FEBS Letters 585 (2011) 1368–1374

journal homepage: www.FEBSLetters.org

Orexin/hypocretin receptor chimaeras reveal structural features important for orexin peptide distinction

Jaana Putula [a,1], Pauli M. Turunen [a,1], Lisa Johansson [b], Johnny Näsman [c], Runar Ra [a], Laura Korhonen [d], Jyrki P. Kukkonen [a,b,d,*]

[a] Biochemistry and Cell Biology, Department of Veterinary Biosciences, P.O.B. 66, FIN-00014 University of Helsinki, Finland
[b] Physiology, Department of Neuroscience, P.O.B. 572, Uppsala University, Uppsala, Sweden
[c] Biochemistry, Department of Biosciences, Åbo Akademi University, Tykistökatu 6, FIN-20520 Turku, Finland
[d] Minerva Institute for Medical Research, Biomedicum 2, Tukholmankatu 8, FIN-00290 Helsinki, Finland

ARTICLE INFO

Article history:
Received 15 March 2011
Accepted 7 April 2011
Available online 15 April 2011

Edited by Maurice Montal

Keywords:
Orexin
Hypocretin
Calcium
Orexin-A
Orexin-B

ABSTRACT

We wanted to analyze the basis for the distinction between OX_1 and OX_2 orexin receptors by the known agonists, orexin-A, orexin-B and Ala^{11}, D-Leu^{15}-orexin-B, of which the latter two show some selectivity for OX_2. For this, chimaeric OX_1/OX_2 and OX_2/OX_1 orexin receptors were generated. The receptors were transiently expressed in HEK-293 cells, and potencies of the agonists to elicit cytosolic Ca^{2+} elevation were measured. The results show that the N-terminal regions of the receptor are most important, and the exchange of the area from the C-terminal part of the transmembrane helix 2 to the transmembrane helix 4 is enough to lead to an almost total change of the receptor's ligand profile.
© 2011 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.

1. Introduction

The native orexin peptides, orexin-A and -B, are neuropeptides, which act via two G-protein-coupled receptors, OX_1 and OX_2 [1,2]. Orexins and orexin receptors took a central position in the sleep regulation soon after their identification and cloning [3–5], but also other functions have been shown, including regulation of appetite, stress response and addiction [6–8]. Most interest, from the pharmacological point of view, probably lies in the development of orexin receptor antagonists as hypnotics, and possibly agonists as research tools and drugs for hypersomnia and narcolepsy. Despite rather intensive drug development approaches from several companies [9], very little is known about the receptor and ligand epitopes determining the interactions. Some mutagenesis studies have been performed with the orexin peptides. The earlier studies applied mainly truncation and alanine scan [10–12]. These studies show that the activity is gradually lost during the N-terminal truncation of the peptide, while the C-terminus is unconditionally required. One interesting finding emerging from these studies is that it seems that each mutation affects the OX_2 receptors less than OX_1. Since the OX_2 receptor does not distinguish between orexin-A and orexin-B, unlike the OX_1 receptor, it would be tempting to suggest that the OX_2-like phenotype is something the receptor–peptide interaction is prone to relax to. However, a later peptide mutagenesis study suggested that one of the agonist peptides, Ala^{11}, D-Leu^{15}-orexin-B (A^{11},DL^{15}-orexin-B), would display significant OX_2 receptor-selectivity [13]. In a recent study, we show that indeed this peptide shows selectivity for the OX_2 receptor, but much weaker than originally reported and variable with respect to different expression systems [14].

Even less is known about the orexin receptor structure. A few natural mutants are known. The frame-shifting (and thus truncating) ones of the OX_2 receptor, found in narcoleptic canines, are completely non-functional [4,15]. In contrast, a point mutation Glu54Lys of OX_2, also found in dog, shows proper membrane localization but a strongly reduced orexin-A binding and orexin-A and -B signaling [15]. Interestingly, this glutamate (and several other amino acids in its vicinity) is conserved also in OX_1 receptor

Abbreviations: A^{11},DL^{15}-orexin-B, Ala^{11}, D-Leu^{15}-orexin-B; C, C-terminus of orexin receptors; Ch1–8, chimaeric orexin receptors 1–8; EC1–3, extracellular loops of orexin receptor 1–3; GFP, green fluorescent protein; IC1–3, intracellular loops of orexin receptor 1–3; N, N-terminus of orexin receptors; TM1–7, transmembrane helices of orexin receptor 1–7

* Corresponding author at: Biochemistry and Cell Biology, Department of Veterinary Biosciences, P.O.B. 66, FIN-00014 University of Helsinki, Finland. Fax: +358 9 191 57033.
E-mail address: jyrki.kukkonen@helsinki.fi (J.P. Kukkonen).
[1] Equal contribution to this work.

