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The orexin 1 receptor modulates kappa opioid receptor function via a JNK-dependent mechanism

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ABSTRACT

The orexin 1 receptor (OX1R) and the kappa opioid receptor (KOR) are two G protein-coupled receptors (GPCRs) previously demonstrated to play important roles in modulating the rewarding effects of drugs of abuse such as cocaine. Using cells heterologously expressing both receptors, we investigated whether OX1R can regulate the function of KOR and vice versa. Activation of OX1R was found to attenuate agonist-activated KOR-mediated inhibition of cAMP production. In contrast, agonist-activated KOR-mediated β-arrestin recruitment and p38 activation were enhanced in the presence of activated OX1R. These effects are independent of OX1R internalization but are blocked in the presence of the JNK inhibitor SP-600125. OX1R signaling does not affect ligand binding by KOR. Taken together, these data suggest that OX1R signaling can modulate KOR function in a JNK-dependent manner, promoting preferential signaling of KOR via β-arrestin/p38 rather than Gαi. Conversely, Gαq coupling of OX1R is unaffected by activation of KOR, suggesting that this crosstalk is unidirectional. Given that KOR Gαi-mediated signaling events and β-arrestin-mediated signaling events are thought to promote distinct cellular responses and physiological outcomes downstream of KOR activation, this mechanism may have important implications on the behavioral effects of KOR activity.

1. Introduction

The neuropeptides orexin A and orexin B (also known as hypocretin-1 and hypocretin-2) are produced from the precursor pre-pro-orexin, which is expressed primarily by a restricted group of cells within the dorsolateral hypothalamus [1–3]. This limited population of orexin neurons projects to many different brain areas and regulates various behaviors including arousal [4], stress/anxiety [5], learning [6] and reward seeking [7]. These effects are mediated via activation of two widely-expressed Gαq-coupled G protein-coupled receptors (GPCRs), namely the orexin 1 and orexin 2 receptors (OX1R and OX2R [8]). Selective antagonism of OX1R reduces the rewarding effects of drugs of abuse such as cocaine [9], establishing OX1R as an important therapeutic target for the treatment of addiction. In contrast, activation of the kappa opioid receptor (KOR), also a GPCR, by its endogenous ligand dynorphin mediates depressive-like behaviors, and stimulation rather than blockade of KOR has been shown to reduce the rewarding effects of drugs [10]. Despite their contrasting functions, orexins and dynorphin can be packaged in the same synaptic vesicles [11], suggesting that they may function as co-neurotransmitters.

Most dopamine neurons in the ventral tegmental area (VTA), an important target for orexin neurons, respond to both orexins and dynorphin [11]. Co-stimulation with both neuropeptides can, however, result in opposing effects on the firing rates of different individual target neurons [11] and it is unclear how these responses are regulated [12]. Intra-VTA infusion of OX1R antagonist SB-334867 reduces cocaine self-administration in rats while intra-VTA infusion of the KOR antagonist norBNI has no effect on drug intake [11]. Interestingly, however, norBNI blocks the ability of SB-334867 to reduce drug intake [11]. Thus, it is conceivable that modulation of KOR function may occur downstream of OX1R activation. Such a relationship between these two GPCRs could explain the observed loss of SB-334867 efficacy in the presence of norBNI and would enhance our understanding of how target cells respond to orexin/dynorphin inputs.

Here we use cell lines heterologously expressing both receptors and show that OX1R signaling via Gαq can attenuate KOR-mediated Gαi signaling. This effect is lost in the presence of SP-600125, an inhibitor of c-Jun N-terminal kinase (JNK), activation of which has previously been associated with negative regulation of KOR [13]. In contrast, dynorphin-induced β-arrestin recruitment to KOR is increased in the presence of OX1R, in an SP-600125-dependent manner. Furthermore, OX1R signaling increases KOR-induced p38 activation, previously shown to require β-arrestin recruitment to KOR [14]. Taken together, our data suggest that OX1R signaling can modulate KOR function in a

