

# OREXIN AND OREXIN RECEPTORS

Takeshi Sakurai \*

## 1. INTRODUCTION

“Reverse pharmacology”, i.e., ligand identification using cell lines expressing orphan receptors, combined with genetic engineering techniques, has increased our understanding of novel signaling systems in the body.<sup>1</sup> Orexin is the first and most successful example of the factors to which such an approach was applied.<sup>2</sup> Our group initially identified orexin A and orexin B as endogenous peptide ligands for two orphan G-protein-coupled receptors found as human expressed sequence tags.<sup>2</sup> Living in post-genome era, the success of the orexin story is driving many researchers to dig up novel bioactive peptides and their receptors for another discovery of new phases in physiology and novel opportunities for clinical treatment. This chapter discusses structures and functions of orexin neuropeptides and their receptors.

## 2. IDENTIFICATION OF HYPOCRETIN AND OREXIN

Orexin/hypocretin peptides were identified by two independent groups utilizing completely different methodologies. de Lecea *et al.* utilized a subtractive-PCR technique to identify transcripts that are expressed specifically in the hypothalamus.<sup>3</sup> They had previously isolated a series of cDNA clones that are expressed in the hypothalamus but not in the cerebellum and the hippocampus. One of these was expressed exclusively by a bilaterally symmetric structure within the posterior lateral hypothalamus. They subsequently cloned cDNAs covering the entire coding region, which encodes a putative secretory protein of 130 amino acids. According to its primary sequence, they predicted that this protein gives rise to two novel peptide products that are structurally related to each other. They named them hypocretin-1 and hypocretin-2.

Around the same time with the report by de Lecea *et al.*, our group reported identification of orexins (orexin-A and orexin-B) by “reverse pharmacological” approach.

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\* T. Sakurai, Institute of Basic Medical Sciences, Departments of Pharmacology, University of Tsukuba, Ibaraki 305-8575, Japan and ERATO Yanagisawa Orphan Receptor Project, Japan Science and Technology Corporation, Tokyo 135-0064, Japan

Structures of orexins were chemically determined by biochemical purification and sequence analysis by Edman sequencing and mass spectrometry.<sup>2</sup> Orexin A and orexin B

constitute a novel peptide family, with no significant homology with any previously described peptides. Orexin A is a 33-amino-acid peptide of 3,562 Da, with an N-terminal pyroglutamyl residue and C-terminal amidation (Fig 1B). Molecular mass of the purified peptide as well as its sequencing analyses indicated that the four Cys residues of orexin A formed two sets of intrachain disulfide bonds. The topology of the disulfide bonds was chemically determined to be [Cys<sup>6</sup>-Cys<sup>12</sup>, Cys<sup>7</sup>-Cys<sup>14</sup>]. This structure is completely conserved among several mammalian species (human, rat, mouse, cow, sheep, dog and pig). On the other hand, rat orexin B is a 28-amino-acid, C-terminally amidated linear peptide of 2,937 Da, which was 46% (13/28) identical in sequence to orexin A (Fig 1B). The C-terminal half of orexin B is very similar to that of orexin A, while the N-terminal half is more variable. The mouse orexin B was predicted to be identical to rat orexin B. The human orexin B has two amino acid substitutions from the rodent sequences within the 28-residue stretch. Pig and dog orexin B have one amino acid substitution from the human or rodent sequences. Other than mammalian species, structures of xenopus and chicken orexin A and orexin B, which have also conserved structures as compared with mammalian sequences, was elucidated (Figure 1A).

The prepro-orexin cDNA sequences revealed that both orexins are produced from the same 130-residue (rodent) or 131-residue (human) polypeptide, prepro-orexin by proteolytic processing. The human and mouse prepro-orexin sequences are 83% and 95% identical to the rat counterpart, respectively (Fig 1A).<sup>2</sup> The majority of amino acid substitutions were found in the C-terminal part of the precursor, which appears unlikely to encode another bioactive peptide (Fig 1A).

It turned out later that prepro-orexin is identical to prepro-hypocretin and that orexin-A and -B correspond to hypocretin1 and 2, respectively. The original description of predicted structures of hypocretin 1 and 2 was incomplete as it did not predict the signal peptide proteolytic site, nor two intrachain disulfate bonds and N-terminal pyroglutamylation of hypocretin-1.<sup>2,3</sup> Nevertheless, hypocretins and orexins currently recognized as same peptides and used as synonyms.