0014-5793/$36.00 © 2011 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.
doi:10.1016/j.febslet.2011.04.020

(Glu46). A number of single nucleotide polymorphisms of orexin receptors are known [6], but have not been consequently investigated. No mutagenesis studies, except for a recent point mutation study [16], have been performed. In this study, several amino acids predicted to contribute to antagonist binding were mutated to alanine. Many of the mutations caused dramatic reduction in the antagonist binding [16].

Orexin receptor subtypes exhibit rather high overall identity and similarity, yet they show distinct pharmacological profile towards the agonists orexin-A, orexin-B and A^{11},$_{D}L^{15}$-orexin-B, and a number of subtype selective antagonists have been developed [9]. Therefore, comparison and exchange of the sequences motifs should allow identification of the sequences important for selective action of native and synthetic ligands. This approach was in the current study realized by a series of orexin receptor chimaeras.

2. Materials and methods

2.1. Drugs and other materials

Human orexin-A, orexin-B and A^{11},$_{D}L^{15}$-orexin-B were from NeoMPS (Strasbourg, France) and the restriction enzymes and polymerases from Finnzymes (Espoo, Finland), Fermentas GmbH (St. Leon-Rot, Germany) and New England Biolabs (Ipswich, MA, USA).

2.2. Cell culture

HEK-293 cells were cultured in high glucose Dulbecco's Modified Eagle Medium (DMEM; Gibco, Paisley, UK) with supplements and otherwise as described in [14]. For Ca^{2+} experiments, the cells were cultured on polyethyleneimine-pretreated (25 μg/ml, 1 h, 37 °C; Sigma) 96-well polystyrene plates (Greiner). The cells were transiently transfected utilizing Fugene HD (Roche Diagnostics GmbH, Mannheim, Germany) – as described in [14] – to introduce different orexin receptor constructs.

2.3. Construction of orexin receptor vectors

Human OX_1 and OX_2 cDNAs used here for cloning are the same as in, e.g. [14]; please also see S3. Cloning was, in part, designed and simulated with the help of Serial Cloner 1.3 (http://www.serialbasics.free.fr/Serial_Cloner.html). In the first round of mutagenesis, we constructed two-component chimaeras with the front part (5′-end of the receptor DNA, N-terminus of the receptor protein) from OX_1 and the rear part (3′-end of the receptor DNA, C-terminus of the receptor protein) from OX_2 (chimaeric orexin receptors (Ch1–3)) and *vice versa* (Ch3–6) (Fig. 2, S1 and S2). No amino acid changes were introduced in the receptors. The stop codon (TGA or TAG) was mutated to GGA in all the constructs to allow fusion of the receptor with C-terminal green fluorescent protein (GFP) – and examination of the expression of the mutant proteins – when cloned into the pEGFP-N3 vector (Clontech, Palo Alto, CA, USA). The receptors were combined from PCR fragments in pEGFP-N3 (S1 and S2). All the PCR products and the final receptors were sequenced. OX_1-GFP was "reconstructed" from Ch2 and Ch4 by exchanging the front part of Ch4 with the front part of Ch2 (HindIII–BstxI-cut). OX_2-GFP was "reconstructed" from Ch1 and Ch5 by exchanging the front part of Ch1 with the front part of Ch5 (HindIII–BstxI-cut). The second round of mutagenesis (Ch7 and Ch8; Fig. 2, S2) was performed without PCR utilizing the native restriction sites for BstxI and MscI. For Ch7, Ch1 was cut with BstxI and BamHI and the 770 bp excised fragment replaced with the corresponding 740 bp fragment from Ch5. For construction of Ch8, the entire Ch2 and Ch4 had to be transferred to pUC18 plasmid (with enzymes HindIII and BamHI), since the backbone of pEGFP-N3 contains restriction sites for MscI. pUC18-Ch4 was cut with MscI and BamHI and the 170 and 580 bp excised fragments replaced with the corresponding 790 bp fragment from Ch2. Finally, Ch8 was transferred back to pEGP-N3 with HindIII and BamHI. DNA and protein sequences of all the constructs are presented in the Supplementary data (S3 and S4, respectively). Following abbreviations are used in context with the orexin receptor peptide sequences: C-terminus of orexin receptors (C); extracellular loops 1–3 of orexin receptor (EC1–3); intracellular loops 1–3 of orexin receptor (IC1–3); N-terminus of orexin receptors (N); transmembrane helices 1–7 of orexin receptor (TM1–7).

2.4. Ca^{2+} measurements

Calcium measurements were performed at 37 °C using FlexStation fluorescence plate reader (Molecular Devices, Sunnyvale, CA, USA) with cells loaded fluo-4 acetoxymethyl ester (Molecular Probes/Invitrogen) as described in [14]. The data were analyzed in Microsoft Excel [14]. The results are presented as mean ± S.E. Each construct was measured in quadruplicate in 4–13 separate transfections.

