British Journal of Pharmacology

DOI:10.1111/bph.12324
www.brjpharmacol.org

Themed Section: Orexin Receptors

REVIEW

Orexin/hypocretin receptor signalling cascades

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Keywords
G-protein-coupled receptor; intracellular Ca^{2+}; phospholipase; endocannabinoid; adenylyl cyclase; plasticity; cell death; non-selective cation channel; K^+ channel; Na^+/K^+ exchanger

Received
11 January 2013
Revised
18 July 2013
Accepted
28 July 2013

Orexin (hypocretin) peptides and their two known G-protein-coupled receptors play essential roles in sleep–wake control and powerfully influence other systems regulating appetite/metabolism, stress and reward. Consequently, drugs that influence signalling by these receptors may provide novel therapeutic opportunities for treating sleep disorders, obesity and addiction. It is therefore critical to understand how these receptors operate, the nature of the signalling cascades they engage and their physiological targets. In this review, we evaluate what is currently known about orexin receptor signalling cascades, while a sister review (Leonard & Kukkonen, this issue) focuses on tissue-specific responses. The evidence suggests that orexin receptor signalling is multifaceted and is substantially more diverse than originally thought. Indeed, orexin receptors are able to couple to members of at least three G-protein families and possibly other proteins, through which they regulate non-selective cation channels, phospholipases, adenylyl cyclase, and protein and lipid kinases. In the central nervous system, orexin receptors produce neuroexcitation by postsynaptic depolarization via activation of non-selective cation channels, inhibition of K^+ channels and activation of Na^+/Ca^{2+} exchange, but they also can stimulate the release of neurotransmitters by presynaptic actions and modulate synaptic plasticity. Ca^{2+} signalling is also prominently influenced by these receptors, both via the classical phospholipase C–Ca^{2+} release pathway and via Ca^{2+} influx, mediated by several pathways. Upon longer-lasting stimulation, plastic effects are observed in some cell types, while others, especially cancer cells, are stimulated to die. Thus, orexin receptor signals appear highly tunable, depending on the milieu in which they are operating.

LINKED ARTICLES

This article is part of a themed section on Orexin Receptors. To view the other articles in this section visit http://dx.doi.org/10.1111/bph.2014.171.issue-2

Abbreviations

2-AG, 2-arachidonoylglycerol; AA, arachidonic acid; AC, adenylyl cyclase; BRET and FRET, bioluminescence and Förster/fluorescence energy transfer, respectively; CB_1 and CB_2, CB_1 and CB_2 cannabinoid receptors, respectively; CHO, Chinese hamster ovary-K1 (cells); CNS, central nervous system; $cPLA_2$, cytosolic (Ca^{2+}-sensitive) PLA_2; DAG, diacylglycerol; DGL, DAG lipase; Dynlt1, dynein light chain Tctex-type 1; ER, endoplasmic reticulum; ERK, extracellular signal-regulated kinase; GPCR, G-protein-coupled receptor; GRK, GPCR kinase; HEK-293, Human embryonic kidney (cells); IP_3, inositol-1,4,5-trisphosphate; $iPLA_2$, intracellular (Ca^{2+}-independent) PLA_2; *ITIM,* immunoreceptor tyrosine-based inhibitory motif; *ITSM,* immunoreceptor tyrosine-based switch motif; Kir channels, inward rectifier K^+ channels; LPA, lysophosphatidic acid; MAPK, mitogen-activated protein kinase; MEK1, MAPK/ERK kinase 1; NCX, Na^+/Ca^{2+}-exchanger; neuro-2a, a mouse neuroblastoma cell line; nPKC, novel PKC; NSCC, non-selective cation channel; OX_1 and OX_2, OX_1 and OX_2 orexin receptors, respectively; PA, phosphatidic acid; PC12, a rat pheochromocytoma cell line; PI3K, phosphoinositide-3-kinase; PI, phosphatidylinositol; PIP, phosphatidylinositol-4/5-phosphate; PIP_2, phosphatidylinositol-4,5-bisphoshate; PIP_3, phosphatidylinositol-3,4,5-trisphosphate; PKA, PKB, PKC and PKD, protein kinase A, B, C and D, respectively; PLA_2, PLC and PLD, phospholipase A_2, C and D, respectively; PTx, pertussis toxin;

pyrrophenone, *N*-[[(2S,4R)-1-[2-(2,4-difluorobenzoyl)benzoyl]-4-[(triphenylmethyl)thio]-2-pyrrolidinyl]methyl]-4-[(Z)-(2,4-dioxo-5-thiazolidinylidene)methyl]-benzamide; RNAi, RNA interference; Src, a protein tyrosine kinase; SHP-2, a protein tyrosine phosphatase; TASK, TWIK-related acid-sensitive K^+ (channel); TRP, transient receptor potential (channel); TRPC, TRP (channel) of the canonical subfamily; VGCC, voltage-gated Ca^{2+} channel; Y2H, yeast-2-hybrid

Introduction

Since the discovery of the orexin (hypocretin) peptides, orexin-A and orexin-B, and their two known G-protein-coupled receptors (GPCRs), OX_1 and OX_2 receptors (the nomenclature according to BJP's CGtP; Alexander *et al.*, 2013), in 1998 (de Lecea *et al.*, 1998; Sakurai *et al.*, 1998), much has been learnt about the organization and function of this system at the molecular, cellular and systems levels. The discovery that orexins are essential for the normal consolidation of sleep and waking and importantly influence homeostatic systems regulating appetite, stress and reward (see Borgland, 2013; Li and de Lecea, 2013) has kindled interest in this system as a target for developing novel pharmacotherapeutics (see Winrow and Renger, 2013). Nevertheless, fundamental questions remain about how this system operates at all levels. Of particular importance for understanding the cellular functions of orexin receptors is knowledge of the signalling cascades utilized and the effectors that are engaged. However, these cascades and effectors are not well understood in native orexin receptor expressing cells. In order to help advance such understanding, we review orexin receptor signalling from two viewpoints. First, we examine what is known about orexin receptor mechanisms and cascades, primarily from work in expression systems. Second, we examine what is known about orexin receptor signalling in different regions of the brain and other tissues. The first viewpoint is presented in this review, while the second is presented in our sister review (Leonard and Kukkonen, 2013).

Early studies in recombinant CHO cells showed that (human) orexin receptors strongly couple to Ca^{2+} elevation (Sakurai *et al.*, 1998; Smart *et al.*, 1999) and to phospholipase C (PLC; Lund *et al.*, 2000; Holmqvist *et al.*, 2002). Thus, orexin receptors were considered to be G_q-protein-coupled GPCRs, which signal via $G\alpha_q \rightarrow$ PLC $\rightarrow$ IP_3 (inositol-1,4,5-trisphosphate) $\rightarrow$ IP_3 receptor $\rightarrow$ Ca^{2+} release from the endoplasmic reticulum (ER), and were expected to induce cell responses mainly via Ca^{2+} and diacylglycerol (DAG)-mediated pathways, such as those resulting from protein kinase C (PKC) activity. However, it was soon found that orexin receptor signalling, both in native tissues and in recombinant cell lines, was considerably more complicated and versatile (see, e.g. Malendowicz *et al.*, 1999; Lund *et al.*, 2000; Karteris *et al.*, 2001; Randeva *et al.*, 2001). In the following sections, we first present the known coupling partners of orexin receptors and then analyze the evidence for the ensuing signal transduction pathways. In each case, we consider the physiological actions resulting from these pathways, if known.

Orexin receptor coupling partners: heterotrimeric G-proteins

Orexin receptors are GPCRs, and as such, are expected to utilize heterotrimeric G-proteins as the main mediators of signal transduction. Both orexin receptors are very promiscuous in their signalling, and so far have been shown – by direct or indirect means – to couple to members of three of the four heterotrimeric G-protein families, namely G_q, $G_{i/o}$ and G_s. Based on the results from expression systems (see below), nothing would preclude either receptor from coupling to any of these effector families, although this issue has not been sufficiently studied. Despite the long history of studying GPCR–G-protein coupling, few methods exist for direct measurements of G-protein activation and subtype-selective G-protein inhibition (Kukkonen, 2004); hence, conclusive results on orexin receptors are scarce.

GTP-azidoanilide methods can be used to selectively label G-proteins for identification. Cells are incubated with GTP-azidoanilide with ^{32}P- or ^{33}P-label, which, upon receptor stimulation, binds to the G-proteins. Exposure to UV light creates a covalent bond between Gα and the label, allowing the $^{32/33}P$-labelled proteins to be identified using antibodies. This technique has revealed the ability of OX_2 orexin receptors to differentially couple to G_q, $G_{i/o}$ and G_s proteins in different tissues (Karteris *et al.*, 2001; Randeva *et al.*, 2001; Karteris *et al.*, 2005; see also *Hypothalamus* and *Adrenal gland* in Leonard and Kukkonen, 2013). It should be noted that this technique requires plasma membrane permeabilization prior to GTP-azidoanilide incubation, which may disrupt the signalling milieu and distort the results. Similar to GTPγS binding (below), GTP-azidoanilide may be more prone to labelling $G_{i/o}$ proteins. The specificity of antibodies to detect the Gα subunits may also be variable. The only studies in the central nervous system (CNS) have involved the hypothalamus, where coupling to G_i, G_o, G_s and G_q has been observed (Karteris *et al.*, 2005).

^{35}S-GTPγS binding is another method for measurement of G-protein activation in permeabilized cells. Due to the GTP/GDP affinity, GDP release rates and expression levels of different G-proteins, agonist-stimulated gross GTPγS binding is an effective measure of $G_{i/o}$ binding only (when used without antibody separation). GTPγS can also be utilized for autoradiography (Laitinen, 2004). Orexin-A stimulates GTPγS binding to rat brain stem sites, putatively indicating receptor (unidentified subtype) coupling to the $G_{i/o}$ proteins (Bernard *et al.*, 2002; 2003).

