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**Review Article**

# Development of structure-activity relationship of orexins: possible roles of three regions of orexin-B

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**Abstract**

Orexin-A and orexin-B are newly identified neuropeptides which are involved in a wide variety of physiological functions. The actions of orexins are mediated by the activation of the orexin-1 and orexin-2 receptors, which have distinct physiological roles. Orexin-B shows a relative selectivity for the orexin-2 receptor. Efforts to address the structure-activity relationship of orexin-B have shown that it is divided into three functional regions based on the potency of the several modified peptides as well as the sequence homology between several species. The C-terminal region is essentially required for receptor activation. The middle region is thought to play a role in receptor selectivity. The N-terminal region may be involved in the difference between the physiological functions of orexin-A and orexin-B. Furthermore, several lines of evidence support the idea that three leucine residues on the middle region could be important for the potency and selectivity of orexins, both functionally and structurally.

## Introduction

Due to extensive genomic research and reverse pharmacology strategies, many novel ligands for orphan G-protein-coupled receptors have been identified (1, 2). Current studies focus on elucidating the physiological and pathophysiological roles of such newly discovered ligands. Of these, orexin-A and orexin-B, also referred to as hypocretin-1 and hypocretin-2, are interesting neuropeptides that have been identified from an extract of rat hypothalamus (3). Based on their expression profiles, orexins were originally considered to be new regulators of feeding behavior (3-5). Currently, orexins are known to be involved in wide variety of physiological mechanisms, including regulation of sleep (6, 7), locomotor activity (8), drinking (9), stress (10), nociception (11), autonomic function (12, 13), gastric acid secretion (14), pituitary hormone secretion (15, 16) and energy metabolism (17). Thus, the orexin pathway is a promising therapeutic target.

The physiological actions of orexins are mediated by the activation of two receptors, the orexin-1 (OX1) and orexin-2 (OX2) receptors. There are several differences between these two receptor subtypes, including the expression patterns in the central nervous system (CNS) (18) and the preference for G-proteins (19) and agonists (3). These observations suggest that the two receptor subtypes mediate distinct physiological functions of orexins. In support of this hypothesis, OX2-deficient mice showed disturbance in sleep architecture whereas OX1-deficient mice did not show any significant phenotypes (20, 21).

Although orexin-B has moderate selectivity for OX2, the precise roles of each receptor subtype are still unknown. Thus, development of more selective ligands is important for further understanding of the roles of the two receptors. In our studies, we have evaluated the importance of each amino acid residue in the potency of orexin-B. In addition, accumulating evidence from several investigators using other types of orexin analog peptides have allowed us to develop a more detailed understanding of the structure-activity relationship (SAR) of orexin peptides. This review focuses on the development of the SAR of orexins, paying particular attention to selectivity for OX2.

## Structural differences between orexin-A and orexin-B

Human orexin-A and orexin-B are 33- and 28-amino acid peptides, respectively, that are proteolytically derived

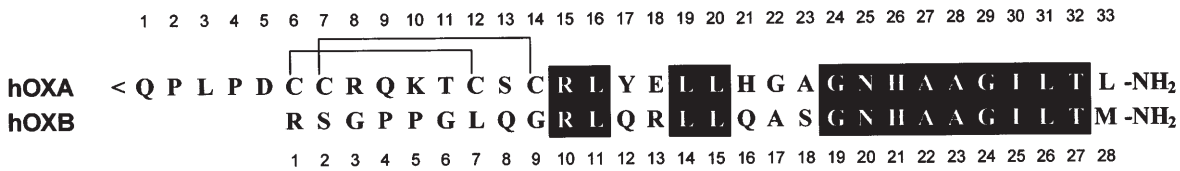


Fig. 1. Amino acid sequences of orexin peptides. Reversed letters indicate amino acids that are identical between orexin-A and orexin-B.