We reported orexins initially as orexigenic peptides.<sup>2</sup> Subsequently they have been reported to have a variety of pharmacological actions (see other chapters). Especially, recent observations implicate orexins/hypocretins in sleep disorder narcolepsy and in the regulation of the normal sleep process. The biological activities of orexins are discussed in other chapters of this book.

### 3. PREPRO-OREXIN GENE, STRUCTURE AND REGULATION OF EXPRESSION

The human prepro-orexin gene, which is located on chromosome 17q21, consists of two exons and one intervening intron distributed over 1432 bp.<sup>4</sup> The 143-bp exon 1 includes the 5'-untranslated region and the coding region that encodes the first seven residues of the secretory signal sequence. Intron 1, which is the only intron found in the human prepro-orexin gene, is 818-bp long. Exon 2 contains the remaining portion of the open reading frame and the 3'-untranslated region.

The human prepro-orexin gene fragment, which contains the 3149-bp 5'-flanking region and 122-bp 5'-non-coding region of exon 1, was reported to have an ability to express lacZ in orexin neurons without ectopic expression in transgenic mice, suggesting that this genomic fragment contains all of necessary elements for appropriate expression

of the gene.<sup>4</sup> This promoter is useful to examine the consequences of expression of exogenous molecules in orexin neurons of transgenic mice, thereby manipulating the cellular environment *in vivo*.<sup>4-6</sup> For example, this promoter was used to establish several transgenic lines, including orexin neuron-ablated mice and rats, and mice in which orexin neurons specifically express green fluorescent protein.<sup>5,6</sup>

The mechanisms that regulate expression of the prepro-orexin gene still remain unclear. Prepro-orexin mRNA was shown to be upregulated under fasting conditions, indicating that these neurons somehow sense the animal's energy balance.<sup>2</sup> Several reports have shown that orexin neurons express leptin receptor- and STAT-3-like immunoreactivity, suggesting that orexin neurons are regulated by leptin.<sup>7</sup> Consistently, we found that continuous infusion of leptin into the third ventricle of mice for 2 weeks resulted in marked down-regulation of prepro-orexin mRNA level.<sup>5</sup> Therefore, reduced leptin signalling may be a possible factor that up-regulates expression of prepro-orexin mRNA during starvation. Prepro-orexin levels were also increased in hypoglycemic conditions, suggesting that expression of the prepro-orexin gene is also regulated by plasma glucose levels.<sup>8</sup> These observations are consistent with our electrophysiological study of GFP-expressing orexin neurons in transgenic mice showed that orexin neurons are regulated by extracellular glucose concentration and leptin.<sup>5</sup>