Chimeric G-proteins, that is, Gα-subunits with the receptor-coupling parts of one G-protein fused with the effector-coupling part of another G-protein, have been used in one study with recombinant human OX_1 receptors expressed in HEK-293 cells (Magga *et al.*, 2006). In this study, different Gα-subunits, $G\alpha_{o1}$, $G\alpha_{11}$ and $G\alpha_{16}$, were directed to $G\alpha_s$ signalling [adenylyl cyclase (AC) stimulation]. Only $G\alpha_{11}$ and $G\alpha_{16}$ fusion proteins, but not the $G\alpha_{o1}$ fusion protein nor the wild-type G_s, were detected to couple to OX_1 receptors. This appeared to contradict co-immunoprecipitation experiments performed in the same study, where G_q but also G_i and G_s, but not G_o were immunoprecipitated together with the OX_1 receptors (Magga *et al.*, 2006). However, chimeric G-proteins may not interact with the orexin receptor or AC in the same way as do native G-proteins.

Indirect methods for determination of G-protein coupling of GPCRs include utilization of toxins, drugs, dominant-negative proteins, blocking peptides, antibodies or knockdown/knockout to block distinct G-proteins. Pertussis toxin (PTx) has until recently been the only commercially available G-protein inhibitor. It ADP-ribosylates $G_{i/o}$ family G-proteins, which results in inactivation. In most cases, PTx may be a fairly reliable tool, but one should still be careful when using it as it may have other effects that are dependent on or independent of its enzymatic activity (Schneider *et al.*, 2007; Mangmool and Kurose, 2011). The use of PTx also requires long incubation (usually at least overnight) to inactivate the entire target G-proteins, and this may induce plastic changes in the cells. We very often observe changes in cell morphology with PTx, which may indicate involvement of proteins other than $G_{i/o}$ family G-proteins (J. P. Kukkonen, unpublished). Some cells may be less sensitive to PTx, either due to weak entry of PTx (low number of 'PTx receptors') or expression of the PTx-insensitive $G_{i/o}$ family member G_z. Thus, the lack of ability of PTx to inhibit a particular response should not directly be interpreted as lack of $G_{i/o}$ involvement; often, the effectiveness of the PTx treatment is not verified by cAMP measurements. Dominant-negative G-proteins have been used in two studies. However, dominant-negative G-proteins often do not show very good specificity (Kukkonen, 2004), which also seems to be reflected in these results (Tang *et al.*, 2008; Ramanjaneya *et al.*, 2009). Drugs, blocking peptides, antibodies or knockdown/knockout have not been utilized to analyze orexin receptor G-protein coupling, except for one study with an anti-G_s antibody (see below).

Yet a more indirect way of determining G-protein coupling is to measure the expected downstream responses mediated by particular G-proteins. Both orexin receptor subtypes efficiently couple to PLC-Ca^{2+} release, suggesting signalling via G_q proteins. This is observed both in multiple recombinant cells of neuronal and non-neuronal type (CHO, HEK-293, neuro-2a, PC12) and in some native cells (see *Phospholipase C*). We consider that G_q, coupling the receptors to PLCβ, is a rather fair conclusion in these cases, but one still has to consider the possibility of engagement of other PLC isoforms via receptor signals other than G_q (reviewed in Kukkonen, 2011; see also *Phospholipase C*). One also has to consider the propensity of orexin receptors to couple to receptor-operated Ca^{2+} influx (see *Ca^{2+}*). AC is another classical GPCR target both for positive and for negative regulation. However, there are many regulating factors, and alterations in AC activity cannot be ascribed to a particular G-protein without further analysis (see *Adenylyl cyclase*). This is especially pertinent to orexin receptors, which prominently elevate Ca^{2+} and activate PKC, both of which are important regulators of ACs. Human OX_1, expressed in CHO cells, appear to couple to G_i family proteins, as revealed by the sensitivity of AC inhibition to PTx, and to G_s family proteins, as revealed by AC stimulation and its inhibition by anti-G_s antibodies or by 'competing' G_s stimulation (Holmqvist *et al.*, 2005). However, in these cells, G_s is activated only at high orexin concentrations, while the G_i and the putative G_q cascades are activated 100-fold more potently (see *Recombinant cells* and figure 3 in Leonard and Kukkonen 2013). This may not be the case in other cell types (see *Hypothalamus* and *Adrenal gland* in Leonard and Kukkonen, 2013).

In conclusion, both orexin receptors are capable of coupling to several G-protein species. Hence, the common conception that OX_1 couples exclusively to G_q, and that OX_2 couples to G_q and $G_{i/o}$ is dubious and other coupling possibilities need to be considered. The direct or less direct methods for assessing G-protein coupling of orexin receptors have been applied in only a few cases, and the conclusions about the importance of particular G-proteins for orexin receptor responses, even in these cases, must be tempered by the limitations in these methods. There are also many responses, as described below and in the sister review (Leonard and Kukkonen, 2013), that cannot be easily ascribed to a particular G-protein. Nevertheless, it is not unreasonable to assume that the G_q–PLC pathway plays an important role in many cases.

Orexin receptor coupling partners: interaction with other proteins

Many, if not all, GPCRs also interact with proteins other than heterotrimeric G-proteins (reviewed in Ritter and Hall, 2009). Novel interactions are often explored utilizing yeast-2-hybrid (Y2H) screening (or a similar method) and further verified by co-immunoprecipitation, glutathione S-transferase (GST)-pull-down, Förster/fluorescence energy transfer (FRET), etc., and even indirect means such as RNA interference (RNAi). These other proteins can transduce GPCR signals, but they can also affect GPCR trafficking or anchoring; however, in many cases, their functional roles are still unclear (reviewed in Ritter and Hall, 2009).

β-Arrestin appears to be an equally common interaction partner for GPCRs (reviewed in Rajagopal *et al.*, 2010), as illustrated in the seminal work of Robert J. Lefkowitz, Nobel Laureate in Chemistry 2012. Classically, β-arrestins are involved in homologous desensitization. GPCR activity would lead to activation of GPCR kinases (GRKs) and receptor phosphorylation, which would recruit β-arrestin, which may, depending on the receptor type, lead to internalization of the receptor. Receptor-containing endosomes would then either recirculate to the plasma membrane or enter lysosomal degradation (reviewed in Rajagopal *et al.*, 2010; Shenoy and Lefkowitz, 2011). However, β-arrestin is not only involved in trafficking but also acts as a signalling scaffold, for example, for the mitogen-activated protein kinase (MAPK) pathways of extracellular signal-regulated kinase (ERK) (reviewed in Rozengurt, 2007; Rajagopal *et al.*, 2010). However, this is not the only way MAPK cascades are regulated by GPCRs (reviewed in Rozengurt, 2007). In transiently OX_1-transfected CHO-K1 cells, human OX_1 receptor activation attracts β-arrestin 1- and 2-GFP to the membrane and, in the continued presence of orexin-A, the fluorescence moves into intracellular puncta (Evans *et al.*, 2001). When investigated using TAMRA (carboxytetramethylrhodamine)-labelled orexin-A, the GFP fluorescence of β-arrestin 1 and TAMRA fluorescence of orexin-A were co-localized (Evans *et al.*, 2001). GRK2 was co-expressed in the cells and the cells were not assessed in the absence of heterologous GRK2. OX_1 receptor–β-arrestin 2 interaction was later investigated in transiently transfected HEK-293 in the absence of heterologous GRK2 (Milasta *et al.*, 2005). OX_1 receptors were shown to co-localize with β-arrestin-GFP (or β-arrestin-red fluorescent protein) and to enter acidified endosomes. The interaction was verified by co-immunoprecipitation. The interaction epitope in the OX_1

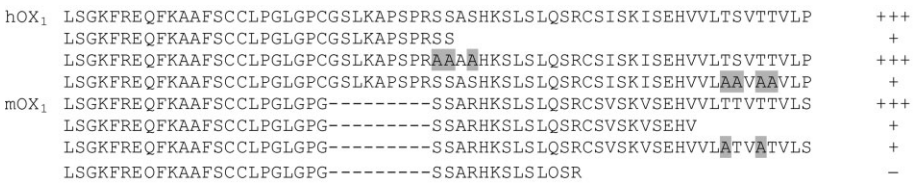


Figure 1

Comparison of the C-terminals of human and mouse OX_1 receptors with respect to the suggested protein–protein interactions. The sequences are aligned to ease the comparison. The wild-type sequences are presented first for each species, and the mutant sequences (truncated and point-mutated) underneath. The human receptor data are obtained from investigations of the interaction of the receptor with β-arrestin-2 by assessment of the co-localization in HEK-293 cells upon endocytosis (Milasta *et al.*, 2005). The mouse receptor data depict the assessment of the interaction of the receptor C-terminus with Dynlt1 with the Y2H method (Duguay *et al.*, 2011). The strength of the interaction is indicated by the symbols after each sequence.

receptor was assessed by truncation and point mutations, which suggest that at least part of the interaction takes place via the receptor's C-terminus; weakened interaction led to a more transient ERK activation (Figure 1). In transiently transfected mouse embryonic fibroblast (MEF) cells from β-arrestin-1/2 double-knockout animals, no OX_1 internalization was observed, unless β-arrestin-2 was reintroduced (Milasta *et al.*, 2005).

OX_1 receptors have been suggested to interact with the protein phosphatase SHP-2 (Voisin *et al.*, 2008; El Firar *et al.*, 2009). This is discussed in detail under *Cell death*.

The Y2H method was utilized to reveal novel interaction partners for the C-terminus of the mouse OX_1 receptor (Duguay *et al.*, 2011). The screen suggested interaction with the dynein light chain Tctex-type 3. A focused testing also identified interaction between the dynein light chain Tctex-type 1 (Dynlt1) and either human orexin receptor. Co-immunoprecipitation in mammalian cells verified the interaction between OX_1 and Dynlt1. The amino acids in either protein important for the interaction were further investigated using the Y2H method and the functional consequences assessed in recombinant HEK-293 cells. OX_1-mediated ERK phosphorylation became more transient upon overexpression of Dynlt1 and more sustained when Dynlt1 levels were reduced using RNAi (Duguay *et al.*, 2011). As dynein is involved in trafficking along microtubules, the effect observed could be related to a difference in receptor trafficking. However, no significant differences were observed in the resting plasma membrane localization or internalization upon agonist exposure (Duguay *et al.*, 2011). It is noteworthy that both Duguay *et al*. (2011) and Milasta *et al*. (2005; see above) identified a partly overlapping region of the OX_1 C-terminus to be involved in protein–protein interaction of OX_1 receptors (Figure 1). In both cases, ERK signalling, but not gross receptor internalization, was affected. However, it seems that β-arrestin promotes ERK signalling, while Dynlt1 inhibits it, and Duguay *et al*. (2011) suggest that this could be due to competition between these proteins for binding to the C-terminus of OX_1. However, there could also be other protein interactions in this domain, contributing to the receptor response. Unfortunately, the β-arrestin and Dynlt1 responses were only tested at high orexin-A concentration (500 nM).