Abbreviations: GPCR, G protein-coupled receptor; OX1R, orexin 1 receptor; KOR, kappa opioid receptor; JNK, c-Jun N-terminal kinase; cAMP, cyclic adenosine monophosphate; VTA, ventral tegmental area; CHO, Chinese hamster ovary; FLIPR, fluorescence *in situ* plate reader; PKA, protein kinase A; MAPK, mitogen-activated protein kinase; FBS, fetal bovine serum; HBSS, Hank's buffered saline solution; DOR, delta opioid receptor; GalR1, galanin receptor 1.

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JNK-dependent manner such that, in response to its ligand dynorphin, KOR now preferentially signals via β-arrestin rather than via Gαi. This could be an important mechanism for determining how a target neuron responds to co-stimulation with orexins and dynorphin. Furthermore, this type of cross-talk may provide a general mechanism by which signaling specificity is regulated downstream of GPCRs.

2. Methods

2.1. Materials

All GPCR constructs were purchased from Missouri S&T cDNA Resource Center. The HA-dDRY-OX1R construct was generated using wildtype HA-OX1R and mutating Arg-144 (CGC) of the DRY motif to Ala (GCC) using the QuikChange II Site-Directed Mutagenesis Kit from Agilent Technologies. (Forward primer: 5′-GCTTCATCG CCCTGGACGC CTGGTATGCCATCTGC-3′, Reverse primer: 5′-GCAGATGGCATACCAGGC GTCCAGGGCGATGA AGC-3′). Note that mutation of the 'DRY motif' to abolish G protein-coupling has been described previously [15]. Dynorphin A (1–17) was purchased from American Peptide and orexin A from Tocris Bioscience. Rabbit phospho-p38, mouse p38 and rabbit phospho-JNK antibodies were purchased from Cell Signaling Technology, rat anti-HA from Roche and mouse anti-actin from Santa Cruz Biotechnology. Secondary antibodies were purchased from LI-COR Biosciences. Dynasore, H89 and norBNI were purchased from Tocris Bioscience, SP-600125 was purchased from Fisher Scientific, PKI was purchased from Santa Cruz Biotechnology and UBO-QIC was purchased from The University of Bonn.

2.2. Cell culture and transfection

CHO cells (ATCC) were grown in F12 media (Gibco) and Drx-KOR cells (DiscoveRx Corporation) in McCoy's media (ATCC) both supplemented with 10% fetal bovine serum (FBS, Gibco). All cells were maintained at 37 °C, 5% CO_2 and passaged every 3 days so that they remained <80% confluent. Transfections were performed using Fugene HD (Roche) as per manufacturer's recommendations. Stable expression of KOR in CHO-KOR cells was maintained using 250 μg/ml G418.

2.3. GloSensor (Promega) cAMP assay

This assay was performed according to the manufacturer's instructions. Briefly, CHO-KOR cells were transiently transfected with pGlosensor-22F and the indicated receptor construct and 24 h later 10,000 cells/well were plated in a white 384-well plate in a volume of 20 μl CO_2-independent media-10% FBS. After 24 h at 37 °C, 20 μl GloSensor substrate/equilibration buffer was added before incubation for 2 h at room temperature. Wells were then pretreated with 5 μl control or 500 nM orexin (Tocris Bioscience) for 10 min prior to treatment with 5 μl of 2 μM forskolin (MP Biomedical) or 2 μM forskolin/1 μM dynorphin (American Peptide) for 15 min. Luminescence was then measured using an EnVision plate reader. Data is normalized to the forskolin signal in the absence of orexin pretreatment for each transfection condition and error bars represent standard deviation from the mean of 3 separate experiments.