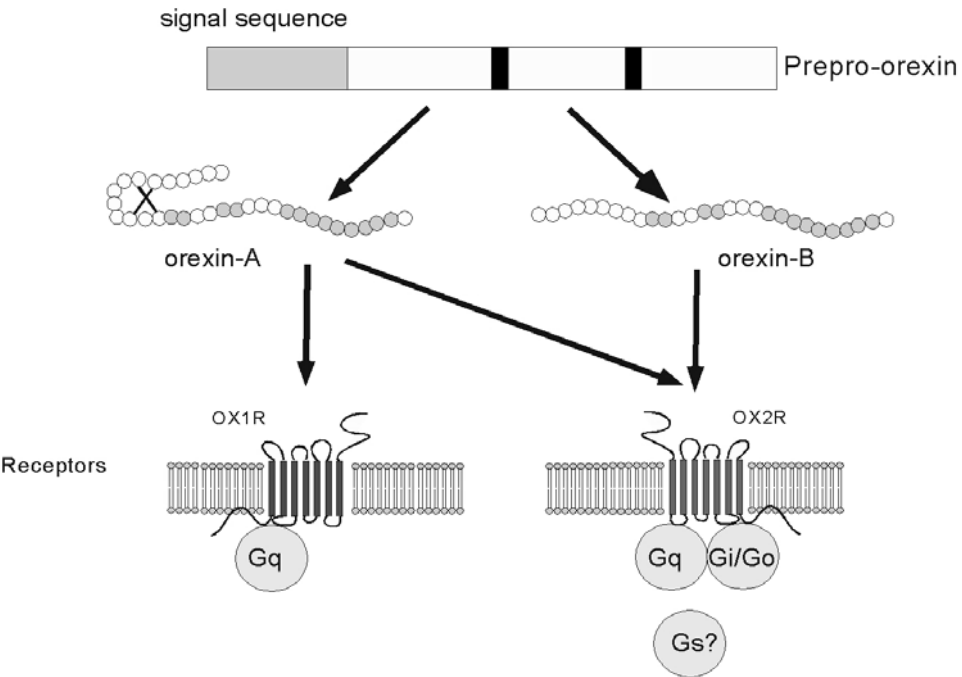
#### 4. STRUCTURES AND PHARMACOLOGY OF OREXIN RECEPTORS

Initially, we used an orphan GPCR termed HFGAN72 (which now named as orexin-1 receptor, OX1R or *hcrtr1* to identify orexins. Since we found that orexin B has significantly lower affinity for the human (OX1R), we sought other orexin receptors for which orexin B has high affinity. A BLAST search of the dbEST database with the OX1R amino acid sequence detected another orexin receptor, orexin-2 receptor OX2R, or *hcrtr2*.<sup>2</sup> The actions of orexins are mediated by these two receptors.<sup>2</sup> Among various classes of G protein-coupled receptors, OX1R is structurally more similar to certain neuropeptide receptors, most notably to the Y2 Neuropeptide Y (NPY) receptor (26% similarity), followed by the thyrotropin-releasing hormone (TRH) receptor, cholestykinin type-A receptor and NK2 neurokinin receptor (25 %, 23% and 20% similarity, respectively).

The amino acid identity between the deduced full-length human OX1R and OX2R sequences is 64%. Thus, these receptors are much more similar to each other than they are to other GPCRs. Amino acid identities between the human and rat homologues of each of these receptors are 94% for OX1R and 95% for OX2R, indicating that both receptor genes are highly conserved between the species. Competitive radioligand binding assays using CHO cells expressing OX1R suggested that orexin A is a high-affinity agonist for OX1R. The concentration of cold orexin A required to displace 50% of specific radioligand binding ( $IC_{50}$ ) was 20 nM. Human orexin B also acted as a specific agonist on OX1R. However, human orexin B has significantly lower affinity compared to human OX1R: the calculated  $IC_{50}$  in competitive binding assay was 250 nM for human orexin B, indicating 2 orders of magnitude lower affinity as compared with orexin. A (Fig 2).

On the other hand, binding experiments using CHO cells expressing the human OX2R cDNA demonstrated that OX2R is a high affinity receptor for human orexin B with  $IC_{50}$  of 20 nM. Orexin A also had high affinity for this receptor with  $IC_{50}$  of 20 nM,

which is similar to the value for orexin B, suggesting that OX2R is a non-selective receptor for both orexin A and orexin B (Fig 2).



**Figure 2.** Schematic representation of orexin system. Orexin-A and orexin-B are derived from a common precursor peptide, prepro-orexin. The actions of orexins are mediated via two G protein-coupled receptors named orexin-1 (OX1R) and orexin-2 (OX2R) receptors. OX1R is selective for orexin-A, whereas OX2R is a nonselective receptor for both orexin-A and orexin-B.

5. GENETICS OF OREXIN RECEPTORS

Genetic studies revealed that dogs with a mutation of *Hcrtr2* gene or OX2R-knockout mice displayed a narcolepsy-like phenotype,<sup>9,10</sup> while OX1R knockout mice did not reveal any obvious abnormality in the sleep/wake states.<sup>10</sup> These studies provide strong evidences for the roles of OX2R in regulating the vigilance state in human and animals. However, the behavioral and electroencephalographic phenotype of OX2R knockout mice is less severe than that found in prepro-orexin knockout mice.<sup>9</sup> OX2R knockouts are only mildly affected with cataplexy-like attacks of REM sleep, whereas orexin knockout mice are severely affected.<sup>9</sup> Double receptor knockout (OX1R- and OX2R-null) mice appear to have the same phenotype of prepro-orexin knockout mice, suggesting that OX1R also has additional effects on sleep/wakefulness. These findings suggest that loss of signaling through both receptor pathways is necessary for emerging all of narcoleptic characteristics.

The phenotypes of orexin receptor knockout mice are more precisely discussed in another chapter.