GPCRs have also been reported to engage in dimerization/oligomerization processes. Such complexes may be homomeric, but heteromeric complexes have also been reported. While the role of homomeric complexes is difficult to assess, heteromeric complexes have sometimes been shown to affect receptor trafficking, signalling or pharmacology (reviewed in Bulenger *et al.*, 2005; Milligan, 2009). Human OX_1 receptors in recombinant HEK-293 cells have been shown, by native gel electrophoresis and FRET studies, to form homomeric complexes, and stimulation with orexin-A has been suggested to increase complex formation (Xu *et al.*, 2011). It is, however, difficult to determine the number of receptors in a complex on the non-denaturing gel used. So far, only CB_1 cannabinoid receptors have been identified as a heterodimerization partner for OX_1 receptors – but it seems that other receptors have not yet been investigated either. Complexes between human OX_1 and CB_1 receptors have been verified by co-immunoprecipitation and fluorescence and bioluminescence energy transfer (BRET and FRET, respectively) in recombinant HEK-293 cells (Ward *et al.*, 2011a). The physiological importance of this, however, has recently been questioned (see *Orexins and endocannabinoids*).

In conclusion, it is difficult to interpret the physiological significance of the putative orexin receptor interaction with proteins other than heterotrimeric G-proteins. One reason for this is that these have not yet been assessed in native orexin receptor-expressing cells, for example, by utilizing RNAi.

Adenylyl cyclase

AC regulation is a classical GPCR response. There are nine plasma membrane-bound AC isoforms, which all show different regulation, and one cytosolic variant (reviewed in Sunahara and Taussig, 2002). A typical feature of membrane-bound ACs is their synergistic regulation by different intracellular signals. The only common regulator is $G\alpha_s$ (AC activator), while the impact of other positive or negative regulators like $G\alpha_i$, $G\beta\gamma$, Ca^{2+} or PKC depends on the AC isoform (reviewed in Sunahara and Taussig, 2002). Orexin receptors are certainly capable of regulating AC (Malendowicz *et al.*, 1999; Randeva *et al.*, 2001; Mazzocchi *et al.*, 2001b; Holmqvist *et al.*, 2005; Karteris *et al.*, 2005; Tang *et al.*, 2008), although this seems less prominent than coupling to the PLC and Ca^{2+} cascades; however, not all tissues and cell types investigated have been examined for cAMP responses. Some of the AC stimulation may relate to the ability of orexin receptors to couple to G_s proteins (*Orexin receptor coupling partners: heterotrimeric G-proteins*, and *Central nervous system and Adrenal gland* in Leonard and Kukkonen,

2013), but also the other significant cascades such as Ca^{2+} and PLC–PKC may play a part. Unfortunately, we usually do not know how AC stimulation by orexin receptors is mediated even at sites where this is known to happen. Nevertheless, AC regulation by orexin receptors has a significant role in some tissue responses like corticosteroid release from adrenal cortex (*Adrenal gland* in Leonard and Kukkonen, 2013).

Phospholipase C

Mammalian phospholipase C, which we here refer to as PLC, is a family of cytosolic phosphoinositide-specific enzymes (reviewed in Kukkonen, 2011). Their most studied substrate is phosphatidylinositol-4,5-bisphosphate (PIP_2), but they also hydrolyze other phosphoinositides, including phosphatidylinositol (PI) and phosphatidylinositol-4-phosphate/phosphatidylinositol-5-phosphate (commonly PIP). Hydrolysis takes place on the phosphoester bond between the phosphate and the glycerol, yielding DAG and inositol phosphates. DAG, as well as IP_3, are the messengers produced upon PIP_2 hydrolysis. It is also noteworthy that PIP_2 itself has emerged as a major regulator of effectors (e.g. voltage-gated M-type K^+ channels [$K_V7.2/3$], N-type Ca^{2+} channels [$Ca_V2.2$] and transient receptor potential [TRP] channels, plasma membrane Ca^{2+} ATPase and Na^+/Ca^{2+}-exchanger [NCX]) (reviewed in Gamper and Shapiro, 2007; Suh and Hille, 2008).

The PLC family is divided into subfamilies of β, γ, δ, ε, ζ and η, with a total of 12 members. The substrate specificity of the members may vary, but this has not been thoroughly investigated. Regulation of the subfamilies and their members is very versatile (reviewed in Kukkonen, 2011). GPCRs can target multiple PLC isoforms. PLCβ is the classical target via $G\alpha_q$ family members, and Gβγ, but also PLCη, can be regulated by Gβγ. GPCRs can regulate, more indirectly, PLCδ and -ζ via Ca^{2+}, PLCγ via Src (a protein tyrosine kinase) or phosphatidylinositol-3,4,5-trisphosphate (PIP_3), and PLCε via Ras and Rho family monomeric G-proteins.

Direct measurements of PLC activity (total inositol phosphate or IP_3 generation, PIP_2 hydrolysis) show that both OX_1 and OX_2 receptors strongly activate PLC in many recombinant expression systems, including CHO, HEK-293, neuro-2a and PC12 cells (Lund *et al.*, 2000; Holmqvist *et al.*, 2002; Putula and Kukkonen, 2012; J. P. Kukkonen *et al.*, unpublished). A reasonable assumption is that this takes place via the G_q family G-proteins, but in the absence of selective pharmacological inhibitors, this has not been verified. Indeed, in recombinant CHO cells, human OX_1 receptors activate two apparently different PLC activities with different specificities for phosphoinositides (Johansson *et al.*, 2008). Among native orexin receptor-expressing cells, PLC activation has been shown in explants of human primary pheochromocytomas (OX_2 mRNA) and in membrane preparations of rat adrenal cortex and hypothalamus and human reproductive tract (mixed receptor populations) (Randeva *et al.*, 2001; Mazzocchi *et al.*, 2001a; Karteris *et al.*, 2004; 2005). Indirect indications of PLC activity are obtained from the use of PLC inhibitors or observation of Ca^{2+} release from the intracellular stores. It should be noted that PLC inhibitors, like U73122, may show non-specific toxicity and inhibit responses independent of their action on PLC (Taylor and Broad, 1998). Ca^{2+} elevation is not always determined to originate from Ca^{2+} release, and not even all Ca^{2+} release is IP_3-dependent (reviewed in Konieczny *et al.*, 2012). For orexin receptor signalling, pharmacological analysis has been used mainly with recombinant cells, where PLC activation is often known from direct measurements. Even more indirect indication for PLC activation has come from evidence of PKC involvement, which is usually rather easily assessed utilizing a suitable panel of pharmacological inhibitors. The members of the conventional (c) and novel (n) subfamilies are activated by DAG (reviewed in Newton, 2010). An nPKC, possibly PKCδ, is involved in human OX_1 receptor stimulation of AC and phospholipase D (PLD) in recombinant CHO-hOX_1 cells (Holmqvist *et al.*, 2005; Jäntti *et al.*, 2012), while cPKC may be involved in the activation of ERK in these cells (Ammoun *et al.*, 2006a). PKC activation is also indicated by PKC translocation upon orexin receptor activation in these cells (Holmqvist *et al.*, 2005; Ammoun *et al.*, 2006a; Johansson *et al.*, 2008; Ekholm *et al.*, 2010). PKC involvement has been assessed, using inhibitors, in some neuronal preparations (Yang *et al.*, 2003; Borgland *et al.*, 2006; Zhang *et al.*, 2010; see *Central nervous system* in Leonard and Kukkonen, 2013). However, PKC inhibitors have been applied surprisingly seldom. It should be noted that DAG is not only generated by PLC activity but can also originate from the PLD or reverse action of sphingomyelin synthase, and it is also generated (although not in the plasma membrane) during phospholipid synthesis (reviewed in Kukkonen, 2011).

DAG is principally metabolized either to phosphatidic acid (PA) by DAG kinase or *sn*2-monoacylglycerol by DAG lipase (DGL) (reviewed in Kukkonen, 2011). Both PA and *sn*2-monoacylglycerol may act as messengers (see *Phospholipase D* and *Orexins and endocannabinoids*).

Prokaryotes and protozoans possess phosphatidylcholine-specific PLC (PC-PLC). Such an enzyme has not been isolated in mammalian cells, but nevertheless, some studies report similar enzymatic activity. The reputed PC-PLC inhibitor, D609, has been used to garner evidence of PLC involvement. However, this inhibitor is not specific for 'PC-PLC' and it definitely is not an inhibitor of PI-PLC, although it has not been tested for all PLC isoforms (reviewed in Kukkonen, 2011; Kukkonen, 2013b). Therefore, even though some orexin responses have been blocked with D609 (reviewed in Kukkonen, 2013b), we think the conclusion of involvement of the putative mammalian PC-PLC in orexin receptor signalling is premature and requires further scrutiny.

In conclusion, PLC activation may be a very central orexin receptor cascade, although in most cases, the coupling has not been adequately assessed by either direct or indirect means. Whether the role of PLC in these cascades is to produce Ca^{2+} elevation (via release or Ca^{2+} channel regulation) or to elevate DAG for possible PKC activation is an important question remaining to be resolved.

Phospholipase D

PLD family enzymes hydrolyze the glycerophospholipid phosphoester bond between the head group and the phosphorus. The common isoforms are PLD1 and -2, which hydrolyze PC generating PA and choline (reviewed in Kukkonen, 2011). Generation of PA is the major signal mediated by PLD1 and -2. PA binds to signalling proteins, but it also has an impact on membrane curvature. PA is metabolized via hydrolysis to either DAG or lyso-PA (LPA), both of

which may have messenger roles. It should be noted that DAG and PA from the PLC and PLD pathways, respectively, have different fatty acid compositions due to their different phospholipid sources and may have different messenger roles. We have recently observed that human OX_1 receptors in CHO cells potently activate PLD (Jäntti *et al.*, 2012). Pharmacological analysis indicates that the isoform activated is PLD1 and that the activation cascade goes via an nPKC but not Rho-family monomeric G-proteins. So far, this is the only report of PLD activation by orexin receptors. However, it should be straightforward to address the potential role of PLD in native orexin receptor function utilizing new commercially available PLD inhibitors (Monovich *et al.*, 2007; Scott *et al.*, 2009; Su *et al.*, 2009).

