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Orexin Receptor Multimerization versus Functional Interactions: Neuropharmacological Implications for Opioid and Cannabinoid Signalling and Pharmacogenetics

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Abstract: Orexins/hypocretins are neuropeptides formed by proteolytic cleavage of a precursor peptide, which are produced by neurons found in the lateral hypothalamus. The G protein-coupled receptors (GPCRs) for these ligands, the OX_1 and OX_2 orexin receptors, are more widely expressed throughout the central nervous system. The orexin/hypocretin system has been implicated in many pathways, and its dysregulation is under investigation in a number of diseases. Disorders in which orexinergic mechanisms are being investigated include narcolepsy, idiopathic sleep disorders, cluster headache and migraine. Human narcolepsy has been associated with orexin deficiency; however, it has only rarely been attributed to mutations in the gene encoding the precursor peptide. While gene variations within the canine OX_2 gene *hcrtr2* have been directly linked with narcolepsy, the majority of human orexin receptor variants are weakly associated with diseases (the idiopathic sleep disorders, cluster headache and polydipsia-hyponatremia in schizophrenia) or are of potential pharmacogenetic significance. Evidence for functional ineractions and/or heterodimerization between wild-type an variant orexin receptors and opioid and cannabinoid receptors is discussed in the context of its relevance to depression and epilepsy.

Keywords: orexin/hypocretin; OX_1 orexin receptor; OX_2 orexin receptor; homo-dimerization; hetero-dimerization; opioid receptor; CB_1 cannabinoid receptor; status epilepticus; feeding behavior; sleep disorder

Introduction

The identification of a role for the orexins in narcolepsy has contributed greatly to the field of orexin genetics [1], but later findings related to other physiological functions, including genetic determinants, also continue to propel this research [2]. The discovery of other functions of the orexinergic system has given fresh rationales for exploring orexin pharmacogenetics. Since the

development of orexinergic drugs as sleep aids [3], a need for a greater understanding of the molecular pharmacology and pharmacogenomics of the orexins has become even more clear [4,5]. To understand orexin pharmacology, it is both practical and informative to examine the putative signaling disruptions in the patient populations world-wide.

Orexin receptors belong to the rhodopsin family of the G protein-coupled receptor (GPCR) superfamily. They have been described as able to interact with several heterotrimeric G protein subfamilies as well as other proteins, which results in a range of cellular responses via, e.g., ion channels, phospholipases and protein kinases, adding up to numerous events such neuronal excitation, synaptic plasticity, and cell death [6–8]. The specific signaling features in different cell types are likely determined by the expression profile of signaling components, signal complexes and other concurrent signals.

An outline of the molecular biology of the orexins, therefore, is introduced prior to examining orexin pharmacology in the context of the functional interactions and/or di/oligomer formation that take place with other receptor systems such as the cannabinoids. Sequencing projects, such as the Exome Aggregation Consortium (http://exac.broadinstitute.org), have revealed that variants of the OX_1 and OX_2 receptors are common in the population (Table 1 and Table 2); the functional significance of variant receptors is discussed. The naturally occurring variants have thus far been only weakly associated with disease and thus the functional characterization of these variants has been given lower priority. However, we will discuss the possibility that co-expression of variant receptors could result in a distinct pharmacology through functional interaction or as a result of the formation of variant heterodimers. We discuss orexin receptor di-/oligomers in the context that homo- or heteromerization of variant orexin receptors may be involved in the development of at least some disease or pharmacogenetic phenotypes.

Table 1. OX_1 orexin receptor variants investigated in disease. aa, amino acid; IC, intracellular loop; SNP, single nucleotide polymorphism; TM, transmembrane helix [2].

OX_1 aa	Location	SNP	Numbering/ Peyron	Numbering/ 0lafsdottir	Frequency	Findings	Ref.
167 Gly/Ser	TM4	rs44603792	-	652 G/A	0002	-	[9]
265 Leu/Met	IC3	rs41501244	793 C/A	-	0.001	-	[10]
279 Arg/Gln	IC3	rs7516785	-	989 G/A	0.008	-	[9]
280 Gly/Ala	IC3	NP_001516	842 *GIA*	995 G/A	0.008	-	[9,10]
281 Arg/His	IC3	rs41439244					
408 Ile/Val	C-term	rs227I933	1222 *GIA*	1375 G/A	0.56	-	[9,10]
-	-	-	-	-	-	1.4 X migraine	[11]
-	-	-	-	-	-	1.6 X mood disorders	[12]
-	-	-	-	-	-	Polydipsia-hyponatremia in schizophrenia	[13,14]
-	-	-	-	-	-	Panic not linked	[15]

Table 2. OX_2 orexin receptor variants investigated in disease. aa, amino acid; IC, intracellular loop; SNP, single nucleotide polymorphism; TM, transmembrane helix; CH, cluster headache [2].

OX_2aa	Location	SNP	Numbering/ Peyron	Numbering/ Olafsdottir	Frequency	Findings	Ref.
10 Pro/Ser	N-term	rs41271310	28 C/T	352 C/T 0.003	0.003	Not linked	[5,9,10]
-	-	-	-	-	-	with narcolepsy	-
11 Pro/Thr	N-term	rs4127312	31 C/A	355 C/A	0.005	Not linked	[5,9,10]
-	-	-	-	-	-	with narcolepsy	-
193 Cys/Ser	TM4	rs41381449	577 T/A	-	0.002	-	[10]
293 Ile/Val	IC3	rs74720047		1201 G/A	0.002	-	[9]
308 Ile/Val	TM6	rs26533	922 G/A	1246 G/A	0.84	-	[9,10]
-	-	-	-	-	-	Ile assoc. with panic	[15]
-	-	-	-	-	-	Migraine not associated	[16–18]
-	-	-	-	-	-	Some association	[18]
-	-	-	-	-	-	with CH	-
-	-	-	-	-	-	No effect on triptan response	[17]
401Thr/lle	C-term	rs41321149	1202 C/T	0.00012	-	-	[10]

The Molecular Biology of the Orexins

In 1998, two research teams, the groups of De Lecea/Sutcfliffe and Sakurai/Yanagisawa, independently published their discovery of novel neuropeptides which are exclusively expressed in a small group of neurons localized to the lateral hypothalamus [15,19,20]. Based on location of origin and sequence similarity to the gut hormone secretin, the De Lecea/Sutcfliffe group named these peptides the hypocretins [20]. As the lateral hypothalamus is classically implicated in the regulation of feeding, the Sakurai/Yanagisawa group assessed the role of this neuropeptide system in appetite regulation; the positive experimental results lead Sakurai/Yanagisawa group to name the peptides "orexins" after the Greek word for appetite [19]. Both groups in parallel identified the precursor peptide, preprohypocretin or preproorexin (PPO), which undergoes cleavage to form two smaller peptides, named hypocretin-1 and -2 or orexin-A and -B, respectively. The gene which encodes for the PPO is located in human chromosome 17q21.2, and codes for a 131 amino acids-long peptide. The processed mature neuropeptides orexin-A/hypocretin-1 and orexin-B/hypocretin-2 are 33 and 28 amino acids in length, respectively.

