleep disorder canine narcolepsy is caused by a mutation in the hypocretin (orexin) receptor 2 gene. *Cell* 1999; **98**: 365–376.
6. Thompson MD, Comings DE, Abu-Ghazalah R, Jereseh Y, Lin L, Wade J, Sakurai T, Tokita S, Yoshida T, Tanaka H, Yanagisawa M, Burnham WM, Moldofsky H. Variants of the orexin2/hcrt2 receptor gene identified in patients with excessive daytime sleepiness and patients with Tourette's syndrome comorbidity. *Am. J. Med. Genet.* 2004; **129B**: 69–75.
7. Rodgers RJ, Ishii Y, Halford JC, Blundell JE. Orexins and appetite regulation. *Neuropeptides* 2002; **36**: 303–325.
8. Farr SA, Banks WA, Kumar VB, Morley JE. Orexin-A-induced feeding is dependent on nitric oxide. *Peptides* 2005; **26**: 759–765.
9. Ferguson AV, Samson WK. The orexin/hypocretin system: a critical regulator of neuroendocrine and autonomic function. *Front. Neuroendocrinol.* 2003; **24**: 141–150.
10. Mobarakeh JI, Takahashi K, Sakurada S, Nishino S, Watanabe H, Kato M, Yanai K. Enhanced antinociception by intracerebroventricularly and intrathecally-administered orexin A and B (hypocretin-1 and -2) in mice. *Peptides* 2005; **26**: 767–777.
11. Sakurai T, Amemiya A, Ishii M, Matsuzaki I, Chemelli RM, Tanaka H, Williams SC, Richardson JA, Kozlowski GP, Wilson S,

Arch JR, Buckingham RE, Haynes AC, Carr SA, Annan RS, McNulty DE, Liu WS, Terrett JA, Elshourbagy NA, Bergsma DJ, Yanagisawa M. Orexins and orexin receptors: a family of hypothalamic neuropeptides and G protein-coupled receptors that regulate feeding behavior. *Cell* 1998; **92**: 573–585.
12. Kukkonen JP, Holmqvist T, Ammoun S, Akerman KE. Functions of the orexinergic/hypocretinergic system. *Am. J. Physiol., Cell Physiol.* 2002; **283**: C1567–C1591.
13. Smart D, Jerman JC, Brough SJ, Rushton SL, Murdock PR, Jewitt F, Elshourbagy NA, Ellis CE, Middlemiss DN, Brown F. Characterization of recombinant human orexin receptor pharmacology in a Chinese hamster ovary cell-line using FLIPR. *Br. J. Pharmacol.* 1999; **128**: 1–3.
14. de Lecea L, Kilduff TS, Peyron C, Gao X, Foye PE, Danielson PE, Fukuhara C, Battenberg EL, Gautvik VT, Bartlett FS 2nd, Frankel WN, van den Pol AN, Bloom FE, Gautvik KM, Sutcliffe JG. The hypocretins: hypothalamus-specific peptides with neuroexcitatory activity. *Proc. Natl. Acad. Sci. U.S.A.* 1998; **95**: 322–327.
15. Sakurai T. Orexins and orexin receptors: implication in feeding behavior. *Regul. Pept.* 1999; **85**: 25–30.
16. Miskolzie M, Lucyk S, Kotovych G. NMR conformational studies of micelle-bound orexin-B: a neuropeptide involved in the sleep/awake cycle and feeding regulation. *J. Biomol. Struct. Dyn.* 2003; **21**: 341–351.
17. Lee JH, Bang E, Chae KJ, Kim JY, Lee DW, Lee W. Solution structure of a new hypothalamic neuropeptide, human hypocretin-2/orexin-B. *Eur. J. Biochem.* 1999; **266**: 831–839.
18. Kim HY, Hong E, Kim JI, Lee W. Solution structure of human orexin-A: regulator of appetite and wakefulness. *J. Biochem. Mol. Biol.* 2004; **37**: 565–573.
19. Lang M, Soll RM, Durrenberger F, Dautzenberg FM, Beck-Sickinger AG. 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. *J. Med. Chem.* 2004; **47**: 1153–1160.
20. Darker JG, Porter RA, Eggleston DS, Smart D, Brough SJ, Sabido-David C, Jerman JC. Structure-activity analysis of truncated orexin-A analogues at the orexin-1 receptor. *Bioorg. Med. Chem. Lett.* 2001; **11**: 737–740.
21. Ammoun S, Holmqvist T, Shariatmadari R, Oonk HB, Detheux M, Parmentier M, Akerman KE, Kukkonen JP. Distinct recognition of OX1 and OX2 receptors by orexin peptides. *J. Pharmacol. Exp. Ther.* 2003; **305**: 507–514.
22. Egashira S-I, Matsuda M, Iwaasa H, Kanatani A. Development of structure-activity relationship of orexins: possible roles of three regions of orexin-B. *Drugs Future* 2004; **29**: 1007–1013.