Phospholipase A_2 cascade

The PLA_2 family comprises a vast number of enzymes with the capability to hydrolyze the *sn*2-ester bond of glycerophospholipids (reviewed in Dennis *et al.*, 2011). Some of these enzymes, most notably the class IV and VI members, are involved in cytosolic signalling. Class IV is also known as cytosolic (Ca^{2+}-sensitive) PLA_2 ($cPLA_2$) and these enzymes are activated by Ca^{2+} and by phosphorylation. Class VI is also known as $iPLA_2$. These enzymes are not activated by Ca^{2+} but are possibly inhibited by it, while they may be activated by ATP, oligomerization, phosphorylation or proteolytic cleavage (reviewed in Balsinde and Balboa, 2005; Dennis *et al.*, 2011). PLA_2 enzymes are well known for the release of arachidonic acid (AA), but many of these enzymes show low fatty acid specificity. The other hydrolysis 'fragment', lysoglycerophospholipid, itself may also be a messenger or lead to messenger generation. AA is classically a substrate for eicosanoid synthesis, but it may also act as a messenger on its own (Kukkonen, 2011). Please note that AA can also result from the breakdown of endocannabinoids (see *Orexins and endocannabinoids*).

We recently identified significant release of ^{3}H-label following orexin-A stimulation of OX_1-expressing CHO and HEK-293 labelled with [^{3}H]-AA (Peltonen *et al.*, 2009; Turunen *et al.*, 2010). We further investigated this in CHO cells and found label residing in both free AA as well as in 2-arachidonoylglycerol (2-AG) (Turunen *et al.*, 2012). 2-AG was produced via the PLC–DGL pathway, while the free AA originated from both the PLA_2 activity and the 2-AG breakdown. In recombinant human OX_1-expressing HEK-293 and neuro-2a cells, orexin receptor stimulation liberated both 2-AG and AA, although the source of AA was not investigated. The PLA_2 isoform in CHO cells was identified as $cPLA_2$ (likely isoform α) (Turunen *et al.*, 2012). The activation mechanism of $cPLA_2$ in these cells remains elusive, but it would be tempting to suggest Ca^{2+} elevation, perhaps by influx, given the Ca^{2+} sensitivity of this isoform. However, orexin receptor-operated Ca^{2+} influx itself appears to require $cPLA_2$ activity, at least in these cells (Turunen *et al.*, 2012), leaving the situation unresolved (see *Ca^{2+}*).

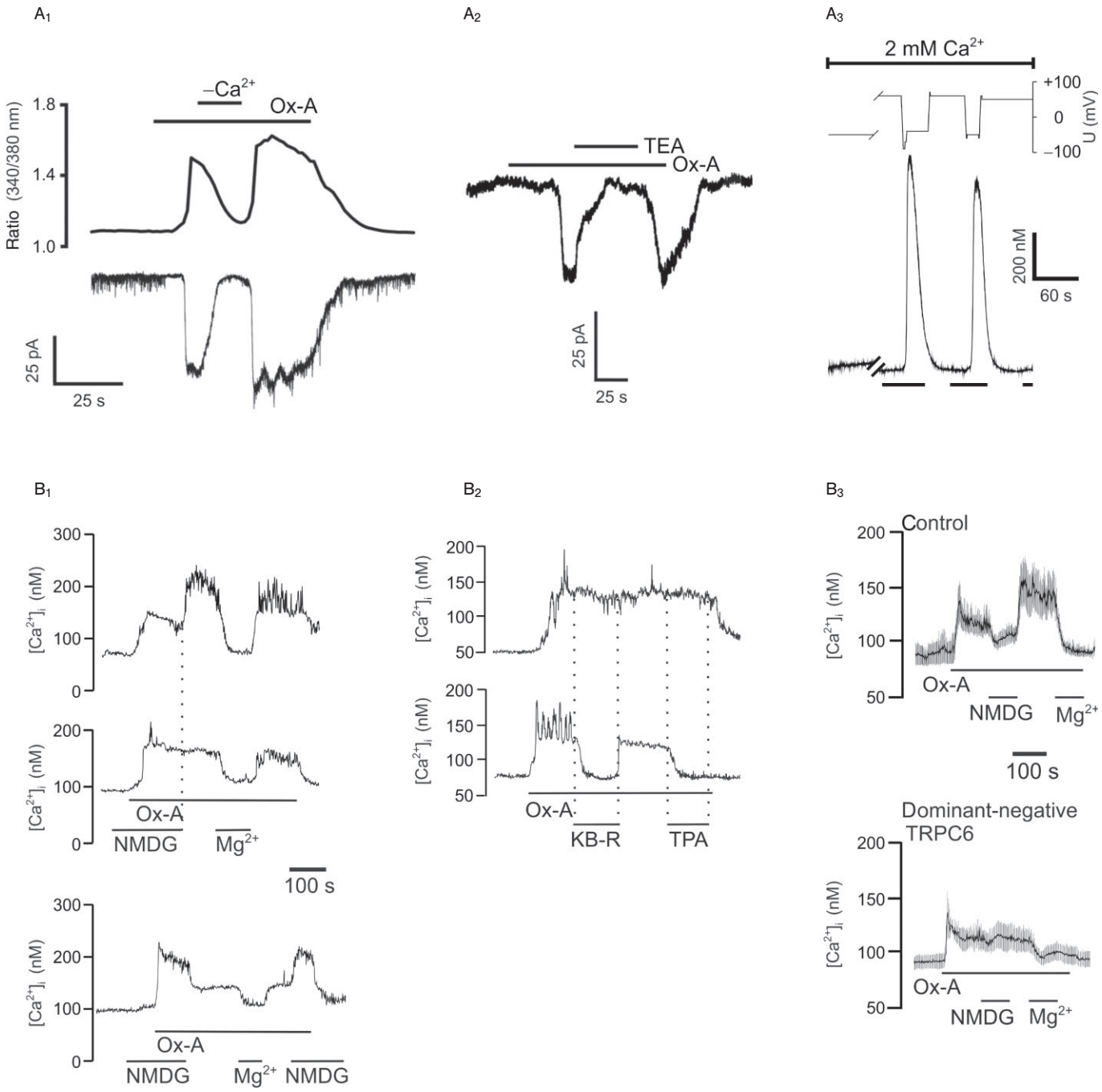
To our knowledge, these are the only reports of PLA_2 activation in orexin signalling, so its role in native orexin receptor operation remains obscure. Similar to PLD, at least the role of $cPLA_2(\alpha)$ in native systems should be easily assessed utilizing the novel pharmacological tool pyrrophenone (*N*-[[(2*S*,4R)-1-[2-(2,4-difluorobenzoyl)benzoyl]-4-[(triphenylmethyl)thio]-2-pyrrolidinyl]methyl]-4-[(Z)-(2,4-dioxo-5-thiazolidinylidene)methyl]-benzamide; Ono *et al.*, 2002).

Ca^{2+}

The earliest reports of orexin receptor activation indicated that these receptors trigger Ca^{2+} elevation in both recombinant and native cells (Sakurai *et al.*, 1998; van den Pol *et al.*, 1998). However, it soon became apparent that this response strongly relied on extracellular Ca^{2+} (Smart *et al.*, 1999; Lund *et al.*, 2000) (Figures 2 and 3).

Classically, PLC activation generates IP_3, which binds ER IP_3 receptors and releases Ca^{2+} into the cytosol via these receptor channels (reviewed in Konieczny *et al.*, 2012). Second, ER Ca^{2+} depletion signals the plasma membrane to allow Ca^{2+} influx to replenish the stores. This 'store-operated' or 'capacitative' Ca^{2+} influx, common in non-excitable cells, was recently shown to involve the ER Ca^{2+} sensor and response transducer stromal interaction molecule 1 (STIM1) and the plasma membrane Ca^{2+} channel orai1, although other proteins (e.g. of the TRP family) may also contribute (reviewed in Konieczny *et al.*, 2012; Yuan *et al.*, 2012). Capacitative Ca^{2+} entry not only recharges the store, but this entire release-influx 'detour' may function to create a high-Ca^{2+} microdomain near the plasma membrane for regulation of Ca^{2+} responsive ion channels, enzymes and other proteins (reviewed in Konieczny *et al.*, 2012). Ca^{2+} influx can also be regulated via other channels and transporters in GPCR signalling. Voltage-gated Ca^{2+} channels (VGCCs) are directly activated by depolarization and can be modulated by second messenger systems. Known receptor-operated Ca^{2+} channels include non-selective cation channels (NSCCs) of the TRP family with a high diversity of activation signals (reviewed in Kukkonen, 2011), the ARC AA-regulated channels, which have recently been suggested to be composed of orai1 and -3 subunits (Shuttleworth, 2009) and the cyclic nucleotide-gated channels. Ca^{2+} influx can also be generated via reversible $Na^+/Ca^{2+}(-K^+)$ exchangers (NCX, NCKX), which can pump in Ca^{2+} by extruding Na^+ (reviewed in Lytton, 2007).

Several lines of evidence indicate that NSCCs contribute to orexin responses. Indeed, orexins depolarize many types of neurons by activation of non-selective cation current (see *Depolarization and synaptic functions*); however, neither the identity of the channels nor their selectivity for Na^+ and Ca^{2+} has been adequately examined. In recombinant human OX_1-expressing CHO cells, these responses have been analyzed in some detail. OX_1 stimulation activates a Ca^{2+} influx, which can be selectively visualized utilizing Mn^{2+} as Ca^{2+} substituent (Lund *et al.*, 2000) and whole-cell patch clamp (Larsson *et al.*, 2005) (Figure $2A_{1-2}$). This Ca^{2+} influx is activated at significantly lower orexin concentrations than Ca^{2+} release and it can be blocked by reversal of the driving force for Ca^{2+} entry (Lund *et al.*, 2000) (Figure $2A_3$) and some TRP channel blockers (Kukkonen and Åkerman, 2001; Johansson *et al.*, 2007) (Figure $2A_2$). This Ca^{2+} influx does not require IP_3 (Ekholm *et al.*, 2007), but instead $cPLA_2$ activity, as inhibition of $cPLA_2$ with methyl arachidonoyl fluorophosphonate or pyrrophenone blocks it (Turunen *et al.*, 2010; 2012). Further studies utilizing dominant-negative TRPC channels suggest that TRPC1 and -3 channels contribute to this response in these cells (Larsson *et al.*, 2005).