The initial focus of the De Lecea/Sutcliffe group was to identify mRNA transcripts selectively expressed in the hypothalamus [20]. Subsequent biological assays with the hypocretin-2/orexin-B peptide showed neuroexcitatory activity on cultured hypothalamic neurons. The work of the Sakurai/Yanagisawa group began with identifying peptide ligands for the putative orphan GPCR, HFGAN72 [19]. Using extracts of rat brain and a cell-based reporter system, the receptor was found to be activated by two peptides now known as orexin-A and -B. The orphan receptor was found to have highest affinity for orexin-A, and coined OX_1 receptor. By making use of homology, an additional orexin receptor, OX_2, was identified, and subsequently shown to have an equal affinity for both orexin peptides. PPO mRNA distribution in the central nervous system (CNS) was mapped. The peptides were found to be linked to the regulation of feeding behavior based on evidence that they stimulated food intake upon intracerebroventricular (ICV) administration, and that PPO mRNA expression in the hypothalamus was increased upon fasting [19].

In addition to appetite, further studies established the role of the orexin/hypocretin system in sleep and wakefulness cycles, metabolic regulation, stress responses, reward/addiction and analgesia [6,19,21,22]. This complexity may reflect the widespread projections of orexin-producing neurons within the CNS. The physiological role for the action of the orexin/hypocretin ligands at the OX_1 and OX_2 receptors in the regulation of wakefulness and sleep is one example. Mignot and co-workers isolated two *hcrtr2* gene (the gene encoding OX_2) frame-shift mutations responsible for hereditary canine narcolepsy [23]. The frame-shifts both generate a premature stop codon. Truncated receptor proteins do not traffic to the plasma membrane and remain localized in the

cytoplasm [24]. Work by Yanagisawa and co-workers concurrently showed that knockout of the precursor peptide, PPO, causes a narcoleptic phenotype in mice [25], which is stronger than the phenotype obtained upon OX_2 knockout [26]. In 2000, a profound decrease in the concentration of orexin-A was reported in the cerebrospinal fluid (CSF) of human narcoleptics with cataplexy [27]. Only a few narcolepsy patients have been shown to harbor T47G (Leu16Arg; the signal peptide) and –C22T (a.k.a. C3250T; 5′ untranslated region) variants of PPO [1,10]. Studies of the Leu16Arg PPO mutant suggested that the processing and trafficking of the PPO are impaired [10], leading to subsequent problems with orexin peptide maturation and release. Although rare, the pathogenic orexin peptide variants identified in narcolepsy provided the rationale for the systematic study of orexin signaling in sleep disorders and with respect to individual differences in drug response.

Orexins play a role in numerous physiological processes including sleep-wake cycle regulation and the stimulation of feeding and regulation of energy homeostasis [28]. Additionally, exogenous orexins stimulate a number of processes in the periphery of the body, including gastric acid secretion [29] and glucocorticoid release [30,31], but the physiological significance of these is not known. Furthermore, in mice orexins exert influence the regulation of feeding and metabolism, and their expression is altered by food deprivation [32,33]. Since there is very limited knowledge as to the effects of orexin peptide and receptor variants from heterologous expression systems, it is difficult to predict a role for these processes in human disease. However, mutagenesis studies, modeling and orexin receptor crystal structures have generated data upon which models of orexin receptor−ligand interaction may be predicted [34–40]

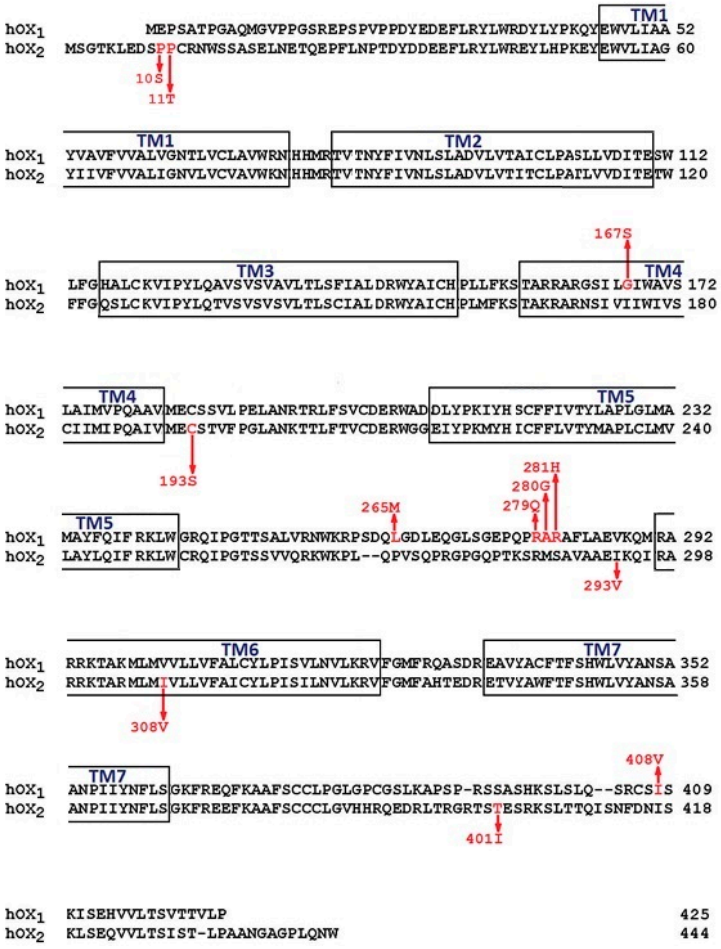

Figure 1. Alignment of the human OX_1 and OX_2 receptor amino acid sequences. The common wild-type sequence of OX_1 receptor is shown (top) and common wild-type sequence of OX_2 is shown (bottom). Arrows mark the location of known amino acid variants in relation to transmembrane regions 1–7 (TM1–TM7). Alignment adapted from Alscript output [41].