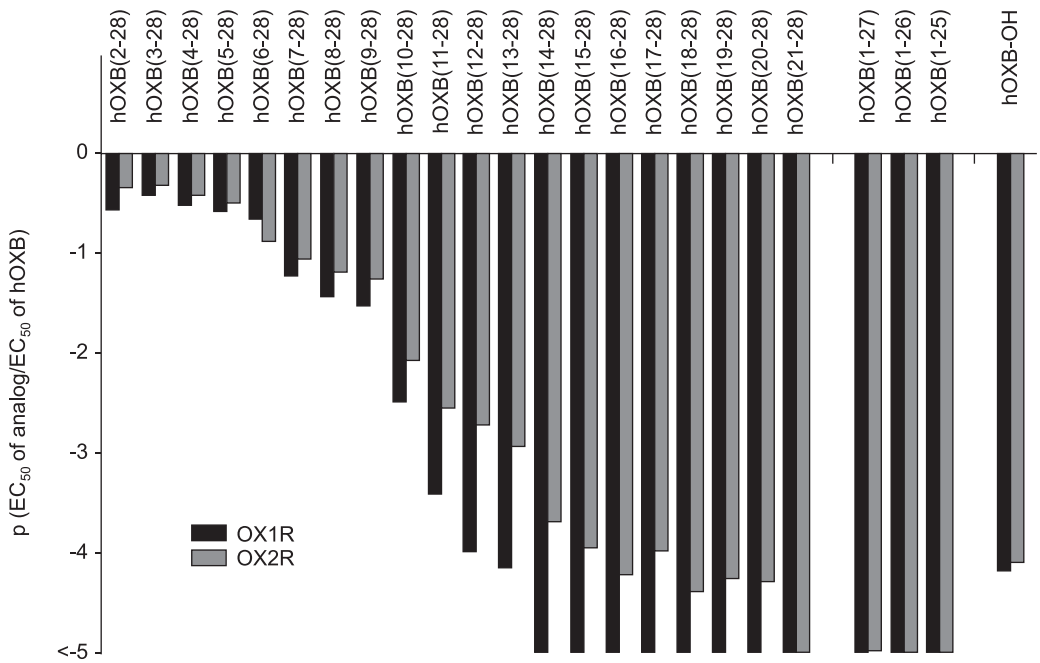


Fig. 2. Relative potency of terminally truncated orexin-B peptides. The values are expressed as the logarithm of the ratio of the $EC_{50}$s for truncated analogs *versus* parent orexin-B. Filled bars and hatched bars show the relative $EC_{50}$ ratios compared to OX1 and OX2, respectively. Orexin-B(1-27), orexin-B(1-26) and orexin-B(1-25) are amidated on their C-termini.

from a 131-amino acid prepro-orexin and are amidated on their C-termini. As shown in Figure 1, orexin-A has an N-terminal pyroglutamyl residue and two intramolecular disulfide bonds (3). In contrast, orexin-B has no additional modifications.

Orexin-A and orexin-B share 46% (13/28) amino acid identity (3). According to sequence similarity, orexins can be divided into three regions. The C-terminal 10 amino acids of both peptides are almost identical and are conserved among several mammals and other vertebrates, such as chicken (22) and *Xenopus* (23). This suggests that the C-terminal region plays an essential role in the activation of both orexin receptors (Fig. 1). The N-terminal portions of both peptides (amino acids 1 to 14 in orexin-A and 1 to 9 in orexin-B) have different lengths and diverse amino acid sequences. The middle part of the orexins consists of 9 amino acids (amino acids 15 to 23 in orexin-A and 10 to 18 in orexin-B) that are partially but regularly conserved between the two orexin peptides. Given that orexin-A has equal affinity and potency for both receptors, whereas orexin-B shows about 10-fold higher affinity and potency for OX2 (3), it appears that the unconserved N-terminal and partially conserved middle regions participate in receptor selectivity.

Because orexin-B has simpler structural properties and moderate receptor subtype selectivity, we have focused on orexin-B to generate subtype selective ligands and to evaluate the roles of the three regions in potency and selectivity.