Näsman *et al*. (2006) also analyzed the responses in differentiated IMR-32 neuroblastoma cells, a model of human sympathetic neurons. Human OX_1 receptors transiently expressed in these cells couple both to Ca^{2+} release and Ca^{2+} influx with Ca^{2+} influx as the primary response at low orexin concentration (1 nM). The pharmacological profile of response inhibition was similar for orexin-A-induced Ca^{2+} influx and dioctanoylglycerol-induced Ca^{2+} influx. Expression of dominant-negative TRPC6 was able to partly inhibit both responses, suggesting that both are mediated by DAG-sensitive TRPC (canonical subfamily of TRP) channels, that is, TRPC3, -6 and/or -7 (Näsman *et al*., 2006). In a further study, the authors found distinct subpopulations of these cells having Ca^{2+} responses that were either dependent on or independent of extracellular Na^+ (Louhivuori *et al*., 2010) (Figure $2B_1$). The Na^+-dependent responses required reverse-mode operation of NCX, which elevates intracellular Ca^{2+} upon elevation of intracellular Na^+ and its extrusion, while the Na^+-independent responses appeared to involve channel-mediated Ca^{2+} influx (Figure $2B_2$). Interestingly, TRPC3 channels may be the source of elevated Na^+, as both RNAi with small hairpin-RNA and expression of dominant-negative TRPC6, which forms inactive complexes with TRPC3, inhibited most of the Na^+-dependent response without inhibiting the Na^+-independent responses (Louhivuori *et al*., 2010) (Figure $2B_3$). These findings suggest a mechanism by which

Figure 2

Ca^{2+} influx in recombinant human OX_1 receptor-expressing cells. (A) Ca^{2+} influx is required for orexin responses in CHO-hOX_1 cells. (A_1) 0.3 nM orexin-A produces Ca^{2+} elevation (top trace) and a simultaneous inward current (lower trace). Removal of extracellular Ca^{2+} ('–Ca^{2+}') attenuates both responses. The current is also effectively attenuated by substituting tetraethylammonium chloride for NaCl (TEA, 70 mM [70 mM NaCl replaced]; A_2). This research (A_{1-2}) was originally published in the *Journal of Biological Chemistry*. Larsson KP, Peltonen HM, Bart G, Louhivuori LM, Penttonen A, Antikainen M, Kukkonen JP, Åkerman, KE (2005). Orexin-A-induced Ca^{2+} entry: evidence for involvement of TRPC channels and protein kinase C regulation. *J Biol Chem*. 2005; 280: 1771–1781. © the American Society for Biochemistry and Molecular Biology. (A_3) Removal of the driving force for Ca^{2+} entry by strong depolarization (see the voltage trace; top) abolishes the Ca^{2+} response (bottom) to 10 nM orexin-A (presence indicated by vertical bars under the Ca^{2+} trace). This research was originally published in the *Journal of Biological Chemistry*. Lund PE, Shariatmadari R, Uustare A, Detheux M, Parmentier M, Kukkonen JP, Åkerman, KEO. The orexin OX_1 receptor activates a novel Ca^{2+} influx pathway necessary for coupling to phospholipase C. *J Biol Chem*. 2005; 275: 30806–30812. © the American Society for Biochemistry and Molecular Biology. (B) IMR-32 neuroblastoma cells transduced with hOX_1 baculovirus show Ca^{2+} responses regulated by extracellular Na^+ likely via NCX. (B_1) Removal of extracellular Na^+ (all NaCl replaced with *N*-methyl-D-glucamine [NMDG]) inhibits orexin-A (1 nM) responses in some cells (upper trace), while in other cells the response is stimulated (lower trace) or not affected (middle trace). 10 mM $MgCl_2$ blocks the response in all three types of cells. (B_2) The NCX blockers KB-R7943 (10 μM; lower trace) and SN-6 (1 μM; not shown) block most of the Ca^{2+} elevation in the cells showing Na^+-dependent elevation whereas the cells with Na^+-independent Ca^{2+} elevation are not affected (upper trace). The Na^+-dependence of the responses is not shown in the figure. Both KB-R7943 and SN-6 are more potent inhibitors of the reverse mode of NCX. The effect of 100 nM 12-*O*-tetradecanoylphorbol-13-acetate (TPA) is also shown in B_2. (B_3) Expression of the dominant-negative TRPC6 subunit abolishes the Na^+-dependent component of the response. Average traces are shown in B_3. Reprinted from Cell Calcium, 48(2–3), Louhivuori LM, Jansson L, Nordström T, Bart G, Näsman J, Åkerman KEO, Selective interference with TRPC3/6 channels disrupts OX_1 receptor signalling via NCX and reveals a distinct calcium influx pathway, pp. 114–123, © 2010, with permission from Elsevier. Figures adapted from Lund *et al.* (2000), Larsson *et al.* (2005), and Louhivuori *et al.* (2010) as indicated. The figures are reproduced with permission.

orexin-stimulated Na^+ entry via TRPC channels drives reverse mode operation of the NCX to elevate intracellular Ca^{2+}. While channels formed by TRPC3 and -6 subunits show some preference for Ca^{2+} over Na^+ (Gees *et al.*, 2010), they likely still allow more Na^+ influx under physiological conditions given the much greater extracellular [Na^+].

In HEK-293 cells, both human orexin receptors also connect to PLC and both Ca^{2+} influx and release (Magga *et al.*, 2006; Tang *et al.*, 2008; Putula *et al.*, 2011; Putula and Kukkonen, 2012). At low orexin-A concentrations (1 nM), regular Ca^{2+} influx-dependent oscillations are observed (Peltonen *et al.*, 2009). OX_1 receptors activate protein kinase D1 and -3 (PKD1 and -3, respectively), as indicated by their translocation to the plasma membrane and phosphorylation at Ser-916 (Peltonen *et al.*, 2010). Influx-dependent Ca^{2+} oscillations are inhibited by dominant-negative constructs of either TRPC3 or PKD3 by 60%, suggesting involvement of these proteins in the process. However, while PKC inhibition with GF109203X inhibits PKD3 phosphorylation at Ser-916 by 70%, it does not affect Ca^{2+} oscillations (Peltonen *et al.*, 2010).

Ca^{2+} measurements utilizing fluorescent probes do not easily distinguish Ca^{2+} influx and release, while electrophysiological techniques may miss/abolish some influx pathways. Some tricks can be utilized to pinpoint the influx in fluorescence measurements, such as removal of extracellular Ca^{2+} or replacement with other divalent cations (e.g. Mn^{2+} mentioned earlier), reduction of the driving force for Ca^{2+} entry (see above) or blocking of the influx with drugs or molecular biological inhibitors. All of these have their own advantages and problems, which cannot be reviewed here. In the absence of potent and specific channel inhibitors, less specific methods, including the drastic means of removal of extracellular Ca^{2+}, must be used. Removal of Ca^{2+} – and less pronouncedly channel block – also alters other orexin receptor responses. In OX_1-expressing CHO cells, the PLC (and Ca^{2+}) response is shifted towards higher orexin-A concentrations and the ERK, cAMP, PLA_2 and PLD responses are almost totally abolished (Ammoun *et al.*, 2006a; Johansson *et al.*, 2007; Turunen *et al.*, 2010; Jäntti *et al.*, 2012). Why is removal of extracellular Ca^{2+} more effective as an inhibitor of these responses than channel block or reduction of the driving force for Ca^{2+} influx? Could extracellular Ca^{2+} be required for orexin binding to its receptors? This has not been assessed by direct experiment, but some indirect evidence is inconsistent with this hypothesis (Lund *et al.*, 2000). Also, if this was the case, it would be unlikely that the sensitivity for extracellular Ca^{2+} is different in different assays and different cell lines. Another hypothesis is that some types of orexin receptor signalling require a priming Ca^{2+} influx, which would allow coupling to other responses. Some circumstantial evidence supports this concept (Ammoun *et al.*, 2006a). However, there is no direct molecular evidence for the hypothesis, and it is often difficult to find the operant and operand; for instance, Ca^{2+} influx seems to be required for PLA_2 activation by orexin receptors, but PLA_2 activity is also required for Ca^{2+} influx (Turunen *et al.*, 2010; 2012). A third explanation is that removal of Ca^{2+} affects the signal pathways downstream of the receptors, but there is no clear evidence to support this. Thus, the Ca^{2+} sensitivity of (some) orexin responses is currently without an unequivocal molecular explanation. Evidently, there also are orexin responses that are robust even when extra- and intracellular Ca^{2+} levels are significantly reduced (Kohlmeier *et al.*, 2008).