2. Orexin Receptor Variants

Genetic variants of the orexin system have been identified. While amino acid sequence variants of human OX_1 and OX_2 receptors (Figure 1) have not been implicated in human narcolepsy [1,10], genetic variants of the OX_1 and OX_2 have been inconsistently and weakly associated with many CNS disorders (Table l, Table 2) including sleep–wake dysregulation, polydipsia in schizophrenia [13,14], panic disorder [15], mood disorders [12], migraine [42,43] and cluster headache [16–18,44,45]. The relevance of human OX_1 and OX_2 receptor variants to disease states, identified originally using candidate gene methodologies [5,46], is subject to confirmation by the methods of next generation sequencing [47] that we pioneered to great effect in rare diseases [48] such as Mabry syndrome [49]. Confirmation that orexin variants are functional, let alone pathogenic, is debatable; however, study of orexin receptor variants has provided insight into orexin receptor signalling that may be relevant to OX_1 and OX_2 functional ineractions and/or heterodimerization.

3. Orexin signaling

The potential for orexin receptor variants to affect receptor homo- and heteromerization is an emerging field of study. The evidence for orexin receptor di-/oligomerization will be presented after we review the available data on the structure and function of the OX_1 (Figure 2) and OX_2 (Figure 3) wild-type and variant receptor signaling in general.

3.1. Coupling to G Proteins and Other Effectors

An understanding of which orexin receptor is able to couple to which G protein is integral to a discussion of orexin pharmacogenetics. The interaction of GPCRs with intracellular signal transducers usually takes place via the 2nd and/or 3rd intracellular loops and/or the C-terminus, however, it can sometimes result from interaction with the 1st intracellular loop [50]. Variations in these regions of orexin receptors could impact signaling directly by eliminating the necessary amino acid motifs or indirectly as a result of alterations in the receptor configuration [5,51,52]. Unfortunately, there are no data available on the actual G protein interaction sites in orexin receptors; β-arrestin (OX_1 and OX_2) and dynein light chain Tctex-type 1 (OX_1) are reported to couple to the orexin receptor C-terminus [53–55] and the Tyr phosphatase SHP-2 to the first intracellular loop (OX_1) [56]. However, based on the knowledge of GPCRs in general, the identified variable sites, OX_1^{265}, OX_1^{279}, OX_1^{280}, OX_1^{281}, OX_1^{408}, OX_2^{293}, and OX_2^{401} (Figure 1) could be implicated in G protein and other protein coupling of orexin receptors.

The orexin receptors bind their synthetic small molecular ligands in a partially hydrophilic, partially hydrophobic cleft [38,39]. The binding of orexin peptides is not known, but is assumed to take place in a similar manner, with the conserved peptide C-terminus diving into the cleft [40]. The N-terminus of the orexin peptide is then predicted to protrude from the cleft [40], and thus the extracellular portions of the receptor may also contribute to the peptide binding that have been useful in developing a pharmacophore model to discover binding modes deduced from the OX2R structure [51].

In recombinant systems, the OX_1 and OX_2 receptors can easily couple to $G_{i/o}$, G_s, and G_q families ($G_{12/13}$ not assessed) and β-arrestin [53,54,57–61]. Similarly, in endogenous cells, orexin receptors are likely capable of coupling to all these G protein families, but the interactions may be subject to species-, tissue- and context-specific regulation [21]. For instance, OX_2 receptors in human adrenal cortex activate G_i, G_s, and G_q proteins [62]. Mixed orexin receptor populations in rat adrenal cortex or hypothalamus couple to G_i, G_o, G_s, and G_q [30]. Factors influencing signaling cascades have been reviewed in detail elsewhere [6,7,21,63,64].

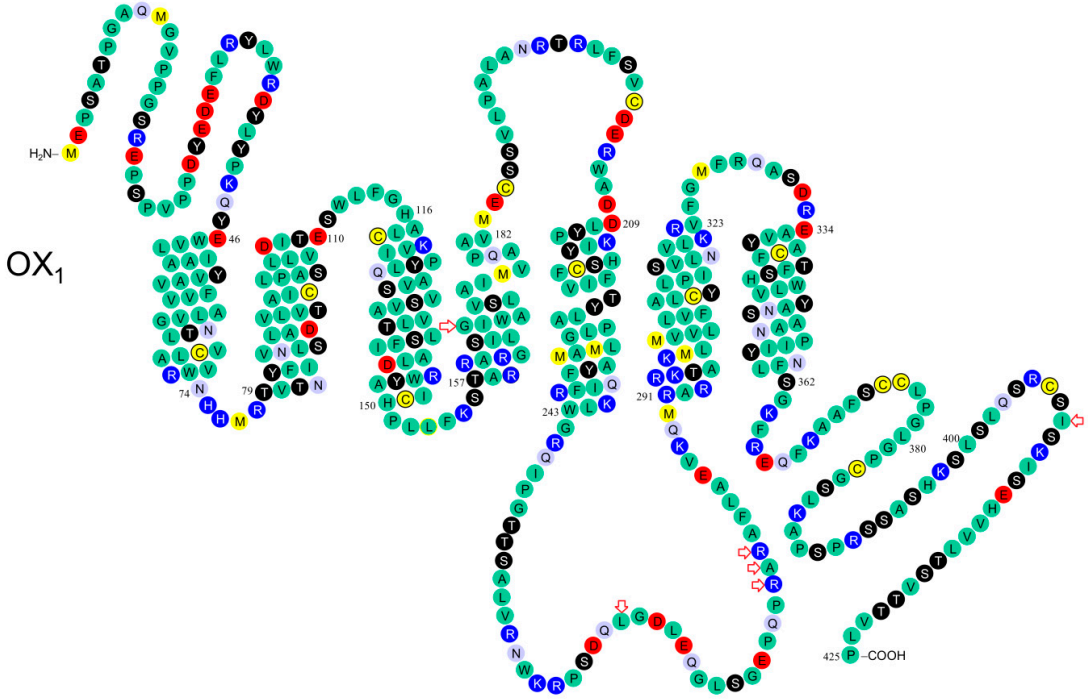

Figure 2. Schematic of the OX_1 receptor in the plasma membrane. Modified from Kukkonen et al. [21]; based on findings of Peyron et al. [10] and Olafsdottir et al. [9]. The variants marked with red arrows are: $OX_1$167 Gly/Ser, $OX_1$265 Leu/Met, $OX_1$279 Arg/Glu, $OX_1$280 Ala/Gly, $OX_1$281 Arg/His, $OX_1$408 Ile/Val [2].