## 6. HOW MANY OREXIN RECEPTOR GENES?

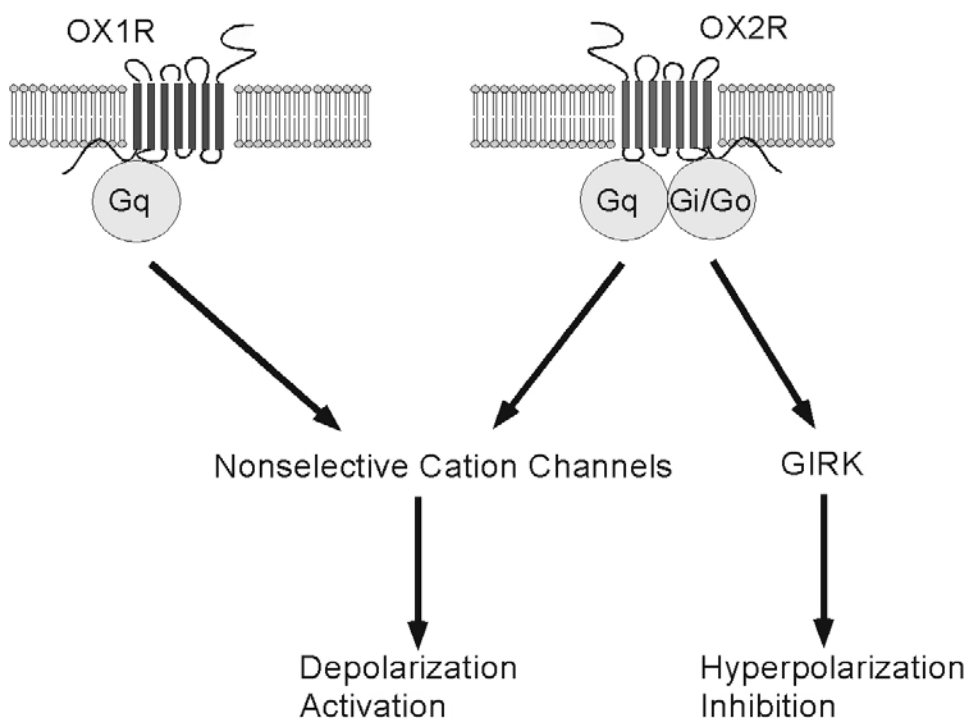
Two genes for orexin receptors have been identified in mammalian species thus far. The phenotypes of OX1R and OX2R double-deficient mice were analyzed and shown to have sleep state abnormalities, which were indistinguishable from that of prepro-orexin gene-deficient mice. This observation suggests that only two receptors for orexins might exist in mammals. However, it is possible that there are other subtypes of receptors produced from OX1R or OX2R genes by alternative splicing. In fact, two alternative C-terminus splice variants of the murine OX2R, termed m OX2alphaR (443 aa) and m OX2betaR (460 aa) have been reported,<sup>11</sup> although orexin A and orexin B showed no difference in binding characteristics between the splice variants.

## 7. SIGNAL TRANSDUCTION SYSTEMS OF OREXIN RECEPTORS

Both OX1R and OX2R are G-protein coupled receptors, which transmit information into cells by activating heterotrimeric G proteins. Activation of the signaling pathways associated with distinct G-proteins may contribute to the diverse physiological roles of orexin in particular neurons. Although many G-protein-coupled neurotransmitter receptors are potentially capable of modulating both voltage-dependent calcium channels and G-protein-gated inwardly rectifier potassium channels (GIRKs), there might be a substantial degree of selectivity in the coupling to one or other of these channels in neurons (Fig. 3). The signal transduction pathways of orexin receptors were examined in cells transfected with OX1R or OX2R. In OX1R-expressing cells, forskolin-stimulated cAMP was not affected by orexin administration. In addition, PTX treatment did not show any effects on orexin-induced increases in  $[Ca^{2+}]_i$ . These results suggest that OX1R does not couple to PTX-sensitive  $G_{i/o}$  proteins.<sup>12</sup> In contrast, orexin inhibited forskolin-stimulated cAMP production in a dose-dependent manner in OX2R-expressing cells. The effect was abolished by pretreatment with PTX. However, orexin-induced increases in  $[Ca^{2+}]_i$  was not affected by PTX treatment in OX2R-expressing cells. These results indicate that the OX2R couples to PTX-sensitive G proteins which were involved in the inhibition of adenylyl cyclase by orexin. These results suggest that OX1R couples exclusively to PTX-insensitive G proteins, and OX2R couples to both PTX-sensitive and -insensitive proteins (Fig 3). The relative contribution of these G-proteins in the regulation of neuronal activity remains unknown. Orexins have been shown to have an excitatory activity in many types of neurons in vivo. For instance, noradrenergic cells of the LC,<sup>13</sup> dopaminergic cells of the ventral tegmental area,<sup>14</sup> and histaminergic cells from the TMN<sup>15</sup> have been shown to be activated by orexins. Because, LC neurons exclusively express OX1R, while TMN neurons exclusively express OX2R, these observations suggest that both OX1R and OX2R signaling are basically excitatory on neurons.