Orexin-mediated Ca^{2+} responses have also been observed in neurons but in only a few cases has the source of the Ca^{2+} elevation been defined (van den Pol *et al.*, 1998; 2001; van den Pol, 1999; Uramura *et al.*, 2001; Lambe and Aghajanian, 2003; Kohlmeier *et al.*, 2004; 2008; Ishibashi *et al.*, 2005; Tsujino *et al.*, 2005). Moreover, Ca^{2+} measurements in neurons have usually been made without simultaneous measurement of membrane potential. As orexin receptors induce depolarization (see *Depolarization and synaptic functions*), the simplest source for Ca^{2+} elevation would be orexin receptor-induced depolarization and subsequent activation of VGCCs. Accordingly, Ca^{2+} influx has been observed (van den Pol *et al.*, 1998) (Figure 3A) and it often depends on the activation of

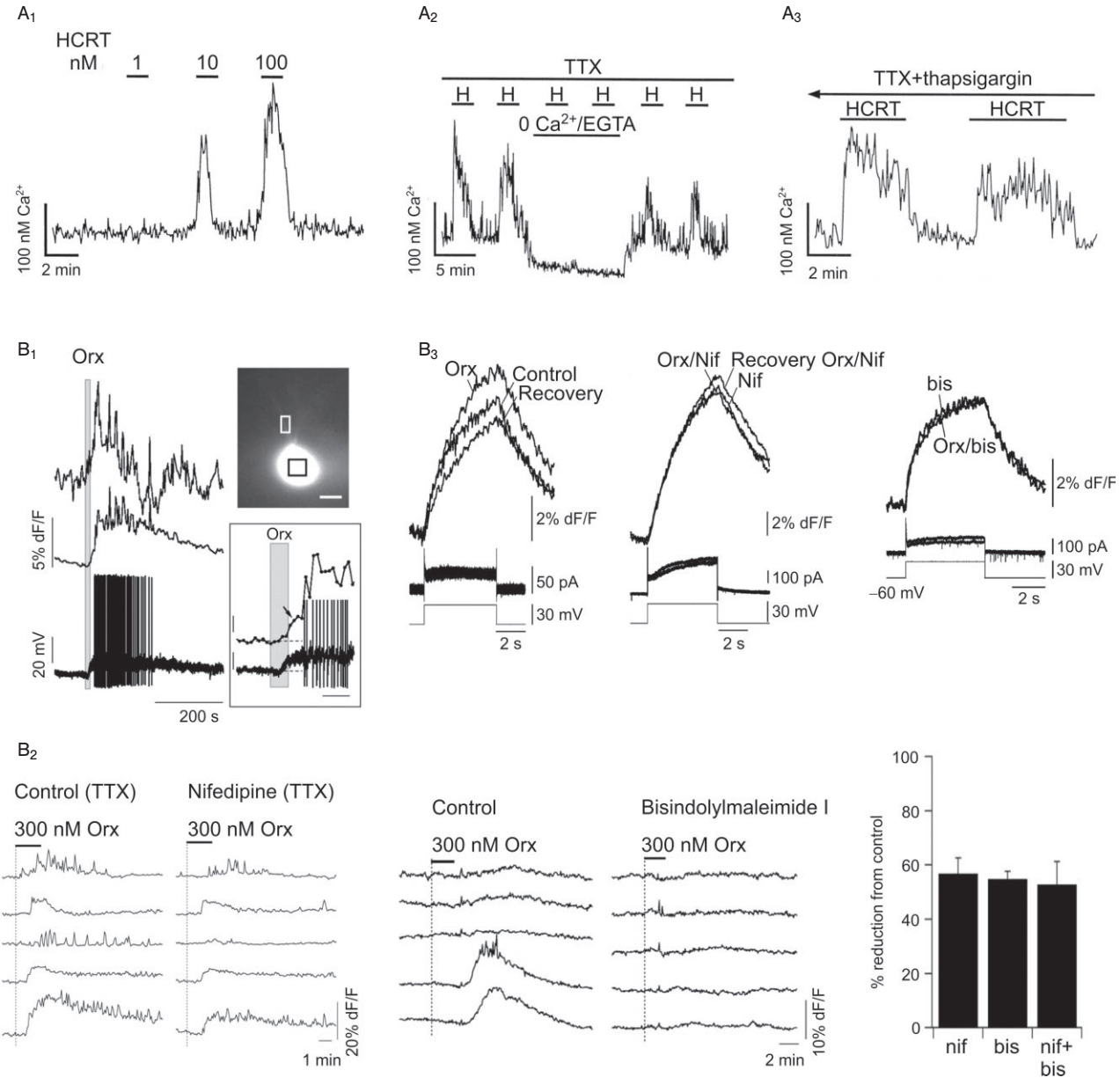


Figure 3

Ca^{2+} influx in native orexin receptor-expressing neurons. (A) Cultured rat hypothalamic neurons loaded with fura-2 AM. (A_1) Response to orexin-B ('HCRT') is concentration-dependent. Ca^{2+} influx rather than release from intracellular stores is likely required for the response since it is blocked by removal of extracellular Ca^{2+} ('0 Ca^{2+}/EGTA'; (A_2) but not by 2 μM thapsigargin (A_3). TTX, tetrodotoxin (1 μM). The response was also blocked by 100 μM Cd^{2+} and 1 μM bisindolylmaleimide I, a PKC inhibitor (not shown). (B) Mouse dorsal raphé neurons recorded in acute brain slices. (B_1) Upper right, a single neuron loaded with the Ca^{2+} indicator *bis*-fura-2 via the patch pipette. Upper left, fluorescence (dF/F) traces (indicating changes in intracellular [Ca^{2+}]) from the same cell. Bottom trace is membrane potential. Middle and uppermost traces are simultaneous Ca^{2+}-dependent fluorescence (average dF/F) from the soma (black box on the cell image) and from a proximal dendrite (white box on the cell image) respectively. The somatic Ca^{2+} trace and the current clamp recording are magnified in the right-hand bottom corner, demonstrating that the depolarization and somatic Ca^{2+} elevation occur before the action potential firing is triggered. The calibration bars indicate 10% dF/F and 10 mV for fluorescence and voltage trace, respectively, and 20 s. Orx, 300 nM orexin-A. (B_2) Ca^{2+}-dependent fluorescence (dF/F) from neurons loaded with fura-2 AM. Orexin-A (300 nM; 'Orx') produces a Ca^{2+} influx that is attenuated by the L-type VGCC blocker, nifedipine (left), and the PKC inhibitor bisindolylmaleimide I (1 μM; right). (B_3) Ca^{2+} -dependent *bis*-fura-2 fluorescence (dF/F) recorded as in B_1 under voltage-clamp conditions. Orexin-A (300 nM; 'Orx') reversibly enhances the somatic Ca^{2+} transient (left column, top trace) produced by a voltage jump from –60 to –30 mV (bottom trace) without changing the total membrane current (left column, middle trace). Nifedipine ('Nif', 1 μM; middle column) and bisindolylmaleimide ('bis', 1 μM; right column) fully attenuate the orexin-enhancement of the Ca^{2+} transient. These data indicate that orexin-A stimulates Ca^{2+} influx both by depolarising these neurons followed by opening of VGCCs and by a PKC-dependent enhancement of Ca^{2+} influx via L-type VGCCs. A_{1-3} adapted from van den Pol *et al.* (1998); B_1 and B_3 adapted from Kohlmeier *et al.* (2008); B_2 adapted from Kohlmeier *et al.* (2004). The figures are reproduced with permission.

VGCCs of N- and L-types (Uramura *et al.*, 2001; Kohlmeier *et al.*, 2004; 2008) (Figure 3B$_{1-2}$). In addition, orexins have also been shown to potentiate Ca^{2+} transients resulting from L-type VGCCs under voltage-clamp conditions (Kohlmeier *et al.*, 2008) (Figure 3B$_3$). Thus, depolarization and activation of both potentiated and un-potentiated influx pathways contribute to the orexin-mediated elevation of intracellular Ca^{2+} in neurons. PKC has been identified in the response in some cases (van den Pol *et al.*, 1998; Uramura *et al.*, 2001; Kohlmeier *et al.*, 2004; 2008) (Figure 3B); however, it remains unclear what is the target of the phosphorylation and how the transients are potentiated (e.g. altered voltage dependence, inactivation, conductance state). Without the ability to control membrane potential, the situation is even less clear since an impact on the membrane potential (e.g. via K^+ channel closure) or directly on Ca^{2+} channels cannot be distinguished. Enhanced activation of VGCCs has also been observed in other excitable cells (Xu *et al.*, 2002; Larsson *et al.*, 2003; Squecco *et al.*, 2011). It is noteworthy that while activation of Ca^{2+} release from intracellular stores is commonly observed in orexin receptor expression systems, there is only one suggestion of this based on direct measurements in neurons (Muroya *et al.*, 2004), although two additional reports find orexin excitation depends on elevation of intracellular Ca^{2+} (Korotkova *et al.*, 2002; Burdakov *et al.*, 2003), suggesting that release may be involved. In other neurons, orexin-mediated Ca^{2+} elevation is insensitive to store depletion (van den Pol *et al.*, 1998; Kohlmeier *et al.*, 2004; 2008), although these Ca^{2+} measurements have focused mainly on the bulk somatic compartment, leaving the dendrites and other cellular compartments virtually unexplored.

In summary, stimulation of Ca^{2+} fluxes appears to be a central orexin response, but several mechanisms are likely involved (PLC-dependent Ca^{2+} release or influx mediated by NSCCs, VGCCs or possibly NCX). In addition, Ca^{2+} transients have been investigated in only a subset of orexin-responding tissues (including neurons). The physiological actions mediated by these Ca^{2+} fluxes are also largely unknown, although they are likely involved in the ability of orexin to promote transmitter release, postsynaptic plasticity and endocannabinoid production (see below). Future experiments should utilize high-resolution microscopy methods to examine dendrites and terminals and additional neuronal cell types to help clarify the relative importance of store release versus influx in orexin-mediated responses.

Orexin signalling in depolarization and synaptic function

Orexins produce direct postsynaptic depolarization in neurons. This depolarization has been studied extensively in brain slices and has been attributed to three mechanisms, which may co-exist in individual neurons: inhibition of K^+ channels, stimulation of electrogenic NCX transport and activation of NSCCs (Figure 4). However, in most cases, the effector proteins underlying these excitatory actions of orexin have not yet been identified due to the lack of experiments with molecular precision (e.g. knockouts or knockdowns) and the general lack of specific pharmacological blockers.

