BIVALENT LIGANDS DISCLOSE GPCR DIMER'S CONFORMATION, SIGNALING AND FUNCTIONS.

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G protein-coupled receptors (GPCRs) constitute the largest family of integral membrane proteins involved in signal transduction and represent a major pharmacological target ensemble. GPCRs have traditionally been considered to exist and function as monomers, however growing biochemical and biophysical evidences have clearly indicated their ability to assemble as dimers and higher-order oligomers.

The aim of this study was to elucidate i) the arrangements of protomers in a dimer, ii) the dimer's signalling and pharmacology and iii) the dimer functions.

We used the oxytocin receptor (OXTR), as a prototypical class A GPCR which is known to be expressed in living cells and native tissues as monomers and homodimers, and bivalent ligands, as chemical tool to study GPCR dimers. Bivalent ligands are synthetic analogs composed of two pharmacophores covalently tethered with a spacer that can simultaneously interact with the two receptor of the target dimer. We synthesized a series of bivalent ligands consisting of two identical oxytocin analogues connected by an aliphatic spacer of increasing length (C6-C14) and we evaluated their effects on OXTR signaling using a G-protein activation assay based on bioluminescence resonance energy transfer biosensors (Busnelli et al. 2016). We demonstrated that oxytocin promoted the direct engagement and activation of Gq and all Gi/o G-protein subtypes, whereas the bivalent ligands behaved as "biased agonists" specifically activating Gq, Gi2 and Gi3. Moreover we obtained for OXTR/Gi2 and OXTR/Gi3 monophasic concentration-responses curves, whereas for OXTR/Gq we obtained biphasic curves with the two bivalent ligands with spacers C8 and C10. The first part of the curve corresponded to an EC50 that was 1,000 times lower that the EC50 of the second phase of the curve and that was similar to OXT and of the other bivalent ligands EC50s. Using docking-modelling, receptor mutagenesis and synthetic peptides that mimicked transmembrane OXTR helices and interferred with the proper dimer formation, we provided evidence for the presence of a specific OXTR dimeric receptor arrangement having a TM1-TM2 interface in which only the two bivalents, with defined spacer length (~25 Å), fit within the channel-like structure that connects the two protomers of the dimer. Because the OXTR are highly abundant in the CNS and regulates many aspects of socio-emotional behavior, we tested the bivalent ligand with C8 spacer in vivo and we observed that it promotes social behavior of mice and zebrafish with a 100- and 40-fold gain in potency as compared to oxytocin and isotocin, the oxytocin-analog in fish, control groups.

All our results demonstrated that OXTRs dimers are expressed in a high affinity state and for the first time, we were able to demonstrate that a GPCR dimer possess a different coupling respect to a monomer. Moreover, in vivo studies, indicated that OXTRs are expressed as dimers in the CNS and contribute to determine the central effects of oxytocin.

In general, our studies demonstrated that bivalent ligands are very promising as new tool to untangle the pharmacology, structure and functional activity of GPCRs dimers.

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