Receptor determinants involved in regulatory and signalling mechanisms of orexin receptors

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The orexin system is involved in the fundamental orchestration between sleep-wake, metabolism and the promotion of behaviour that stimulates psychochemical reward. This system mediates these activities through the action of endogenous orexin neuropeptides, orexin A and B (OxA and OxB), on two GPCRs, orexin receptors 1 and 2 (OX_1R and OX_2R). In addition to G protein-coupling, largely via $G_{q/11}$, these receptors interact with β-arrestins; a set of ubiquitously expressed multi-adaptor proteins that are critically involved in regulatory and signalling properties of GPCRs.

A significant orexin receptor subtype-specific difference is the temporal stability of receptor-arrestin proximity over sustained periods of stimulation as observed through the use of bioluminescence resonance energy transfer (BRET), a biophysical assay that measures protein proximity in live cells and in real-time. Specifically, OX_2R displays more sustained proximity with β-arrestins than OX_1R **[1]**. Similarly, profiles were observed that differentiated the receptor subtypes when ubiquitination of orexin receptor complexes and ERK1/2 phosphorylation were measured **[1]**. The molecular mechanism of this difference in stability was investigated by evaluating the contribution of the C-terminus of orexin receptors, a key binding region for β-arrestins.

Hypothesized G protein-coupled receptor kinase (GRK) phosphorylation determinants in the C-terminus of OX_2R were investigated for their contribution to β-arrestin binding. Using BRET, the data indicated that unlike OX_1R **[2]**, disruption of two putative GRK phosphorylation sites at amino acid positions 406-409 (SLTT) and 427-431 (TSIST) of the OX_2R C-terminal tail by mutation of S/T residues to alanine (SLTT→ALAA, (A406); TSIST→AAIAA, (A427)) was necessary to destabilize OX_2R-β-arrestin interactions. BRET values between OX_2R and the OX_2R A406-A427 mutant were significantly different from 3-60 min post OxA (0.6 μM) stimulation, measured using two-way ANOVA and Bonferroni multiple comparison post-tests ($p<0.05$, n=3). Further mutation of S/T residues in the more proximal putative GRK phosphorylation site (399-403, TSTES→AAAEA (A399)) did not have any further effect on reducing the OX_2R-β-arrestin BRET signal, and the mean BRET values of this mutant (OX_2R A399-A406-A427) were similarly significantly lower compared to OX_2R over the same measurement period (two-way ANOVA; $p<0.05$, n=3). Measurement of inositol phosphate (IP) indicated that G protein-coupling of the mutant was not compromised, but instead resulted in a significant increase in potency (pEC_{50} values were as follows: 8.15±0.09 (OX_2R), 8.69±0.10 (OX_2R A399-A406-A427 mutant); mean potency values were analyzed using a paired *t*-test ($p<0.05$, $n=5$)). This increase may be due to the decreased ability of the mutant to recruit β-arrestin and desensitize G protein-mediated signalling pathways involving IP production. This study provides evidence for the molecular mechanism of this interaction that may be responsible for subtype-specific signalling and regulatory function with respect to β-arrestin, a better understanding of which may help us to develop the therapeutic potential of these receptors.

1. Dalrymple MB, Jaeger WC, Eidne KA, Pfleger KD (2011). Temporal Profiling of Orexin Receptor-Arrestin-Ubiquitin Complexes Reveals Differences between Receptor Subtypes. *J Biol Chem* **286**(19)**:** 16726-16733.

2. Milasta S, Evans NA, Ormiston L, Wilson S, Lefkowitz RJ, Milligan G (2005). The sustainability of interactions between the orexin-1 receptor and beta-arrestin-2 is defined by a single C-terminal cluster of hydroxy amino acids and modulates the kinetics of ERK MAPK regulation. *Biochem. J.* **387**(Pt 3)**:** 573-584.