## N- and C-terminal truncation of orexin-B

To analyze the requirement for regional structure in orexin-B, we have synthesized several truncated analogs of orexin-B (24) (Fig. 2). Only a few amino acid deletions from the C-terminus of orexin-B significantly reduced the potency of orexin-B for both orexin receptors, with over 50,000-fold reduction (24, 25) (Fig. 2). Additionally, a lack of C-terminal amidation also resulted in marked loss of

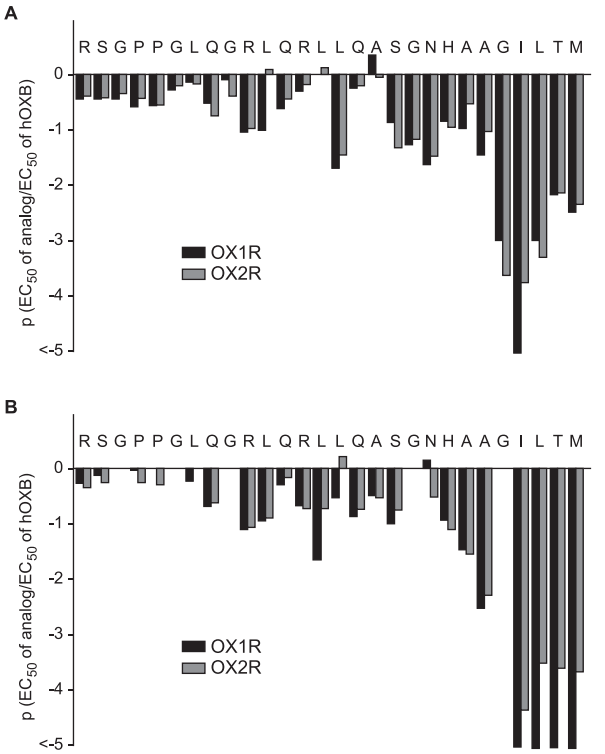


Fig. 3. Relative potency of orexin-B peptides with amino acid substitutions. The values are expressed as the logarithm of the ratio of the $EC_{50}$s for analog peptide *versus* parent orexin-B. Filled bars and hatched bars show relative $EC_{50}$ ratios compared to OX1 and OX2, respectively. (A). Each letter on the X-axis represents an amino acid of orexin-B that was replaced by alanine. Alanine residues of intact orexin-B were replaced by glycine. (B). Each letter on the X-axis represents an amino acid of orexin-B that was replaced by the corresponding D-amino acid.

activity for both receptors, suggesting that, like other C-terminally amidated neuropeptides, the C-terminal amino acid is required for receptor activation (24, 25) (Fig. 2). Furthermore, as shown in Figure 3, amino acid replacement in the C-terminal regions of orexin-A and orexin-B also caused a significant reduction in potency (24-28). These observations confirm the idea that the highly conserved C-terminal region is required for receptor activation.

In contrast, truncation within N-terminal five amino acids of orexin-B resulted in a moderate (up to 10-fold) reduction in potency for both receptors (Fig. 2) but did not change the intrinsic receptor selectivity. These results show that this region is unlikely to play a key role in receptor activation or selectivity. Similar observations have been reported by others for orexin-B (25) as well as for orexin-A (25, 28). However, this does not indicate that the N-terminal regions of orexin peptides are unnecessary for their physiological function. For instance, several reports have described a difference in brain and cerebrospinal fluid concentrations of the two orexin peptides even though they are derived from the same precursor (29-32). These observations suggest that the N-terminal region might be involved in solubility and proteolytic stability and, accordingly, be related to the difference in physiological function (33).

We also assessed the middle regions of orexin-B using additional truncated peptides from orexin-B(7-28) to orexin-B(16-28). We observed that the potencies for both receptors were gradually reduced depending on the extent of truncation (Fig. 2). The reduction was more remarkable for OX1 than for OX2 (Fig. 2), and a similar observation was also reported for orexin-A (28). In addition, both of the C-terminal decapeptides originating from orexin-A and orexin-B retained activity for OX2 but not OX1 (28) (Fig. 2). These results indicate that the extent of middle region of orexin-B is required for the activation of both receptors and also that the region is more strongly involved in activation of OX1 than OX2. Thus, these results suggest a possible role of the middle region in receptor selectivity.