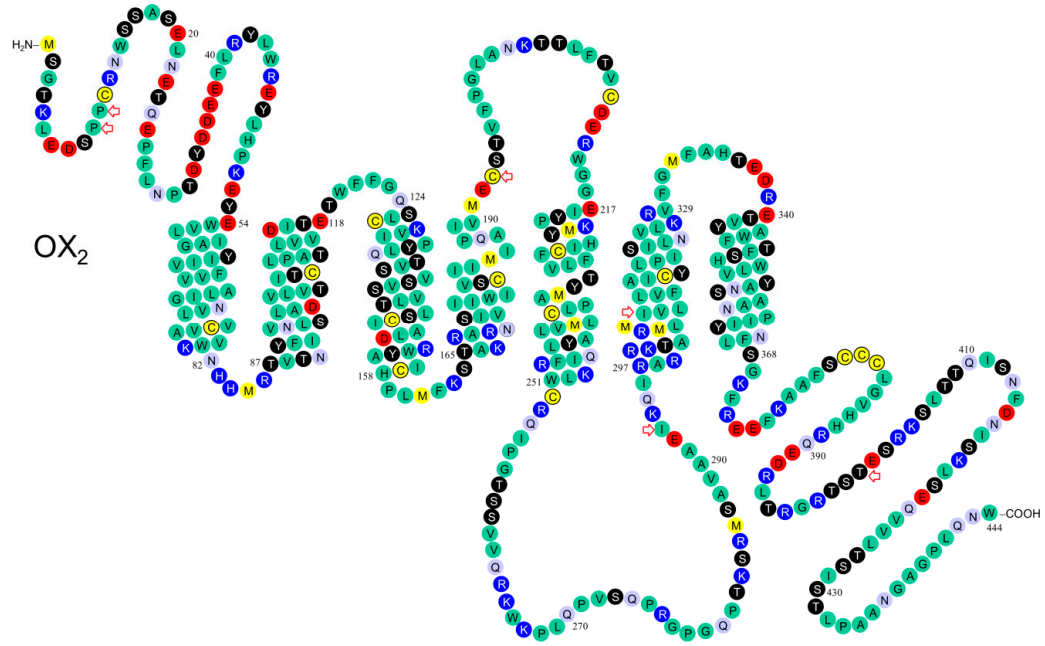

Figure 3. Schematic of the OX_2 receptor in the plasma membrane. Modified from Kukkonen et al. [21]; based on findings of Peyron et al. [10] and Olafsdottir et al. [9]. The variants marked with red arrows are: $OX_2$10 Pro/Ser, $OX_2$11 Pro/Thr, $OX_2$193 Cys/Ser, $OX_2$293 Ile/Val, $OX_2$308 Ile/Val, and $OX_2$401 Thr/Ile [2].

3.2. Post-Translational Modifications

It is difficult to predict sites affecting receptor folding. In principle, however, every residue can influence receptor folding at the local or a more general level. A major change in the amino acid size, conformation, polarity and, especially, charge is likely to have a more pronounced effect of this type. Glycosylation, found on the extracellular orexin GPCR surfaces, could be affected by the availability of Asn and Ser/Thr residues (and other sites in the putative consensus sequence) [2,65]. This could be relevant for the OX_2^{10} Pro/Ser, OX_2^{11}Pro/Thr, and OX_2^{193}Cys/Ser variants (Figure 1).

In humans, polymorphisms in the N-terminal of OX_2 include the Pro10Ser and Pro11Thr variants (Figures 1 and 2) described previously [5,66]. These variants may directly affect ligand binding, or indirectly affect ligand binding by altering receptor structure. However, the effects are difficult to predict, since the termini and loops connecting transmembrane helices are variable in comparison to the transmembrane core of especially rhodopsin family GPCRs, and no consequent mutagenesis studies have been conducted on orexin receptor N-termini.

We review the findings on the pharmacology of the OX_2 Pro10Ser variant before suggesting a rational for examining the OX_2^{193} Cys/Ser (transmembrane helix 4 (TM4)) variant, the OX_1^{167} Gly/Ser (TM4) variant and the less interesting OX_2^{308} Ile/Val (TM6) variant [2]. Thompson et al., identified the OX_2 Pro11Thr variant in two DQB1*0602-negative excessive daytime sleepiness (EDS) patients and the OX_2 Pro10Ser variant in a Tourette's syndrome patient comorbid with attention deficit hyperactivity disorder (ADHD) and probable EDS (Figure 2) [5]. While the OX_2 Pro10Ser and OX_2 Pro11Thr variants were reported to be more common in human leukocyte antigen (HLA) DQB1*0602-negative narcoleptics compared with controls, Peyron et al., found these variants to be benign with respect to narcolepsy [10].

These variants, however, are known to be rare in the general population (Table 1). The Pro10Ser variant (rs41271312) and the Pro11Thr variant (rs41271310) have been found to have approximate allele frequencies of 0.46% and 0.26%, respectively. In addition, even rarer polymorphisms have been identified at OX_2^{10} (a Pro10His substitution) and OX_2^{11} (a Pro11Ala substitution). The rarity of these variants in non-EDS populations suggested that their function should be evaluated *in vitro* [5].