However, these studies only examined effect of orexins on receptor-expressing cell bodies. There is a possibility that orexin receptors are also on presynaptic terminals, because Li *et al.*<sup>16</sup> reported that orexin increases local glutamate signalling by facilitation of glutamate release from presynaptic terminals. Therefore, it is possible that activation

of PTX-sensitive G proteins in the downstream of OX2R might be involved in functions other than activation of neurons, such as in the tips of developing neurites and on presynaptic nerve terminals, leading to growth cone collapse and enhanced synaptic release of the transmitter. Alternatively, OX2R mediated activation of Gi might result in inhibition of some population of neurons. In fact, orexin was recently reported to inhibit proopio-melanocortin neurons in the arcuate nucleus *in vitro*.<sup>17</sup>



**Figure 3.** Schematic drawing of the intracellular signal transduction systems of orexin receptors in neurons. OX1R is coupled exclusively to the Gq subclass of heterotrimeric G proteins, whereas OX2R may couple to Gi/o, and/or Gq.

The other reports also suggested that in adrenocortical cells, orexins stimulate corticosterone secretion through the activation of the adenylate cyclase-dependent signaling via an activation of OX2R.<sup>18-20</sup> The induction of cAMP in the adrenal cortex might be a cell-type dependent phenomenon, since in PC12 cells, orexins inhibited the PACAP-induced increase in the cAMP.<sup>21</sup> These differences in G-protein coupling might be influenced by receptor density and/or densities in each G-proteins.

Recently, several studies showed molecular interactions and cross-talks between orexin receptors and other receptors. Hilairt et al. showed that when the cannabinoid receptor (CB1) and OX1R are co-expressed, there is a CB1-dependent enhancement of the orexin A potency to activate the mitogen-activated protein kinase pathway. It was also shown that CB1 and OX1R are closely apposed at the plasma membrane to form

**Table 1:** Distribution of OX1R and OX2R in the rat brain. The relative density of labeling is classified as: absent (-); sparse (+); moderate (++); extensive (+++); very extensive (++++)

Region	Ox1 r ir	Ox1r mRNA	Ox2r mRNA
<b>Telencephalon</b>			
<i>Olfactory system</i>			
Anterior olfactory nu	+++	++	-
Piriform cortex	+++	-	++
Tenia tecta	++	+++	+
<b>Cerebral cortex</b>			
Granular insular cortex	+	+	-
Neocortex layer 6	+	+	++
Neocortex layer 5	+	+	-
Neocortex layer 2	-	-	++
Clastrum	+	+	-
Cingulate/Retro splenial cortex	++	++	-
<b>Basal Ganglia</b>			
Caudate putamen	+	-	-
Globus palidus	++	-	+
Substantia nigra, pars compacta	++	+	+
Subthalamic Nucleus	++	+	++
Nucleus accumbens, rostral	-	-	+
<i>Hippocampal formation</i>			
CA1 region	+	+	-
CA2 region	++	++	-
CA3 region	+	-	+++
Dentate gyrus	+++	++	+
<b>Amygdala</b>			
Amygdaloid nuclei	++	++	++
Substantia innominata	++	+	+
<i>Septal and basal magnocellular</i>			
Bed nucleus of the stria terminals	+++	+++	++
Lateral septal nucleus, dorsal part	++	-	+
Medial septal nucleus	++		+++
Nucleus of the horizontal limb of the diagonal band	++	++	+++
Nucleus of the vertical limb of the diagonal band	+	+	++
<b>Thalamus</b>			
Anteromedial thalamic nucleus	+++	++	+
Centrolateral thalamic nucleus	+++	+	++
Centromedial thalamic nucleus	+	+	++
Paracentral thalamic nucleus	++	-	+
Paraventricular thalamic nucleus	+	+++	++
Reticular thalamic nucleus	+++	++	-
Zona incerta	+++	++	++
Lateral & medial geniculate nuclei	++	-	-
<i>Hypothalamic Preoptic Nuclei</i>			
Anteroventral preoptic area		+	
Magnocellular preoptic area	+	+	++
Medial preoptic nu.	-	-	++
medial preoptic nucleus	++	++	++
supraoptic nucleus	+	+	+
ventrolateral preoptic area	+	+	+
ventromedial preoptic area	-	-	+
<b>Hypothalamus</b>			
Anterior hypothalamic area	+++	++	+
Arcuate hypothalamic nu	+++	-	+++
Dorsomedial hypothal. nu	+	+	+++
Lateral hypothal. area (LHA)	++	+	+++
Magnocellular preoptic nu	+	+	+
Medial mammillary nu	++	-	++
Paraventricular hypothalamic	+++	-	++
Posterior hypothal. area	++	++	++
Premammillary nu	++	++	+++
Supraoptic nu	+++	+	+
Suprachiasmatic nu	+++	-	-
Ventromedial hypothalamic nu	+++	++	-
<b>Mesencephalon</b>			
Dorsal tegmental nu	+++	++	+
Inferior colliculus	+	+	+
Interpeduncular nu	++	+	++
Periaqueductal grey	++	++	++