K^+ channel closure is typically identified by an increase in input resistance and current reversal at the K^+ equilibrium potential. However, K^+ channel subtype identities have not been widely scrutinized; at present, there is evidence that orexin turns off Kir and TASK family channels (inward rectifier and TWIK-related acid-sensitive K^+ channels, respectively) (Hoang *et al.*, 2003; 2004; Doroshenko and Renaud, 2009). Kir3 channels are targets for GPCR inhibition via the G_q

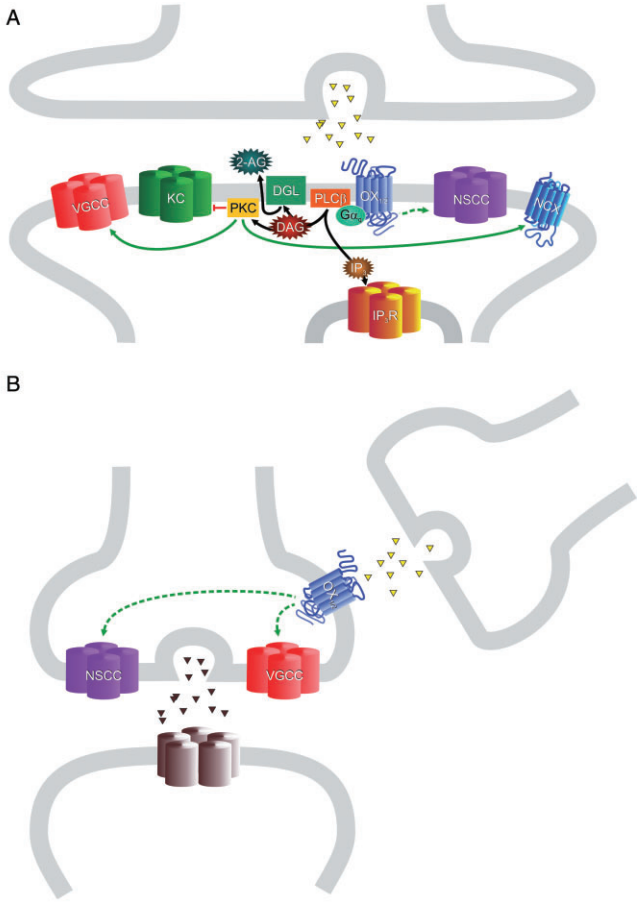


Figure 4

Schematic representation of possible orexinergic mechanisms in synaptic signalling. (A) Postsynaptic orexin signalling mechanisms. Ion fluxes are not shown. The PLC pathway may be active as suggested by some PKC-dependent effects as well as release of the endocannabinoid 2-AG, although there is little direct evidence for IP_3-mediated Ca^{2+} release from ER in neurons. Inhibition of leak/inward rectifier/voltage-gated K^+ channels (2P/Kir/Kv) ('KC') at least sometimes utilizes the G_q–PLCβ–PKC pathway. PKC could also be involved in the activation of NCX, but NCX may also act passively, driven by Ca^{2+} elevation (influx or release from ER; forward mode) or Na^+ elevation (reverse mode). There also is some evidence for PKC-dependent activation of postsynaptic L-type VGCCs. The putative role of PIP_2 is not depicted in the figure: PIP_2 is required by NCX, some K^+ and other channels, and PLD and cPLAα, and thus hydrolysis of PIP_2 (by PLC) would lead to inhibition of these while elevated PIP_2 (via, e.g. the PLD pathway) would stimulate these. Different NSCCs may be positively or negatively regulated by PIP_2. (B) Presynaptic orexinergic modulation of glutamate- or GABAergic terminal signalling by orexinergic enhancement of VGCC-mediated Ca^{2+} influx. Indirect evidence suggests that orexin stimulation may be able to trigger transmitter release by depolarization or other means (depicted as NSCC, although not directly identified in this response).

pathway both by PKC activation and by PIP_2 degradation (Sadja *et al.*, 2003; Luscher and Slesinger, 2010), while GPCR regulation of TASK channels may differ from this (Chen *et al.*, 2006; Lindner *et al.*, 2011; reviewed in Mathie, 2007).

Evidence for involvement of NCX (as distinguished from NSCCs) (Eriksson *et al.*, 2001; Wu *et al.*, 2002; Burdakov *et al.*, 2003; Wu *et al.*, 2004; Acuna-Goycolea and van den Pol, 2009; Zhang *et al.*, 2011), in most cases, has relied on the sensitivity to NCX inhibitors. However, these inhibitors (Ni^{2+} and KB-R7943) may significantly inhibit other targets including TRPC channels (Kraft, 2007), while the Li^+ permeability of TRP channels is generally not known. Additional evidence sometimes presented in support of NCX activation, such as no change of input resistance or a sensitivity to strong buffering of intracellular Ca^{2+}, also have alternative explanations [e.g. channels both opening and closing, rectification and Ca^{2+} stimulation of channels (like TRPC3; Nilius *et al.*, 2007)]. We therefore suggest involvement of NCX currents in orexin receptor-mediated depolarization in CNS neurons be considered provisional until more definitive analyses (e.g. more selective NCX blockers, knockouts or knockdowns) have been performed.

Evidence for NSCCs has come mainly from decreased membrane resistance, *I–V* curves, that reverse positive to resting membrane potential (some near 0 mV), a sensitivity to changing extracellular [Na^+] and a large increase in membrane current noise, which is thought to represent current fluctuations from channels opening and closing (e.g. Brown *et al.*, 2002; Burlet *et al.*, 2002; Liu *et al.*, 2002; Kohlmeier *et al.*, 2008). Nevertheless, the underlying NSCC identities or their activation mechanisms have not been determined in central neurons. An important next step will be to test whether TRPC channels, which contribute to currents in recombinant cells (see *Ca^{2+}*), are involved here. If TRPC channels correspond to the orexin-activated NSCCs, multiple known orexin receptor signals, including Ca^{2+}, DAG, DAG → PKC, free fatty acids or their metabolites, lysophosphatidylcholine, or PIP_2 decrease, could mediate their activation (reviewed in Nilius *et al.*, 2007; Venkatachalam and Montell, 2007; Leonard and Kukkonen, 2013; Kukkonen, 2013a). However, many other TRP channel types are also activated by these messengers, suggesting that messenger identification alone will not unequivocally identify the effectors.

In some neurons, orexins increase the miniature excitatory and/or inhibitory postsynaptic current frequency (van den Pol *et al.*, 1998; Li *et al.*, 2002; Smith *et al.*, 2002; Davis *et al.*, 2003; Borgland *et al.*, 2008; Ono *et al.*, 2008; Acuna-Goycolea and van den Pol, 2009; Borgland *et al.*, 2009; Dergacheva *et al.*, 2012), which is measured after blocking action potentials with TTx. This is usually interpreted to mean that activation of presynaptic terminal orexin receptors increases the probability of vesicle release. It should be noted that orexins can also promote TTx-sensitive transmitter release through receptors at or near presynaptic terminals by stimulating terminal (aka ectopic) action potentials (Lambe and Aghajanian, 2003) or by modulating presynaptic VGCCs (see above) (Burlet *et al.*, 2002).

Thus, orexins have diverse actions on central neurons. The most obvious response is postsynaptic stimulation and it is exciting that several mechanisms are utilized. However, there are also other prominent orexin responses in neurons including modulation of presynaptic transmitter release and changes in synaptic plasticity (Selbach *et al.*, 2004; Borgland *et al.*, 2006; Selbach *et al.*, 2010; see also Leonard and Kukkonen, 2013). Nevertheless, the mechanisms underlying orexin receptor signalling in the CNS are largely unknown, especially with respect to the NSCC activation, which is an important class of responses for many GPCRs. Identification of both signalling mechanisms and channel targets in the CNS will be important future advances. As selective pharmacological inhibitors are scarce, the use of knockouts, knockdowns and more reduced culture systems should be exploited.

The evidence for the different mechanisms by which orexins may impact the electrical activity of neurons and other excitable cells is discussed in detail in Leonard and Kukkonen (2013).

Phosphoinositide-3-kinase

PI3K pathway is one of the central pathways regulating cell growth and survival. GPCRs can regulate PI3K of class Ia and b – phosphorylating PIP_2 to PIP_3 – via, for example, Ras or Gβγ (reviewed in Wymann *et al.*, 2003). PIP_3 activates phosphoinositide-dependent kinase 1 (PDK1), which helps activate other target kinases like protein kinase B (PKB). In glucagon-secreting InR1-G9 cells from Syrian golden hamster insulinoma cells, orexin-A (unknown receptor subtype) activates PI3K, leading to PIP_3-regulated kinase cascades and inhibition of proglucagon mRNA production (Göncz *et al.*, 2008; see *Endocrine and exocrine pancreas* in Leonard and Kukkonen, 2013). PI3K is also activated by orexin-A in differentiated mouse white adipocyte-like 3T3-L1 cells (OX_1 and OX_2 mRNA), leading to glucose uptake and triglyceride synthesis (Skrzypski *et al.*, 2011; see *White and brown adipose tissue* in Leonard and Kukkonen, 2013). The mechanism utilized by orexin receptors to activate PI3K is unknown.

Cell death

Persistent orexin receptor stimulation induces programmed cell death. This occurs both in recombinant CHO cells and in cancer cell lines and primary cancer cells (Ammoun *et al.*, 2003; Rouet-Benzineb *et al.*, 2004; Ammoun *et al.*, 2006b; Voisin *et al.*, 2006; Voisin *et al.*, 2011; see also *Cell lines natively expressing orexin receptors* and *Recombinant cells* in Leonard and Kukkonen, 2013). A cell death response is manifested via molecular markers such as loss of plasma membrane polarity, caspase activation, mitochondrial cytochrome C leakage into the cytosol, and nuclear fragmentation and condensation. The details of this process have mainly been investigated in the recombinant CHO cells. Two different pathways for cell death in human OX_1-expressing CHO cells have been suggested. It has to be pointed out that the clones used are different and it is possible that the background cells, too, are different, namely one group is using regular CHO-K1 cells (Ammoun *et al.*, 2006b) and the other CHO-S suspension-adapted subclones (Rouet-Benzineb *et al.*, 2004; Voisin *et al.*, 2008; El Firar *et al.*, 2009); this may be reflected in the fact that cell death is blocked by serum in the former but not in the latter cells. In the detailed studies of Laburthe and co-workers, the signal cascade was suggested to involve G_q, Src (or a related kinase), phosphorylation of OX_1 and