Variations in the sequence of the receptors may have effects apart from ligand binding. Previously, Thompson et al., tested the functional significance of the Pro10Ser and Pro11Thr variants in transfected COS-7 cells by measuring calcium elevation [5].The results suggested that OX_2 Pro10Ser variant may be an example of a pharmacogenetic variant, though we cannot rule out the possibility that decreases in efficacy and potency of orexin peptides at these variant OX_2 receptors result from altered receptor expression levels. However, there is a lack of conservation at these amino acid positions in the dog, rat and mouse compared with the human wild-type and each variant, suggesting degeneracy at these positions. The conservation of proline residues in general [67] and between species [68] suggest they may influence receptor structure and function; certainly, proline residues are known to induce kinks in peptide chains and disrupt α-helix and β-sheet structures.

3.3. Binding Pocket

Out of the 11 amino acid variants discussed in this review, the majority are not found within the small molecule binding pocket [38,39]. Variant OX_1^{167} harbors an alteration on TM4 close to the intracellular site of the receptor, OX_2^{193} which results in an alteration in TM4 and OX_2^{308} variation is located on TM6 (Figure 1). The latter two sites are less likely to affect ligand binding directly as they point away from the binding cavity [38,39].

Variations of the orexin receptors have also been found in regions located outside the predicted binding cavity that may also have consequences on the binding affinities measured in pharmacological assays. These variants include the canine OX_2 Glu54Lys mutation [23] which has been identified in narcoleptic animals. It is possible that human variants of the N-terminal part of the receptor, such as at OX_2^{10}, may also influence binding indirectly due to conformational changes or the altered capacity of receptors to homo- and heterodimerize.

The different role of the two orexin peptides and the two orexin receptors has yet to be fully explained. The respective roles of each peptide may be distinguished by expressing variant copies of one or more orexin receptor polymorphism. In addition to ligand binding differences, co-expression of the variant receptors in heterologous expression systems may allow identification of the domains in each receptor that define homo- and heteromerization.

It is possible that phosphorylation events that regulate receptor desensitization in response to ligand may be altered in a number of orexin receptor variants. Hydroxyl group-containing amino acids Ser, Thr and Tyr are putative substrates for phosphorylation, while other amino acids variations may affect the kinase consensus sequences (Lamey et al. 2002). Though these sites have not yet been targeted by point mutagenesis studies, a Scansite (http://scansite.mit.edu/) motif search suggests that polymorphisms at OX_1^{167}, OX_1^{265}, OX_1^{279}, OX_1^{280}, OX_1^{408}, and OX_2^{401} (Figure 1) may be worth examining.

4. Modelling Orexin Receptor Hetero- and Homodimerization

Our understanding of the trafficking, ligand interaction and signaling of the orexin receptors may be impacted by the fact that GPCRs have been shown to dimerize [50,69]. GPCRs were originally assumed to exist as monomers, a view that has since been revised. In fact, some models predict that all functional GPCRs form dimers and oligomers [50,69]. In recombinant expression systems, orexin receptors may form homodimers, heterodimers or even higher order oligomers [70–73]; the number of monomers in each complex is difficult to assess based on the methods utilized. It should also be noted that most studies have been performed upon heterologous receptor expression.

If dimerization takes place, it may influence the pharmacological properties of OX_1 and OX_2 receptors. One level of potential impact is the affinity for native and synthetic ligands, while the other levels include signaling, localization and trafficking. The common pharmacogenetic variants of both OX_1 and OX_2, may provide an opportunity to examine the receptor domains necessary for dimerization. This is based on the hypothesis that variant receptors, such as the $CysLT_1$ and $CysLT_2$ receptors [74–77] may contribute to a phenotype through epistasis made manifest by functional interaction or heterodimerization [78]. In the absence of such data, however, we will discuss the limited evidence for such interactions in native cells in the context of the likely functional significance of OX_1–OX_2 variant interactions.

Orexin pharmacology may be modulated by the formation of OX_1–OX_2 heteromers; however, there is currently little physiological evidence of its significance. Since there is evidence of orexin OX_1 and OX_2 receptor co-expression in at least some tissues [79] there is a possibility that heterodimerization takes place. Even in cells expressing predominantly a single receptor subtype, the cell may express orexin receptor heterodimers, if the individual is heterozygous for a polymorphism. Since there are many GPCRs expressed in any given cell, there are plenty of potential heteromerization partners for orexin receptor. The limitations of the research of homo- and heteromerization and the occasional inconsistency in results, sometimes even within a single study, are discussed in this context.

The most often used resonance energy transfer-based techniques, fluorescence resonance energy transfer (FRET) and bioluminescence resonance energy transfer (BRET), are better used to assess dimerization and cannot be used to show or exclude higher order complexes. While these methods imply that orexin receptors can make both homomeric and heteromeric complexes, heteromers may have radically different pharmacology, signaling and trafficking properties as compared to homomeric ones [6,50]. Secondly, FRET and BRET methods do not easily indicate the efficiency of the complex formation. Resonance energy transfer efficiency is dependent on the orientation and distance of the donor and acceptor. Any change in receptor conformation, irrespective of whether it leads to receptor activation or not, may or may not affect the efficiency of energy transfer [70,80–83], and thus a difference in efficiency of energy transfer or lack thereof may be of little predictive value.

The impact of naturally occurring variants, such as OX_1^{408} Ile/Val and OX_2^{401} Thr/Ile (Figure l), on receptor dimerization is unknown; however, the polymorphic sites are located within the

receptor C-terminus and proximal to potential palmitoylation sites. Palmitoylation at the cytosolic end of the third intracellular loop has been shown to promote dimerization of μ opioid receptors while the oligomerization of β_2 adrenoceptors occurs partially via the C-terminus [84,85]. However, other GPCRs may dimerize/oligomerize via other domains.

The importance of dimerization/oligomerization for most family A GPCRs is unclear [50,69]. Notable exceptions to this are the opioid receptors, whose pharmacology, signaling and trafficking are significantly affected by dimerization [86]. With respect to orexin receptors, early evidence suggested that heterodimerization between CB_1 cannabinoid receptors and the OX_1 may strongly potentiate orexin receptor signaling. However, as we will discuss shortly, the signal potentiation more likely depends on the ability of OX_1 receptor signaling to produce 2-AG, a CB_1 receptor ligand, and thus co-signaling of the OX_1 and CB_1 receptors [64,87–90]. While this does not preclude dimerization, based on current evidence, it may suggest that the phenomenon is of limited physiological significance.