2.5. Data analysis

Student's two-tailed *t*-test with Bonferroni correction was used in all statistical comparisons. Microsoft Excel was used for the nonlinear curve-fitting used for the determination of the pEC_{50} values. The significances are as follows: ns (not significant); $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. The symbol "†" is used for the second comparison (NS stands for not significant) and "‡" for the third (*NS* stands for not significant).

3. Results and discussion

We have previously expressed wild-type GFP-tagged OX_1 and OX_2 orexin receptors in HEK-293 cells receptors [14]; as already confirmed in that study, the pharmacology of the receptors was very much as expected from previous studies (see e.g. [2]) with respect to the endogenous ligands orexin-A and orexin-B, i.e. the ligands were approximately equipotent on the OX_2 receptor (orexin-B 1.6-fold more potent) (Fig. 3B–D), whereas orexin-A was clearly more potent (5.5-fold) on the OX_1 receptor (Fig. 3A, C, and D). The putative OX_2-selective ligand, A^{11},$_{D}L^{15}$-orexin-B, was also clearly more potent on the OX_2 receptors than on the OX_1 receptors (Fig. 3). Thus, OX_1 and OX_2 receptors display distinct agonist potency profiles (orexin-A > orexin-B $\gg$ A^{11},$_{D}L^{15}$-orexin-B and orexin-B $\geqslant$ orexin-A > A^{11},$_{D}L^{15}$-orexin-B, respectively).

In the first round of mutagenesis, we replaced the OX_2 receptor subtype sequence with the OX_1 sequence from N-terminus to C-terminus in three steps and *vice versa*, i.e. approximately at the 1/4 (N–TM2; Ch1, Ch4), 1/2 (N–TM4; Ch2, Ch5) and 3/4 of the length (N–TM6; Ch3, Ch6) (Figs. 1 and 2, S2). In Ca^{2+} experiments, the subtype-non-selective agonist, orexin-A, did not show significant difference in potency between OX_1 and OX_2. Among the other clones, marked differences in the potency of orexin-A were seen (pEC_{50} between 8.15 and 6.37; Fig. 4A).

Differential expression (or signal coupling) would cause problems in the analysis of the absolute agonist potencies. Luckily, the profile of the three agonists could be followed instead of absolute potencies, due to the "internal control" offered by the non-selective agonist orexin-A. The exchange of the N- or C-terminal quarter (N–TM2 or TM6–C, respectively) did not change the profile of the receptor, i.e. Ch1 and Ch6 are similar to OX_2 and Ch3 and Ch4 similar to OX_1 (Figs. 2 and 4). A dramatic effect on the receptor