These observations obtained from truncated peptides demonstrate that orexin-B can be functionally separated into three parts that are likely to be compatible with structural segmentation according to sequence similarities. The C-terminal regions of orexin peptides are functionally important as expected from their high sequence conservation. Unexpectedly, the highly diverse N-terminal region seems to be less important for intrinsic selectivity and potency, but may play a key role in their differential distribution patterns. In addition, the partially conserved middle region may help direct orexin receptor recognition and intrinsic selectivity.

## Amino acid modifications affecting receptor selectivity

Next, we performed two kinds of replacement studies, alanine scanning and D-amino acid scanning, to address the role of particular residues in the activity and selectivity of orexin-B. Alanine replacement is frequently performed to evaluate the importance of the side chain with minimum impact on three-dimensional structure. On the other hand, D-amino acid replacement is used to analyze the importance of the side chain orientation, and it causes minimal changes in overall hydrophobicity and charge. Although nearly all of the replacements had no significant effects on selectivity, we identified three amino acid residues that are important for receptor recognition (Fig. 4).

A replacement of $Leu^{11}$ with Ala retained potency for OX2 but not for OX1 (Fig. 3A). Thus, [$Ala^{11}$]orexin-B showed remarkable selectivity for OX2. The selectivity value, which is the logarithm of the ratio of the EC50 for OX1 to that for OX2, is 2.08 (Fig. 4). This suggests that the side chain at this position plays different roles in the activation of OX1 and OX2. For further investigation of the side chain requirements for both receptors, we performed replacement studies with other amino acids.

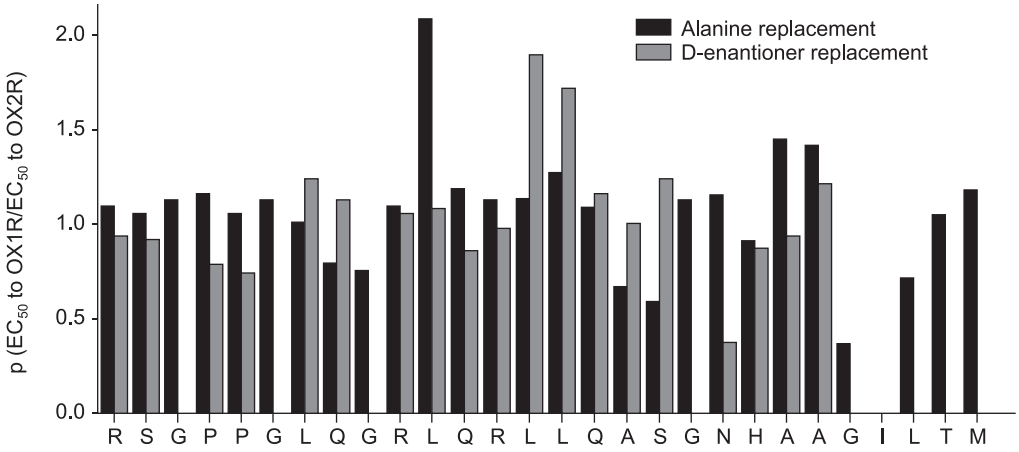


Fig. 4. Receptor selectivity of orexin-B analogs in two replacement studies. The values are expressed as the logarithm of the ratio of the $EC_{50}$s to OX1 *versus* OX2R. Positive values represent selectivity for OX2. Filled bars show results from alanine replacement, and hatched bars show results from D-enantiomer replacement. The value of intrinsic selectivity of orexin-B is 1.01.

Replacement of Leu[11] with the hydrophobic amino acids Ile and Val resulted in almost the same profiles as intact orexin-B (our unpublished data). Although Trp is a bulky residue compared to Leu, [Trp[11]]orexin-B showed only moderate reduction in the potency for both orexin receptors (27). In contrast, the analogs replaced with hydrophilic residues such as Arg, His, Glu (our unpublished data) or Ser (27) at this position revealed marked reductions in potency for both receptors. These results indicate that a hydrophobic property of the side chain at this position is required for the activation of both receptors. The Leu[11]-substituted analogs that have enhanced OX2 selectivity showed a selective reduction in potency for OX1. For example, [Trp[11]] and [Ser[11]]orexin-B (selectivity values = 1.51 and 2.08, respectively) reduced the potency for OX1 more markedly than for OX2 (27). This suggests that OX1 recognizes the side chain of Leu[11] more strictly than OX2. In contrast, [D-Leu[11]]orexin-B had equally reduced potency for both receptors (Fig. 3B), suggesting that the D-type side chain at this position might sterically interfere with association of the orexin-B peptides and both OX1 and OX2.