4.1. Evidence for Orexin Receptor Dimerization

The significance of disrupting motifs necessary for family A GPCR dimerization [91–93] is not clear by comparison with evidence of the deleterious effects of disrupting motifs critical to family C GPCRs dimerization [94,95]. One reason is the limited availability of anti-GPCR antibodies in general and such towards orexin receptors in particular. Usually, antibodies to orexin receptors either do not bind well or they bind equally well to cells regardless of receptor expression.

The study by Xu et al., provided some evidence for ligand-enhanced OX_1 homomerization. Orexin-A was found to enhance di-/oligomerization, while antagonists reduced di-/oligomerization below basal [70]. OX_1 activation enhanced the FRET signal only under conditions of low receptor expression levels. Dimerization/oligomerization is a dynamically regulated process in orexin receptor signaling, as has previously been reported for a number of GPCRs [93,96], though it has been difficult to prove any significant agonist-mediated impact on the process. In some other family A GPCRs, the process of heterodimerization has also been implicated in the maturation and cell surface trafficking of [97,98].

The two splice variants of the mouse OX_2 receptor, $OX_{2\alpha}$ and $OX_{2\beta}$ [99] differ by 17 amino acids in the C-terminus. Wang et al., demonstrated that splice variants heterodimerize much more efficiently than homodimerize, as indicated by BRET studies [80]. Since studies of the functional interactions between human orexin receptor variants are not available, the data from Wang et al., on mouse orexin receptors may provide insight into the human system.

Wang et al., suggest that the role of the C-terminus of the OX_2 receptor in heterodimerization may be similar to opioid receptor heterodimerization. A mutant δ-opioid receptor with a 15-residue C-terminal deletion reported by Cvejic et al., was not able to dimerize [100]. The data from Wang et al. [80] suggests that, like for the δ-opioid and somatostatin receptors [100,101], OX_2 dimerization may predominantly involve the C-terminal domain. These experiments may define a role for the C-terminal domain in the putative dimerization of orexin receptors that occurs in nature, assuming the lessons from the heterodimerization of mouse OX_2 receptor variants are relevant to human orexin receptors. Functional mOX_2 heterodimers elevated intracellular Ca^{2+} and activated ERK. The interaction of either human orexin receptor subtype with GPR103 as identified by BRET and FRET, was also found to result in ERK1/2 phosphorylation when receptor co-expressing cells were stimulated by either orexins or the GRP103 agonist, QRFP, and the effect of each peptide was blocked by orexin receptor antagonists [80].

Evaluating the physiological relevance of receptor multimers is important. Even though tools such as selective ligands, membrane-permeant peptides or antibodies can sometimes detect receptor dimers and oligomers, it is possible that heterodimerization is not always possible. For instance, $mOX_{2\alpha}$ and $mOX_{2\beta}$ are differentially expressed in the brain.

4.2. Evidence for OX_1-CRF_1 Receptor Dimerization

The study by Navarro et al., presents an analysis of the complex receptor interaction between OX_1, CRF_1 corticotropin-releasing factor receptor and the non-GPCR σ1 receptor [102]. The OX_1−CRF_1 complex, shown by FRET, was shown to be subject to cross-antagonism (i.e. the response to agonist of either receptor was blocked by antagonists towards either receptor. By contrast, activation of each receptor resulted in negative crosstalk between the receptor responses, despite the fact that both activation of each receptor alone stimulated coupling to β-arrestin and phosphorylation of protein kinase B and ERK1/2. These effects were shown to be blocked by cell-permeable peptides composed of either the OX_1 receptor TM1 or -5 [102].

Evidence of the physiological relevance of the interaction between OX_1 and CRF_1 was consistent with dopamine release from ventral tegmental area (VTA) upon orexin-A stimulation; a response that was inhibited by antagonists of both OX_1 and CRF_1 receptors, and by CRF itself. When σ1 receptors were stimulated, OX_1 and CRF_1 receptors were freed from cross-antagonism and negative crosstalk, suggesting a native OX_1−CRF1−σ1 interaction. Upon activation of the σ1 receptors, CRF_1 no longer inhibited the OX_1-stimulated dopamine release, and the total dopamine release was higher upon stimulation of both σ1 and OX_1 receptors than of OX_1 receptors alone [102], stressing the physiological and pharmacological significance of the interaction. Collectively, these results suggest that OX_1 receptor complex formation with CRF_1 or GPR103 may be very efficient.

4.3. OX_1 Orexin Receptor and κ Opioid Receptor (κOR) Heteromerization

Evidence of receptor co-localization suggests the possibility of OX_1−κOR receptor heteromerization that may have clinical relevance. The balance of orexin peptide signaling through either the OX_1 or the OX_2 may determine anti-depressant or pro-depressant effects [103]. Physiological evidence implicates orexins in emotional state. Reduced cerebrospinal orexin levels and reduced diurnal orexin fluctuations have been reported in patients with depression [104] and decreased OX_1 activity may worsen depression in a chronic unpredictable mild stress (CUMS) mouse model [105].

In this context, it is interesting that excessive activation of κOR induces not depression but anxious and fearful behavior in both humans and rodents [106]. While the contribution of variant receptors to these processes is unknown, it is possible that the system that may be regulated by heterodimerization [103] and/or functional interaction [6]. For example, putative orexin receptor heterodimerization with κOR may modulate pain, stress responses and conditions such as addiction, depression, and schizophrenia [107]. These effects might be indirect. It has been hypothesized that κOR stimulation may act to inhibit OX_1 activation in dopaminergic neurons [108].

Some studies suggest that the functional interaction occurs through a purely intracellular signaling pathway in which the OX_1 receptor interaction enhances c-Jun N-terminal kinase (JNK)—mediated phosphorylation of κOR in CHO-K1 cells, inhibiting κOR signaling to AC and enhancing its signaling via β-arrestin and p38 MAPK, apparently via $G_{q/11}$ [109]. While this may be consistent with evidence that orexin and dynorphin neuropeptides negatively regulate the reward-regulating function of the other through opposing effects in the ventral tegmental area [110], whether this is a functional significant mechanism remains unclear. An additional aspect to take into consideration is that essentially all orexinergic neurons co-express dynorphin (though the reverse is not true).