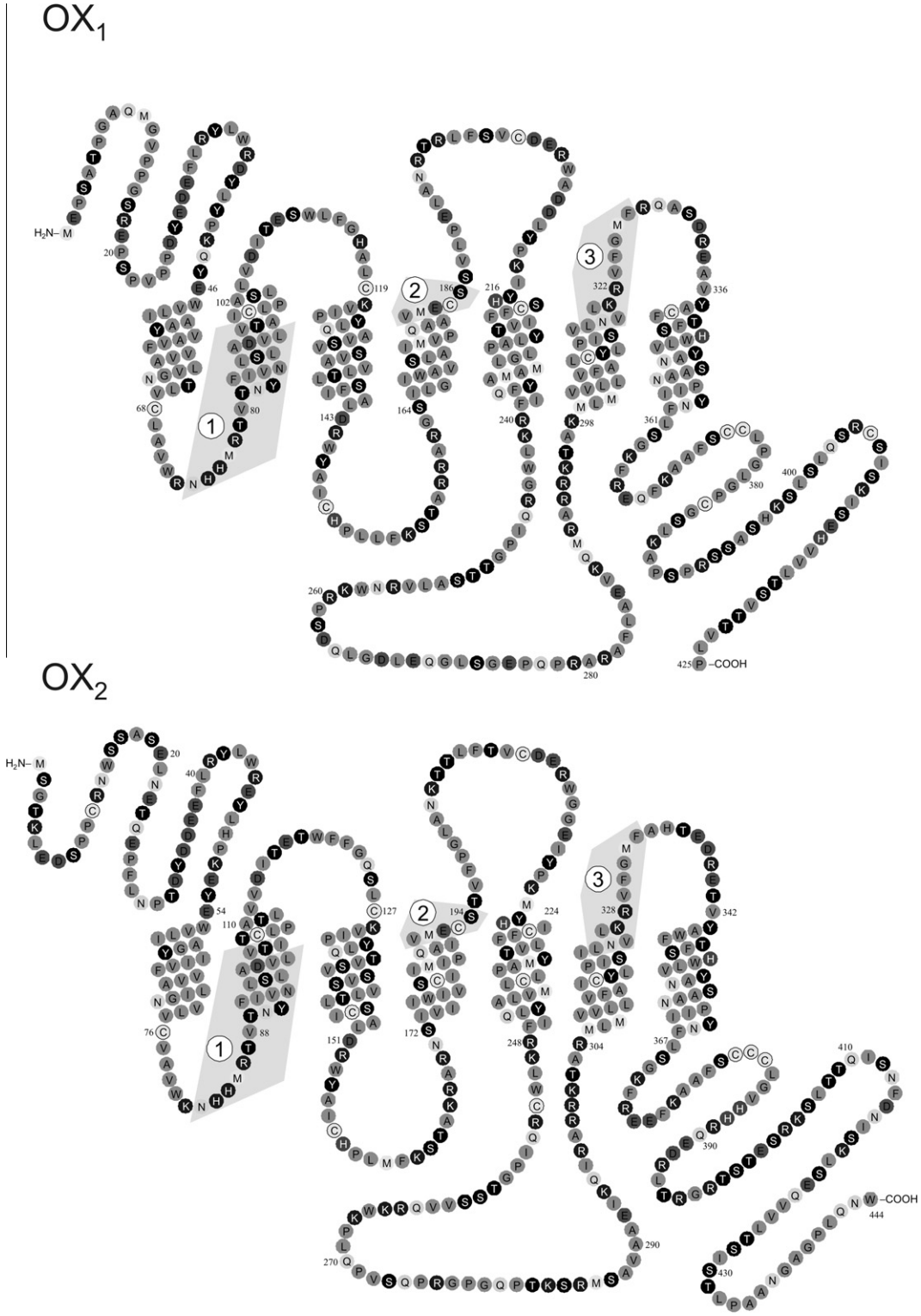

Fig. 1. Representation of the cut-points for the construction of the chimaeric orexin receptors in snake-like plots of OX_1 and OX_2 receptor. There are significant stretches of complete amino acid identity in the transition zones, and therefore, similar to this figure, the exact point of transition from one subtype to another cannot be pointed out, but is represented by the gray boxes numbered 1–3.

profile was, instead, seen when the N-terminal half of the receptor was exchanged. Ch2, with the N-terminal half from OX_1 and the C-terminal from OX_2 (Fig. 2), effectively adopted a profile of OX_1 receptors, though with somewhat increased potency of A^{11},$_D L^{15}$-orexin-B (Fig. 4). Similarly, the profile of Ch5, with the N-terminal half from OX_2 (N–TM4) and the C-terminal (TM4–C) from OX_1 (Fig. 2), is essentially indistinguishable from that of OX_2 (Fig. 4).

The data from the first round of mutagenesis thus indicates that the most important part for the agonist-selectivity lies in the 2nd quarter of the receptors (TM2–TM4); since there is high homology in this area between the receptor subtypes, the area with sequence