Principal sensory trigeminal nucleus	++	-	++
Raphe nuclei	++	++	++
Substantia nigra, pars compacta	++	++	++
Superior colliculus	+	+	+
<b>Rhombencephalon</b>			
Facial nucleus	+++	-	++
Locus coeruleus	+++ +	++++	-
Pontine reticular nucleus	++		++
Spinal trigeminal	++	-	++

nucleus			
<b>Cerebellum</b>			
Cerebellar cortex			
Deep cerebellar nuclei	++		
<b>Spinal cord and dorsal root</b>			
Spinal cord (grey matter, dorsal and ventral horn)	+++		
DRG	+++		

heterodimers.<sup>22</sup> It was also showed that OX1R and OX2R are capable of forming a homo- or heterodimer<sup>23</sup>. These observations suggest complex signaling cascade might exist in the downstream of orexin receptors, although we have to be careful about interpretation of these results from in vitro experiments.

## 8. DISTRIBUTION OF OREXIN RECEPTORS

Although orexin receptors are basically expressed in a pattern consistent with projections of orexin-producing neurons, mRNA for OX1R and OX2R were shown to be differentially distributed throughout the brain (Table 1). For instance, within the hypothalamus, a low level of OX1R mRNA expression is observed in the dorsomedial hypothalamus (DMH), while a higher level of OX2R mRNA expression is observed in this region. Other areas of OX2R expression in the hypothalamus are the arcuate nucleus, paraventricular nucleus (PVN), LHA, and most significantly, the tuberomammillary nucleus (TMN)<sup>24</sup>. In these regions, there is little or no OX1R signal. In the hypothalamus, OX1R mRNA is abundant in the anterior hypothalamic area and ventromedial hypothalamus (VMH).

Outside the hypothalamus, high levels of OX1R mRNA expression are detected in the tenia tecta, hippocampal formation, dorsal raphe nucleus, and most prominently, the locus coeruleus (LC). OX2R mRNA is abundantly expressed in the cerebral cortex, nucleus accumbens, subthalamic nucleus, paraventricular thalamic nuclei, anterior pretectal nucleus, and the raphe nuclei.

Within the brain, OX1R is most abundantly expressed in the LC, while OX2R is most abundantly expressed in the TMN, regions highly important for maintenance of arousal. The raphe nuclei contain both receptor mRNAs. These observations suggest strong interaction between orexin neurons and the monoaminergic systems. More precise description of the distribution of orexin receptors is discussed in other chapters.

## 9. STRUCTURE-ACTIVITY RELATIONSHIPS

Activities of synthetic orexin B analogs in cells transfected with either OX1R or OX2R were examined to define the structural requirements for activity of orexins on their

receptors.<sup>25</sup> The ability of N- or C-terminally truncated analogs of orexin B to increase cytoplasmic  $\text{Ca}^{2+}$  levels in the cells showed that the absence of N-terminal residues had little or no effect on the biological activity and selectivity of both receptors. Truncation from the N-terminus to the middle part of orexin B resulted in moderate loss of activity, in the order of peptide length. In particular, deletion of the conserved sequence between orexin A and orexin B caused a profound loss of biological activity, and the C-terminally truncated peptides were also inactive for both receptors. These results suggest that the consensus region between orexin A and orexin B is important for the activity of both receptors.

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