A significant reduction in potency for both receptors was observed in [D-Leu[14]]orexin-B (Fig. 3B). The reduction for OX1 was more marked than for OX2; thus, the substitution enhanced selectivity for OX2 (selectivity value = 1.89) (Fig. 4). In contrast, replacement of Leu[14] residue with Ala left the potency for both receptors unchanged, indicating that the Leu side chain at this position does not play an important role in receptor activation. This also indicates that the reduction in potency of [D-Leu[14]]orexin-B is not likely to be caused by lack of L-type Leu side chain, but rather might be due to steric hindrance caused by D-Leu side chain. The enhanced OX2 selectivity of [D-Leu[14]]orexin-B indicates that OX1 is more susceptible to D-amino acid replacement at this position compared to OX2.

The replacement of Leu[15] with Ala caused a significant reduction in potency for both OX1 and OX2 (47- and 27-fold, respectively) (Fig. 3A), indicating that the leucine residue at this position might be essential for the activation of both receptors. In support of this, the corresponding orexin-A analog, [Ala[20]]orexin-A, also noticeably reduced the potency for both receptors (28). In contrast, isomerization of L-Leu[15] to D-Leu had no effect on the activation of OX2 and caused a slight reduction in OX1 activity (selectivity value = 1.72) (Fig. 4), indicating that the orientation of the Leu[15] side chain might not be critical for either receptors, despite the importance of the leucine residue itself. A hydrophobic interaction between the Leu residue and the relatively large hydrophobic space in orexin receptors could play an important role in orexin receptor association.

Interestingly, all key amino acids identified from the replacement studies are leucine residues that have some common characteristics. For example, all of them are located in the middle part of orexin-B, which is likely to be involved in their selectivity as deduced from the truncated peptides. Also, they are highly conserved between orexin-A and orexin-B as well as among several species. And finally, enhanced selectivity of all analogs for OX2 are mainly due to a reduction in potency for OX1.

Based on these facts as well as accumulating evidence reported by other laboratories, we attempted to elucidate the role of the three leucine residues based on the proposed three-dimensional structure of orexin-B.

## Functional structure consisting of three leucine residues

The three-dimensional structure of orexin peptides has been characterized by NMR spectroscopy (34-36). The orexin-B peptide is thought to contain two helix

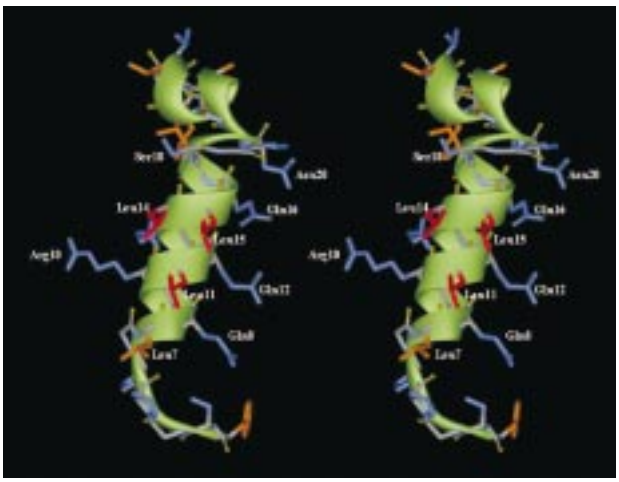

Fig. 5. Three-dimensional model of orexin-B. The three-dimensional model of orexin-B (PDB ID: 1CQ0) was generated using the Viewer Light 5.0.

structures, helix I, at amino acids 7 to 19, and helix II, at amino acids 23 to 28 (34). The helix arrangements on orexin-B are consistent with the domains identified by sequence similarity and by the sensitivity to amino acid truncation and replacement. Helix I is located in the middle part of orexin-B, wherein a gradual reduction in potency was observed with progressive truncation, and helix II is located in the highly conserved C-terminal region.