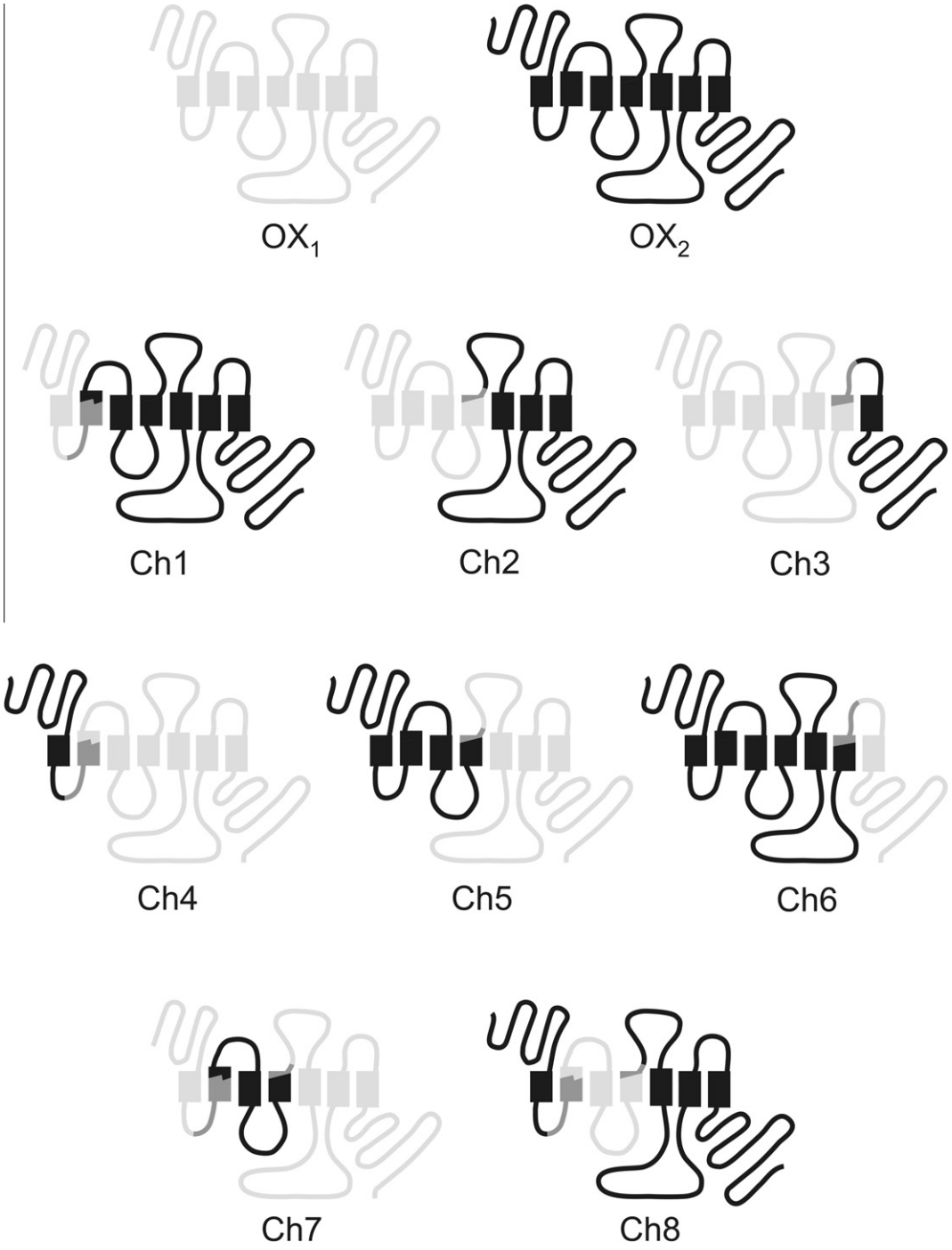

Fig. 2. Representation of the design of each chimaeric orexin receptor in snake-like plots. OX_1 sequence is marked in light gray and OX_2 in black. Similar to Fig. 1, there are full sequence homologies in each stretch where the exchange was made and therefore the exact point of transition cannot be pointed out; these areas are marked in darker gray.

diversity effectively only spans from the C-terminal part of TM2 to the C-terminal part of TM4 (Figs. 1 and 2). We therefore constructed two additional chimaeric receptors, Ch7 and Ch8, where only this part (the sequence between the "cut-point areas" 1 and 2 of Fig. 1) was exchanged (Fig. 2). Indeed, Ch7 with most of the sequence from the OX_1 receptor and TM2–TM4 from OX_2 (Fig. 2), adopted a profile very similar to OX_2 with only slightly lower potency of A^{11},$_D L^{15}$-orexin-B (Fig. 4). Ch8 with most of the sequence from the OX_2 receptor and TM2–TM4 from OX_1 (Fig. 2), changed it profile to one much more like OX_1 than OX_2, but it still retained higher relative potencies of both orexin-B and A^{11},$_D L^{15}$-orexin-B than OX_1 (Fig. 4). However, the overall functionality of Ch8 is questionable; very low overall GFP fluorescence (i.e. receptor expression level) was observed (not shown), which also is likely reflected in the low potency of orexin-A.

The mutagenesis thus identifies the area spanning from TM2 to TM4 as almost the sole determinant of agonist selectivity. This area comprises the C-terminal part of TM2, EC1, TM3, IC2 and most of TM4 of the receptor. However, also other parts of the receptor seem to act in concert with these parts, and thus the complete ligand profile is likely determined by the receptor as an entity. In comparison of all the chimaeras (Fig. 4), it seems that a fully OX_1-like profile cannot be obtained with any of the chimaeras, of which even the closest ones (Ch2, Ch3 and Ch4) show a somewhat