While κOR monomers signal mainly through activation of $G_{i/o}$ (Borsodi et al. [111]), their dimerization with OX_1 may result in "opposite" signaling through G_s [108]. Co-localization findings suggest that normal OX_1 and κOR homo- and heterodimerization may coordinate signaling responses through the co-release of peptides in regions such as the hypothalamus [65]. This conclusion is consistent with evidence that OX_1 and κOR co-expression in hippocampal neurons is less extensive in depressed rats than in normal rats [112]. In this context, it is intriguing that Chen et al., reported that heteromerization reduced the coupling of OX_1 and κOR to G_q and G_i, respectively, while it instigated coupling of the receptor heteromers to G_s and thus to cAMP elevation in HEK293

cells. Similarly, Li et al., showed that heterodimerization of the apelin receptor and κOR increased cAMP accumulation after treatment with either class of ligand [82].

Having presented a critical review of the homo- and heterodimerization literature for orexin and other systems, such as opioid and CRF_1 receptors, we discuss the evidence for the functional interaction of OX_1 and the CB_1 cannabinoid receptor before discussing its potential clinical relevance in epilepsy.

4.4. Interaction of OX_1 Receptors and CB_1 Cannabinoid Receptors

Lipid metabolites such as 2-arachidonoyl glycerol (2-AG) and anandamide are native ligands of CB_1 cannabinoid receptors [113]. CB_1 receptor has been shown to heterodimerize with other class A GPCRs, including D_2 dopamine [92], opioid [114] and AT_1 angiotensin [115,116] (103,104) receptors, and this has been assessed also with respect to human OX_1 and OX_2 receptors [64,73]. In contrast to the more discrete hippocampal expression of the orexins, the widespread expression of the endocannabinoid system and the CB_1 receptors allows ample opportunity for co-expression of the receptors and other interactions of the systems.

Physiologically, orexins and endocannabinoids may have overlapping functions. Endocannabinoids regulate many physiological functions including analgesia, appetite, learning and memory [113]. Furthermore, orexin receptor signaling may trigger production of 2-AG, leading to the activation of CB_1 receptor [64,87–89]. *In vitro*, the human OX_1 forms heteromeric complexes with CB_1 receptors [72,117] that, if present *in vivo*, may be influenced by orexin receptor variants. Furthermore, heteromultimerization of CB_1 and OX_1 receptors has been reported to be subject to orexin-A feedback involving the both receptors [72].

The data from Jäntti et al., further suggested that both orexin receptor subtypes are capable of forming constitutive homo- and heteromeric complexes with one-another and with CB_1 cannabinoid receptors [81]. However, while dimerization is possible as such, the data suggest that the downstream effect on signaling may result from a functional and not molecular interaction of the receptors [64,88]. In the case of OX_1–CB_1 interaction, Jäntti et al., reported that the major part, if not all, of the interaction results from orexin-promoted 2-AG production and not heterodimerization [64]. However, no receptor dimerization-blocking peptides have been assessed for this interaction.

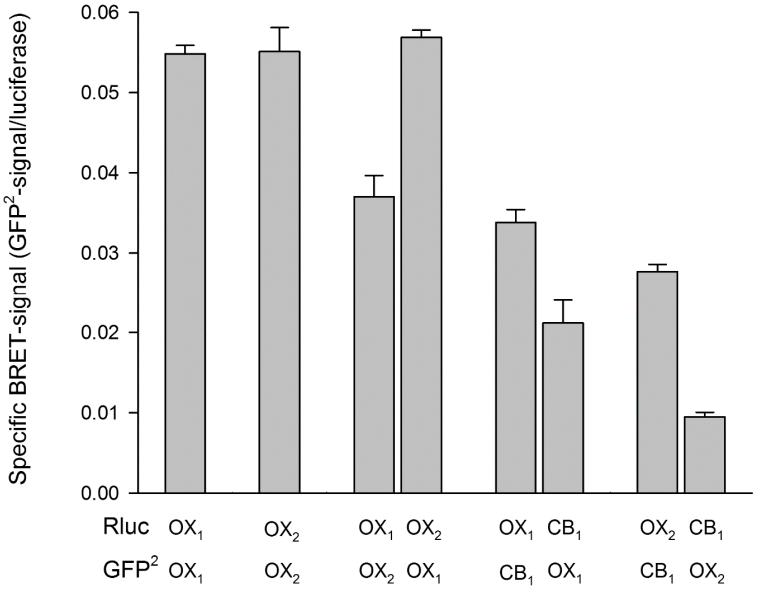

Figure 4. Human orexin and cannabinoid receptor interactions as measured utilizing BRET ($BRET^2$) between renilla luciferase (Rluc) and GFP^2 fused to the receptors' C-termini. The Rluc background has been subtracted from the values. The data presented are the mean ± SE; n refers to the number of cell batches. Each experiment was performed at least three times in 4–6 replicates. The data originate from Jantti et al. [64]

Using a BRET assay, Jäntti et al., found that CB_1 receptors readily homodimerize and heterodimerize with OX_1 and OX_2 receptors (Figure 4). Homomeric complexes of OX_1, OX_2 and CB_1 showed the best BRET efficacy in this study. While the evidence for OX_1–CB_1 receptor interaction was similar to that for OX_1 and OX_2 receptors, however, the evidence for CB_1 and OX_2 receptor interaction was weaker (Figure 4) [81]. These studies highlight the issue of GPCR dimer stability. When constitutive CB_1–OX_2 complexes were tested for a possible change upon orexin stimulation, only a minor (< 10%) reduction in BRET was reported. However, these data may suggest that the OX_1-CB_1 complexes formed are stable. Since FRET or BRET efficiency cannot be used to determine the affinity for complex formation for geometric reasons, different receptor combinations cannot be compared quantitatively; this is clearly illustrated in Figure 4, which shows how the BRET efficiency can be very different depending on which partner of the heteromer harbors which BRET component. While these results establish that such complexes can be formed they do not indicate that they are actually present and function in cells *in situ*. In conclusion, both orexin receptor subtypes and CB_1 cannabinoid receptors are capable of forming constitutive homo- and heteromeric complexes that may be relevant to variant orexin signaling. Whether these have physiological function or whether they would be dynamically regulated, however, remains to be shown. A possible way of determining the physiological significance is presented by the so-called bivalent ligands. Although ligands, such as those designed for the CB_1 receptor dimers [118,119], may show some selectivity for the receptor homo- or heterodimers, no agonists specific to putative orexin–CB_1 heterodimers exist currently. In fact, the data suggest that the effect of orexin potentiation of CB_1 signaling may not be result of heterodimerization but as the result of orexin-promoted 2-AG production (see above). The extent to which the functional interaction can explain the interaction of orexin and cannabinoid systems better than receptor heterodimerization, is important due to the potential for such an interaction to influence physiological processes which underlie anticonvulsant drug effects.