Interestingly, the three critical leucine residues whose modification enhanced OX2 selectivity are clustered on helix I and form a hydrophobic face on the structure (Fig. 5). Around the hydrophobic leucine residues, there are hydrophilic residues, including $Gln^{8}$, $Gln^{12}$, $Arg^{13}$, $Gln^{16}$, $Ser^{18}$ and $Arg^{10}$, and they form an amphipathic α-helix (36). According to this information from the proposed three-dimensional structure, we hypothesized that the leucine residues contribute to stabilize the structure of orexin-B which is required for the receptor activation. Previous reports indicate that the hydrophobicity of side chains in the nonpolar face of an amphipathic α-helical peptide contributes to its stability and that L- to D-amino acid replacements have destabilizing effects in an amphipathic α-helix (37, 38). Therefore, the three Leu residues, which form the hydrophobic face on helix I of orexin-B, might play an important role in helix stabilization. An important role of the helix I structure in the orexin-B peptide is strongly supported by the involvement of the middle region of orexin-B in receptor selectivity. In addition, not only the D-amino acid replacement but also the Pro replacement of these leucine residues, which are also known to interfere with helix formation, lowered the potency for OX1 more significantly than for OX2 (25, 27). These results suggest an important role of the L-type Leu residues in receptor activation, especially for OX1, possibly through helix stabilization. On the other hand, as mentioned previously, replacement studies show the importance of leucine residues themselves for receptor activation, indicating that these residues could also directly participate in the activation of orexin receptors. Therefore, it could be concluded that these Leu residues play important functional and structural roles in orexin receptor activation.

Because all of the corresponding leucine residues in orexin-A are completely conserved in mammals (Fig.1), it is difficult to explain the mechanisms of intrinsic selectivity of orexin-B by functions of these leucine residues. However, there is a critical difference in helix conformation in the middle region of orexin-A (35) and orexin-B (36), while all three leucine residues are located in helix structures. Therefore, the difference residing in the middle region of two orexin peptides might play a key role in demonstrating the intrinsic receptor selectivity of each peptide.

## Summary and future perspectives

Here we summarize the structure-activity relationship of orexin-B and possible roles of three regions of the peptide. N- and C-terminal truncation studies clarified three functional regions of the orexin-B peptide, which are also distinguished by their primary structures. The C-terminal region is essentially required for receptor activation. The middle region is thought to play a role in receptor selectivity. The N-terminal might be involved in solubility and proteolytic stability and related to the difference in physiological function of the two peptides. Further replacement studies identified three leucine residues in the middle part as functional and structural key residues for activation of OX1. As a result, the modification of these leucine residues caused an enhancement of OX2 selectivity. These three leucine residues are integrated in a helix structure in orexin-B. The proposed three-dimensional structure also supports the importance of these leucine residues in the conformation of the functional structure.

For further understanding, identification of ligand recognition sites of both orexin receptors remains to be elucidated. The analysis using point mutated and chimeric receptors should be beneficial for this purpose. In addition, receptor binding assay systems are very important for investigating the mechanisms of orexin receptor association. However, currently there is no reliable binding assay system possibly due to the adhesive properties of the orexin peptides. Recently, several groups have developed small-molecule antagonists for orexin receptors (39-41). These new ligands might be useful to develop the binding assay systems.

Based on the information from the SAR analysis described in this article, we have tried to develop a more OX2-selective analog by combining several replacements each of which produce enhanced selectivity (27). As a result, we successfully identified [$Ala^{11}$, D-$Leu^{15}$]orexin-B, which shows 400-fold selectivity for OX2 over OX1. It should be noted that [$Ala^{11}$, D-$Leu^{15}$]orexin-B is as potent in activating OX2 as intact

orexin-B and, therefore, should be a promising tool for elucidating the physiological roles of OX2.

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