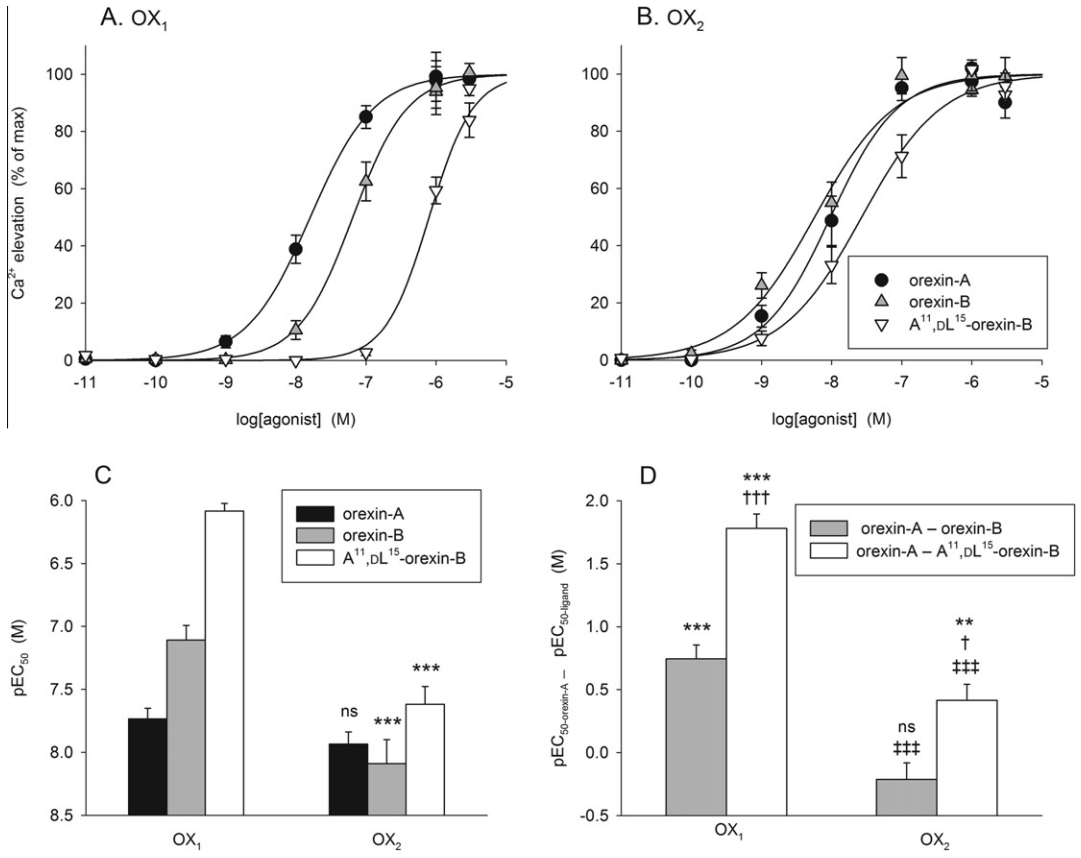

Fig. 3. Concentration–response relationships for the orexin peptides on native orexin receptors. (A and B) The average concentration–response curves from 4 batches of cells. (C) The averaged pEC_{50} values from the same data set. The comparison is for the same ligand between OX_1 and OX_2. (D) Comparison of the same data normalized to the pEC_{50} of orexin-A in each batch. The values are equal to the logarithm of the fold difference in potency ($pEC_{50\text{-orexin-A}} - pEC_{50\text{-ligand}} = \log(EC_{50\text{-ligand}}/EC_{50\text{-orexin-A}})$). The first comparison ("∗") is for the other agonists to orexin-A (=0), separately for each receptor subtype. The second comparison ("†") is similarly between orexin-B and A^{11},DL^{15}-orexin-B. The third comparison ("‡") is for the same ligand (orexin-B or A^{11},DL^{15}-orexin-B) between OX_1 and OX_2.

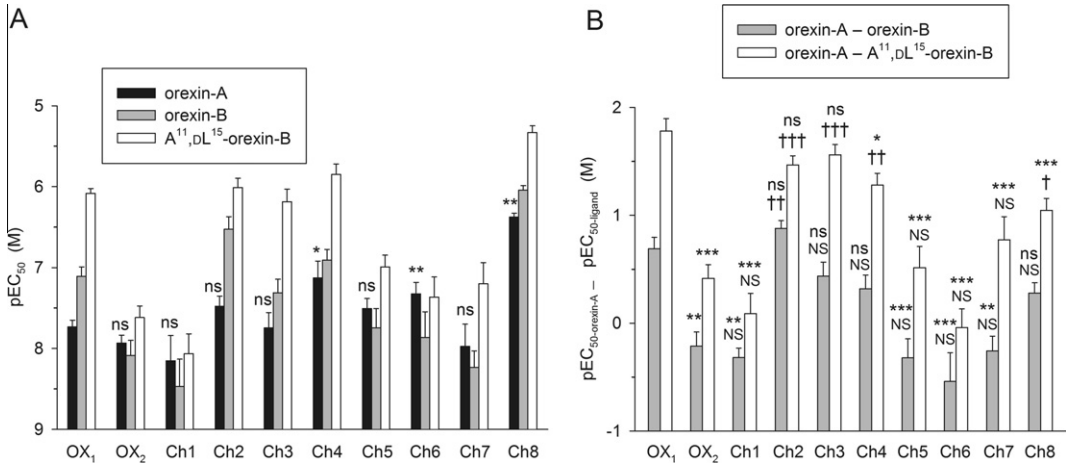

Fig. 4. Responses to the activation of receptors of the first round and second round of mutagenesis. (A) The averaged "raw" data. The comparison is for orexin-A between different constructs. (B) The same data were normalized to the orexin-A in each batch of cells (as in Fig. 3D. The first comparison ("∗") is for the other agonists to orexin-A (=0), separately for each receptor subtype. The second comparison ("†") is similarly between orexin-B and A^{11},DL^{15}-orexin-B.