2.4. Calcium/FLIPR assay

10,000 cells/well were plated in a 384-well black clear-bottomed microtitre plate and incubated for 18 h at 37 °C, 5% CO_2. Growth media was then replaced with 25 μl assay buffer (HBSS, 20 mM HEPES, 0.77 mg/ml probenecid, pH7.4). After a further 30 min at 37 °C, 25 μl of 'Calcium 5' dye (Molecular Devices) was added to each well and plates were incubated at room temperature for 2 h in the dark. Following addition of orexin (10 μl, various concentrations), fluorescence was measured using a fluorescence imaging plate reader (FLIPR; Molecular Devices) and maximum response reads were divided by the baseline read taken before peptide addition for each well. Data is plotted as a % of the maximum read on the plate and error bars represent standard error of the mean of 3 replicates. Data shown is representative of 3 separate experiments.

2.5. Radioligand binding assay

To prepare membranes, cells were trypsinized and harvested in cold PBS. After 1 wash in cold PBS, cells were incubated in cold assay buffer (25 mM HEPES, 5 mM $MgCl_2$, pH7.4) + 10 mM EDTA for 45 min. Then, after centrifugation at 45,000 *g* at 4 °C for 30 min, the supernatant was removed and the pellet was washed with cold assay buffer and resuspended in cold assay buffer + protease inhibitors (1 ml per T75 starting material). The suspension was then passed through a 27-gauge needle and protein concentration determined. 10 μg of membrane/well of a 96-well plate was incubated with various concentrations of [^{3}H]-U69 (Perkin Elmer) for 30 min at 37 °C in a total volume of 100 μl assay buffer + 0.6% BSA. This was performed in the presence or absence of norBNI to allow for the subtraction of non-specific binding. Samples were then filtered using filters presoaked with 0.3% polyethyleneimine. Filters were then washed 5 times with assay buffer and allowed to dry before scintillation counting. Error bars represent standard error of the mean of 3 replicates and curves shown are representative of at least 3 separate experiments.

2.6. β-arrestin recruitment assay (PathHunter ™; DiscoveRx Corporation)

Direct interaction between a GPCR and β-arrestin can be monitored using enzyme fragment complementation between two inactive deletion mutants of β-galactosidase; one of the inactive β-gal fragments is fused to the carboxyl-terminus of the receptor and the other to β-arrestin. Recruitment of β-arrestin to the GPCR promotes restoration of functional β-gal enzyme, which can be detected using a luminescent substrate. This was performed according to manufacturer's instructions. Briefly, 5000 cells/well were plated in a white 384-well plate in a volume of 20 μl optimem supplemented with 2% charcoal-treated FBS. Following overnight incubation at 37 °C, 5 μl dynorphin (various concentrations) was added to the plate and cells were incubated for a further 90 min at 37 °C. 12 μl of the chemiluminescent PathHunter™ detection reagent was added and plates were incubated at room temperature in the dark for 1 h before luminescence was measured using an EnVision plate reader. Error bars represent standard error of the mean of 3 replicates and curves shown are representative of at least 3 separate experiments.

3. Results

3.1. OX1R signaling attenuates KOR-mediated inhibition of forskolin-stimulated cAMP production

KOR couples primarily to Gαi, activation of which inhibits adenylate cyclases, resulting in reduced intracellular levels of cyclic adenosine monophosphate (cAMP) [16]. To monitor KOR-mediated Gαi activation, CHO cells stably expressing KOR (CHO-KOR) were transiently transfected with 'pGloSensor-22F' cAMP biosensor (Promega). GloSensor is a form of firefly luciferase genetically modified by insertion of a cAMP-binding moiety such that its luciferase activity/light output is dependent on it binding to cAMP [17]. An EC_{90} (2 μM) of forskolin, a direct activator of adenylate cyclases, was used to increase the cellular cAMP/luciferase signal and provide an assay window for detecting a dynorphin-induced reduction of cellular cAMP levels.

Treatment of pGloSensor-transfected forskolin-treated CHO-KOR cells with 1 μM dynorphin for 15 min reduced the luciferase signal by 77 ± 4.8% compared to that observed in cells treated with 2 μM forskolin alone, demonstrating a dynorphin-mediated reduction in

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