23. Asahi S, Egashira SI, Matsuda M, Iwaasa H, Kanatani A, Ohkubo M, Ihara M, Morishima H. Development of an orexin-2 receptor selective agonist, [Ala(11), D-Leu(15)]orexin-B. *Bioorg. Med. Chem. Lett.* 2003; **13**: 111–113.
24. Haynes AC, Jackson B, Chapman H, Tadayyon M, Johns A, Porter RA, Arch JR. A selective orexin-1 receptor antagonist reduces food consumption in male and female rats. *Regul. Pept.* 2000; **96**: 45–51.
25. Porter RA, Chan WN, Coulton S, Johns A, Hadley MS, Widdowson K, Jerman JC, Brough SJ, Coldwell M, Smart D, Jewitt F, Jeffrey P, Austin N. 1,3-Biarylureas as selective non-peptide antagonists of the orexin-1 receptor. *Bioorg. Med. Chem. Lett.* 2001; **11**: 1907–1910.
26. Hirose M, Egashira S, Goto Y, Hashihayata T, Ohtake N, Iwaasa H, Hata M, Fukami T, Kanatani A, Yamada K. N-acyl 6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline: the first orexin-2 receptor selective non-peptidic antagonist. *Bioorg. Med. Chem. Lett.* 2003; **13**: 4497–4499.
27. McAtee LC, Sutton SW, Rudolph DA, Li X, Aluisio LE, Phuong VK, Dvorak CA, Lovenberg TW, Carruthers NI, Jones TK. Novel substituted 4-phenyl- [1,3]dioxanes: potent and selective orexin receptor 2 (OX(2)R) antagonists. *Bioorg. Med. Chem. Lett.* 2004; **14**: 4225–4229.
28. Gnad F, Reiser O. Synthesis and applications of beta-aminocarboxylic acids containing a cyclopropane ring. *Chem. Rev.* 2003; **103**: 1603–1623.
29. Bubert C, Cabrele C, Reiser O. Novel strategies for the synthesis of peptides containing cis- or trans-b-aminocyclopropanecarboxylic acids. *Synlett* 1997; **7**: 827–829.
30. Beumer R, Bubert C, Cabrele C, Vielhauer O, Pietzsch M, Reiser O. The synthesis of diastereo- and enantiomerically pure beta-aminocyclopropanecarboxylic acids. *J. Org. Chem.* 2000; **65**: 8960–8969.
31. Koglin N, Zorn C, Beumer R, Cabrele C, Bubert C, Sewald N, Reiser O, Beck-Sickinger AG. Analogues of neuropeptide Y containing beta-aminocyclopropane carboxylic acids are the shortest linear peptides that are selective for the Y1 receptor. *Angew. Chem. Int. Ed. Engl.* 2003; **42**: 202–205.
32. Beck W, Jung G. Convenient reduction of S-oxides in synthetic peptides, lipopeptides and peptide libraries. *Lett. Pept. Sci.* 1994; **1**: 31–37.
33. Bufe B, Hofmann T, Krautwurst D, Raguse JD, Meyerhof W. The human TAS2R16 receptor mediates bitter taste in response to beta-glucopyranosides. *Nat. Genet.* 2002; **32**: 397–401.
34. De Pol S, Zorn C, Klein CD, Zerbe O, Reiser O. Surprisingly stable helical conformations in alpha/beta-peptides by incorporation of cis-beta-aminocyclopropane carboxylic acids. *Angew. Chem. Int. Ed. Engl.* 2004; **43**: 511–514.
35. Tyndall JD, Pfeiffer B, Abbenante G, Fairlie DP. Over one hundred peptide-activated G protein-coupled receptors recognize ligands with turn structure. *Chem. Rev.* 2005; **105**: 793–826.
36. Yang JT, Wu CS, Martinez HM. Calculation of protein conformation from circular dichroism. *Meth. Enzymol.* 1986; **130**: 208–269.
37. Okumura T, Takeuchi S, Motomura W, Yamada H, Egashira S, Asahi S, Kanatani A, Ihara M, Kohgo Y. Requirement of intact disulfide bonds in orexin-A-induced stimulation of gastric acid secretion that is mediated by OX1 receptor activation. *Biochem. Biophys. Res. Commun.* 2001; **280**: 976–981.
38. Wieland HA, Soll RM, Doods HN, Stenkamp D, Hurnaus R, Lammle B, Beck-Sickinger AG. The SK-N-MC cell line expresses an orexin binding site different from recombinant orexin 1-type receptor. *Eur. J. Biochem.* 2002; **269**: 1128–1135.
39. Voisin T, Rouet-Benzineb P, Reuter N, Laburthe M. Orexins and their receptors: structural aspects and role in peripheral tissues. *Cell. Mol. Life Sci.* 2003; **60**: 72–87.