elevated potency of orexin-B or A^{11},DL^{15}-orexin-B. Curiously, both Ch1 and Ch6, i.e. the receptors with the largest part from OX_2 but with either N- or C-terminal quarter from OX_1 (Fig. 2), were actually even more like the "classical" OX_2, i.e. with higher potency of orexin-B and A^{11},DL^{15}-orexin-B (see e.g. [2]), than OX_2 itself (Fig. 4). Although A^{11},DL^{15}-orexin-B is close to orexin-B, it does not show completely similar requirements of receptor determinants for receptor binding/activation. For instance, orexin-B shows the same relative potency for Ch2 as for OX_1, whereas the potency of A^{11},DL^{15}-orexin-B for Ch2 is somewhat elevated. Insertion of the 2nd quarter (TM2–TM4) of the OX_2 receptor in the OX_1 receptor (Ch7) produces a receptor that is indistinguishable from OX_2 with respect to orexin-B, but with a reduced potency of A^{11},DL^{15}-orexin-B. In contrast, Ch8 is rather half-way between OX_1 and OX_2 with

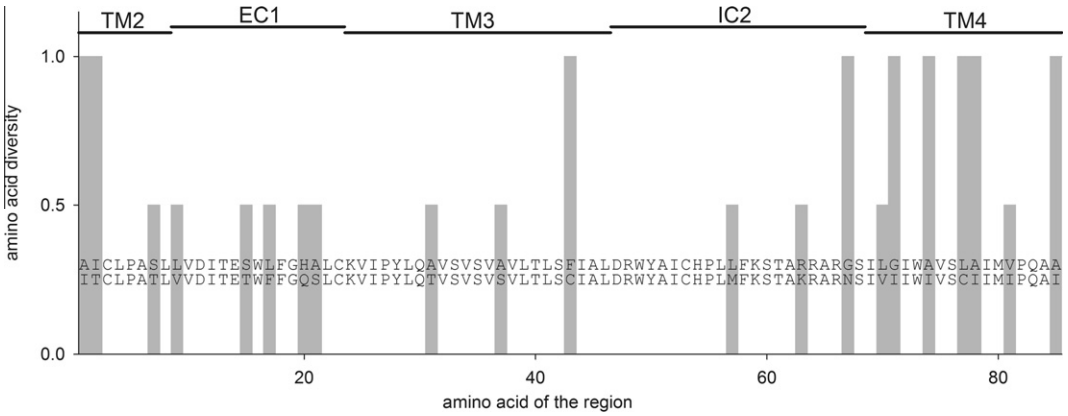

Fig. 5. Amino acid diversity between OX_1 and OX_2 in the region determining most of the peptide agonist selectivity, between the cut-points 1 and 2 (see Fig. 1). The alignment was performed with ClustalW (http://www.align.genome.jp/) and the scaling is based on that of given by this program. Strongest diversity is indicated with 1, while some similarity (":" and "." of ClustalW) gives 0.5. Printed on the bars, the sequences of OX_1 (above) and OX_2 (below) in this region.

respect to both ligands; however, Ch8 may not work optimally (see above). In conclusion, it seems that although TM2–TM4 represents the most important part the receptor, the entire receptor makes an entity, for instance so that the more N- and C-terminal parts have adapted to an interaction with each other. Secondly, orexin-B variants are, somewhat unexpectedly, not equal in their binding/activation properties. Thirdly, it seems that the receptor is prone to "relaxing" towards the agonist non-selective conformation (or slightly higher potency of orexin-B than orexin-A) as it is easier to get a more OX_2-like profile than an OX_1-like profile.

If the TM2–TM4 area is the most important part for agonist peptide distinction, what determinants then within this domain could be important? Even when the 2nd intracellular loop is eliminated as unlikely for the agonist binding, this domain contains the 2nd extracellular loop and (at least parts of) the transmembrane helices 2, 3 and 4, all of which may affect the agonist binding, based on the very little information there is available on the peptide binding to peptide receptors. Analysis of the amino acid sequence variation in this region does not reveal any obvious site; the sequences are mostly identical and the diversity is rather evenly distributed along the amino acid chain except for some concentration in the TM4 (Fig. 5). For further analysis of receptor determinants, modeling of the receptor structure would probably be necessary. However, this is not a trivial task, despite development of the algorithms and the four already available crystal structures, as also illustrated by the results of a recent study on orexin receptors [16]. Receptor mutagenesis is also not a straight-forward approach, as the correct folding may be compromised in the mutant receptors. To compensate for this, we used functional Ca^{2+} responses in intact cells, which should take place through the properly folded receptors only. Some of the constructs, in particular Ch4 and Ch8, showed generally weak expression, but even these constructs produced functional responses. Another parameter of interest would be the binding affinity. Unfortunately, the only commercially available radioligands, ^{125}I-orexin-A and -B, are not very useful because of assay problems such as very high glass-fiber binding and the general complications associated with agonist binding to (intact) cells, including the fact that this also is affected by the receptor–G-protein-interaction [17]. We actively work to develop a binding assay devoid of or less-affected by these problems.