5. Relevance of CB_1/OX_1 Expression in Hippocampus to Disease

A number of studies have demonstrated molecular and functional cross-talk between CB_1 and OX_1 receptors in heterologous expression systems and functional crosstalk in neurons. This may be relevant to neuron membranes at nerve terminals in many brain regions. It is especially true in the hippocampus [120], where co-expression of CB_1 and OX_1 is pronounced. In view of this, changes in CB_1/OX_1 expression may impact CNS disease states associated with certain regions.

For example, the kainic acid model of status epilepticus (SE) has been shown to result in changes in CB_1/OX_1 mRNA expression in the hippocampus [120]. It has been hypothesized that changes in GPCR expression in kainic acid model of SE may present putative therapeutic targets selective to SE. There is significant interest in developing new means to stop SE and limit the brain damage that develops as seizure duration increases due to pharmacoresistance [121,122]. This is not only the case for traditional benzodiazepine drugs; over 40% of SE cases are refractory to initial treatment with two or more medications [121], rendering SE a life-threatening neurological disorder.

Recently, the problem of this pharmacoresistance has resulted in a wide interest in compounds that target CB_1. While safety concerns have historically led to restrictions in the medicinal use of marijuana [117], evidence of cannabinoid involvement in epileptogenesis is increasingly suggesting that cannabinoids may be useful interventions in epilepsy. In human subjects, downregulation of CB_1 receptors has been reported in human epileptic hippocampal tissue [118], Consistent with this, Falenski et al., found reduced CB_1 levels in the rat hippocampus following SE in the pilocarpine model [119]. However, Bojnik et al., found elevated CB_1 expression in rats in the kainic acid model [120] that may be consistent with evidence that CB_1 agonists protect against the excitotoxic injury induced by kainic acid [121].

To some extent physiological studies indicate that endogenous cannabinoids in the hippocampus act as retrograde messengers from depolarized postsynaptic neurons to presynaptic terminals [122]. This may result in depolarization-induced suppression of inhibition (DSI); although it is also relevant to homo- and heterosynaptic depolarization-induced suppression of excitation

(DSE), and cannabinoid-mediated plasticity. Orexinergic neurons themselves are under control of presynaptic CB_1 receptor-regulated inputs [84,90].

The long-term effects of kainic acid-induced SE on CB_1 and OX_1 expression in rat hippocampus has been examined in order to establish whether these changes reflect a causative mechanism of epilepsy or whether they influence treatment efficacy. CB_1 expression in the hippocampus increased after SE, as measured by immunohistochemistry and RT-PCR [119]. Zhu et al., did not report any increase in OX_1 expression after SE, however it is known that antibodies to OX_1 may not always perform well (personal observations). Nevertheless, it was reported that the increased CB_1 expression and the potential involvement of OX_1 may not only reflect the pathophysiology of SE but also represent pharmacological targets for SE [119].

Unlike the cannabinoids, data showing the seizure-modulating role of orexins are still emerging. For example, Doreulee et al., reported a putative involvement of the orexinergic system in antiepileptic mechanisms [9]. On the other hand, another study reported behavioral seizure activity when orexin peptides were injected ICV into rats [28].

The overlap in CB_1 and OX_1 distribution in nerve terminals of the hippocampus may be worth examining with respect to their role in heterodimer formation and epileptogenesis [120]. However, alternative explanations may include the following: (1) The OX_1/CB_1 heterodimers in SE merely represent biomarkers that may be of limited specificity in SE or its treatment, and (2) The OX_1/CB_1 heterodimers in SE have more relevance as novel therapeutic targets, although they do not necessarily reflect the pathobiology of SE. CB_1 and OX_1 "cross-talk" in SE, however, may only become clinically useful with the advent of anticonvulsant drugs developed to target either OX_1 or CB_1.

6. Conclusions

This review focuses on the discussion of evidence for functional interaction and/or homo- and heterodimerization with systems such as the opioid and cannabinoid systems. We presented perspectives on orexin signaling in the context of other neurotransmitter systems implicated in epilepsty and neurodegeneration that could shape further studies.

Human OX_1 orexin receptors have been shown to homodimerize and interact functionally with other receptor types. In particular, the evidence for heterodimerization versus functional interaction for OX_1 and κOR and the CB_1 cannabinoid receptors is discussed. While dimerization may be important for orexin receptor responses and trafficking, many physiological effects can be explained through the evidence for functional interactions (i.e. interactions of the receptor signal pathways rather than receptors themselves). For example, we review the evidence whereby orexin receptor signaling via endocannabinoid production acts at CB_1 receptors.

By contrast, dimerization may be a means whereby signaling is optimized for a given receptor type. For example, complexes may form when optimal cannabinoid concentrations are available for cannabinoid receptors. While orexin receptor subtypes may readily form homo- and heteromeric complexes, as suggested by significant BRET signals, it is unclear whether this takes place *in situ*: except for one remarkable study, in which this has been assessed [102]. As pointed out in this study tools such *ex vivo* preparation may be a useful means of assessing this in future studies.

The pharmacology and of the known orexin receptor variants, especially those located in the carboxyl terminal, such as OX_1^{408} and OX_2^{401} (Tables 1 and 2), are discussed in the context of GPCR signaling; however, their potential impact of orexin receptor homo- and heterodimerization is not yet known. Since co-expression of OX_1 and OX_2 receptor variants is relatively common, however, the functional significance of variant receptor signaling is discussed. Given that state of the present knowledge it is unclear if co-expression results in, A. distinct pharmacology through functional interaction, B. distinct pharmacology as a result of the formation of variant heteromers, or C. no change in pharmacology. The structural insights resulting from resolving orexin receptor crystal structure may resolve these moot points [30–32]. We discussed the hypothesis that homo- or heteromerization of orexin receptors with other systems such as cannabinoid receptors may present useful targets in the pharmacological treatment of some neurological conditions such as epilepsy and depression.

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