In conclusion, the results of the chimaeric mutagenesis studies suggest that the most significant distinction between orexin peptides occurs in the region from the C-terminal end of TM2 to the C-terminal end of TM4 of the receptor. This explains most, but not all the selectivity, and it seems that the receptor subtypes may have evolved to stabilize their internal structures.

Acknowledgements

We gratefully acknowledge Dr. Ari Koivisto (Orion Corporation Orion Pharma R&D, Turku, Finland) for access to the FlexStation plate reader and Pirjo Puroranta for technical assistance. This study was supported by the Academy of Finland, the Sigrid Jusélius Foundation, the Magnus Ehrnrooth Foundation, the University of Helsinki Research Funds, the Novo Nordisk Foundation, the Swedish Research Council and the Uppsala University. Jaana Putula is supported by the Drug Discovery Graduate School (DDGS).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2011.04.020.

References

[1] de Lecea, L. et al. (1998) The hypocretins: hypothalamus-specific peptides with neuroexcitatory activity. Proc. Natl. Acad. Sci. USA 95, 322–327.
[2] Sakurai, T. et al. (1998) Orexins and orexin receptors: a family of hypothalamic neuropeptides and G protein-coupled receptors that regulate feeding behavior. Cell 92, 573–585.
[3] Chemelli, R.M. et al. (1999) Narcolepsy in orexin knockout mice: molecular genetics of sleep regulation. Cell 98, 437–451.
[4] Lin, L. et al. (1999) The sleep disorder canine narcolepsy is caused by a mutation in the hypocretin (orexin) receptor 2 gene. Cell 98, 365–376.
[5] Nishino, S., Ripley, B., Overeem, S., Lammers, G.J. and Mignot, E. (2000) Hypocretin (orexin) deficiency in human narcolepsy. Lancet 355, 39–40.
[6] Kukkonen, J.P., Holmqvist, T., Ammoun, S. and Åkerman, K.E. (2002) Functions of the orexinergic/hypocretinergic system. Am. J. Physiol. Cell. Physiol. 283, C1567–C1591.
[7] Boutrel, B., Kenny, P.J., Specio, S.E., Martin-Fardon, R., Markou, A., Koob, G.F. and de Lecea, L. (2005) Role for hypocretin in mediating stress-induced reinstatement of cocaine-seeking behavior. Proc. Natl. Acad. Sci. USA 102, 19168–19173.
[8] Harris, G.C., Wimmer, M. and Aston-Jones, G. (2005) A role for lateral hypothalamic orexin neurons in reward seeking. Nature 437, 556–559.
[9] Roecker, A.J. and Coleman, P.J. (2008) Orexin receptor antagonists: medicinal chemistry and therapeutic potential. Curr. Top. Med. Chem. 8, 977–987.
[10] Darker, J.G., Porter, R.A., Eggleston, D.S., Smart, D., Brough, S.J., Sabido-David, C. and Jerman, J.C. (2001) Structure–activity analysis of truncated orexin-A analogues at the orexin-1 receptor. Bioorg. Med. Chem. Lett. 11, 737–740.
[11] Okumura, T. et al. (2001) 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. 280, 976–981.
[12] Ammoun, S., Holmqvist, T., Shariatmadari, R., Oonk, H.B., Detheux, M., Parmentier, M., Åkerman, K.E. and Kukkonen, J.P. (2003) Distinct recognition of OX1 and OX2 receptors by orexin peptides. J. Pharmacol. Exp. Ther. 305, 507–514.
[13] Asahi, S., Egashira, S., Matsuda, M., Iwaasa, H., Kanatani, A., Ohkubo, M., Ihara, M. and Morishima, H. (2003) Development of an orexin-2 receptor selective agonist, [Ala(11),D-Leu(15)]orexin-B. Bioorg. Med. Chem. Lett. 13, 111–113.

[14] Putula, J., Turunen, P.M., Jäntti, M.H., Ekholm, M.E. and Kukkonen, J.P. (2011) Agonist ligand discrimination by the two orexin receptors depends on the expression system. Neurosci. Lett. 494, 57–60.
[15] Hungs, M., Fan, J., Lin, L., Lin, X., Maki, R.A. and Mignot, E. (2001) Identification and functional analysis of mutations in the hypocretin (orexin) genes of narcoleptic canines. Genome Res. 11, 531–539.
[16] Malherbe, P., Roche, O., Marcuz, A., Kratzeisen, C., Wettstein, J.G. and Bissantz, C. (2010) Mapping the binding pocket of dual antagonist almorexant to human orexin 1 and orexin 2 receptors: comparison with the selective OX1 (SB-674042) and OX2 (EMPA) antagonists. Mol. Pharmacol. 78, 81–93.
[17] Kukkonen, J.P. (2004) Regulation of receptor-coupling to (multiple) G-proteins. A challenge for basic research and drug discovery. Receptors Channels 10, 167–183.