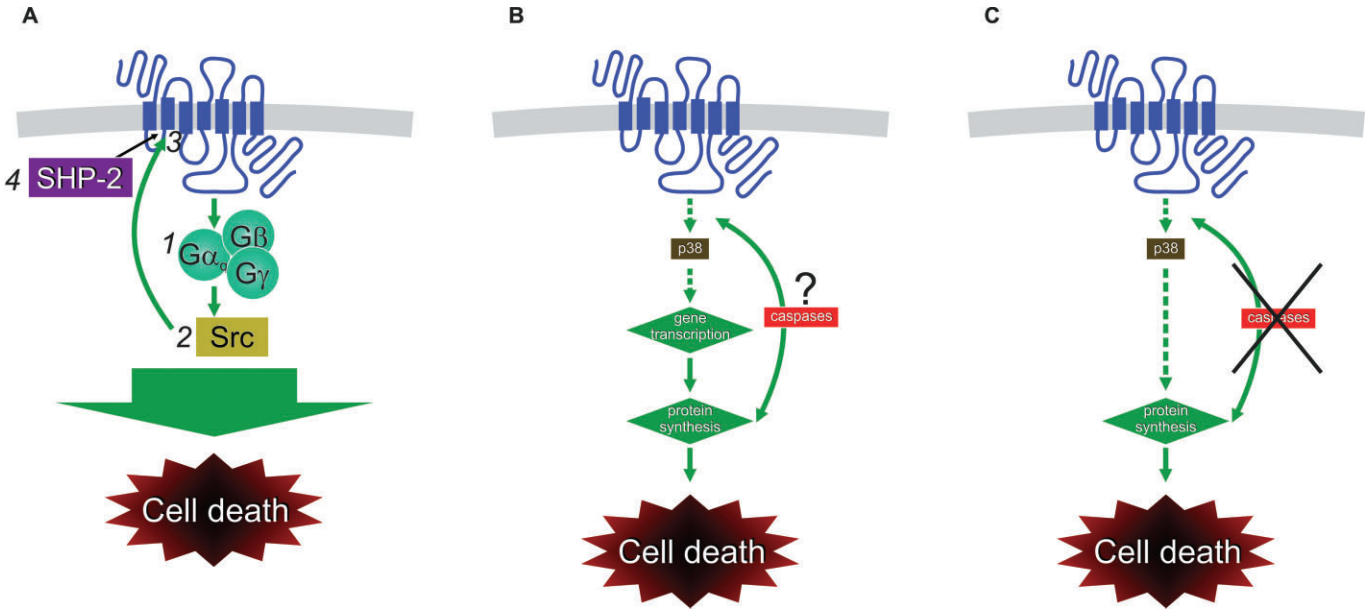


Figure 5

The OX_1 receptor activation-mediated cell death pathways mapped in CHO cells. (A) the SHP-2-dependent cascade (Voisin *et al.*, 2008; El Firar *et al.*, 2009); (B) the p38-mediated cascade (see Ammoun *et al.*, 2006b). Please note that the site of action of caspases in the cascade has not been determined. (C) The altered cell death response upon caspase inhibition (Ammoun *et al.*, 2006b).

recruitment of the protein phosphatase SHP-2 (Figure 5A). The studies identify two known phosphorylation/SHP-2 interaction motifs in orexin receptors, called *ITIM* (immunoreceptor tyrosine-based inhibitory motif; Thr-Asn-Tyr-Phe-Ile-Val) and *ITSM* (immunoreceptor tyrosine-based switch motif; Ile-Ile-Tyr-Asn-Phe-Leu) (Voisin *et al.*, 2008; El Firar *et al.*, 2009). As discussed in detail in Kukkonen (2013b), we believe that *ITSM*, in the transmembrane helix 2, is likely to be the correct site, as the putative *ITIM* sequence overlaps with the classical Asn-Pro-X-X-Tyr-motif, required for structural integrity of any GPCR. Hence, any exchange of this Tyr for another amino acid, which constitutes essential evidence in Voisin *et al.* (2008), disrupts the receptor structure, as is also indicated in these results upon careful reading. To firmly link the phosphorylation to Tyr-83 of the *ITSM* sequence, a phosphopeptide analysis of orexin receptor would be required. In the studies of Kukkonen and co-workers, p38 MAP kinase was identified as the carrier of the cell death response (Ammoun *et al.*, 2003; 2006b) (Figure 5B). Cell death also required mRNA and protein synthesis and caspase activity. Upon inhibition of the caspases, however, the cell death takes a 'shortcut' (Figure 5C). p38 MAPK has been associated with orexin responses in some other cell types, as well (see *Cell plasticity*).

Both orexin receptor subtypes are capable of activating cell death as observed in recombinant CHO-S cells and in native cancer cell lines of colon carcinoma, neuroblastoma and pancreatic acinar tumour (Rouet-Benzineb *et al.*, 2004; Voisin *et al.*, 2006). Primary colorectal tumours (but not normal colon epithelium) also express OX_1 receptors, and orexin receptor activation has been suggested to hold promise for treating chemotherapy-resistant carcinoma (Voisin *et al.*, 2011).

There are several open questions relating to orexin-triggered cell death. Is this a physiological response? Cell death seems to require not extremely high but still rather significant orexin levels over a long time. Could such exposure take place in a normal physiological context or would 'orexin overflow' represent a pathological mechanism? Would only some cell types display the cell death response or is this a general mechanism for orexin receptors? While the former questions are currently rather difficult to answer, the latter one could be easily assessed using cultures of native orexin receptor-expressing cells (e.g. neurons).

Cell plasticity

Orexin receptors are in principle able to activate some classical plasticity-regulating cascades such as PKC, PI3K and ERK, and p38 MAPK. PKC activation has been described above (see *Phospholipase C*). ERK is activated in OX_1- and OX_2-expressing CHO cells and OX_1-expressing neuro-2a cells (Ekholm *et al.*, 2007). GPCRs can regulate ERK and other MAPK pathways via multiple mechanisms (reviewed in Rozengurt, 2007). In OX_1-expressing CHO cells, ERK activation can be partially inhibited by inhibiting PLC, cPKC, Src and PI3K, which appear to converge at Ras; however, Src, PIK or Ras activity has not been directly assessed in these cells (Ammoun *et al.*, 2006a). ERK signalling is also observed in recombinant HEK-293 cells (Milasta *et al.*, 2005; Tang *et al.*, 2008). In these cells, OX_2 receptors activate AC and PLC; both protein kinase A (PKA) and PKC are suggested to contribute to ERK activation (Tang *et al.*, 2008). p38 MAPK is activated in OX_1-expressing CHO cells (Ammoun *et al.*, 2003; 2006b), OX_2-expressing HEK-293 cells (Tang *et al.*, 2008) and native H295R cells (Ramanjaneya *et al.*, 2009); the activation cascades are not known. In recombinant cells, no long-range effects have been assessed except

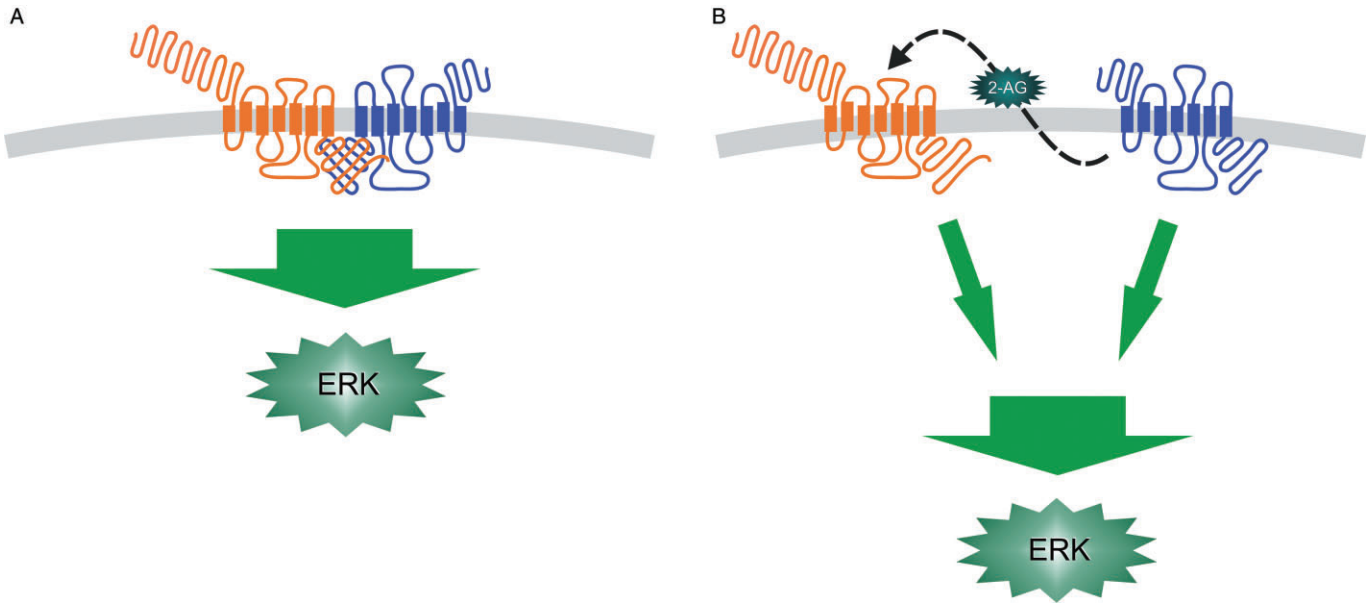


Figure 6

Suggested interaction schemes in ERK signalling upon OX_1 orexin receptor (blue) and CB_1 cannabinoid receptor (orange) co-expression in the same cells. (A) Dimerization of OX_1 and CB_1 receptors enhances the signalling/ligand potency (Hilairet *et al.*, 2003; Ellis *et al.*, 2006). (B) Enhanced signalling is obtained by OX_1 receptor-mediated production of the CB_1 ligand 2-AG and subsequent co-signalling of these two receptors (Turunen *et al.*, 2012; Jäntti *et al.*, 2013). See *Orexins and endocannabinoids* for details.

that ERK may partially inhibit p38-induced cell death (see above) in CHO-hOX_1 cells (Ammoun *et al.*, 2003).

ERK and PKC have also been associated with synaptic plasticity. Orexin-A stimulated AMPA receptor trafficking to the plasma membrane in co-cultures of rat prefrontal cortex and striatum and the response was fully inhibited by removal of extracellular Ca^{2+} or by a MAPK/ERK kinase 1 (MEK1) inhibitor (Shin *et al.*, 2009). Similar findings were obtained in a striatal slice preparation, but MEK1 inhibition was not tested (Shin *et al.*, 2009). In contrast, PKC mediates the increased NMDA receptor trafficking to the plasma membrane in rat ventral tegmental area slices (Borgland *et al.*, 2006). Both neuronal responses are observed within 10–20 min and therefore cannot represent new protein synthesis but likely result from plasma membrane fusion of pre-formed channel-harbouring vesicles.

Some long-term plastic effects have also been reported for orexin receptor activation. ERK is activated in native H295R cells (mainly OX_2 mRNA), mediated entirely via PKC (Ramanjaneya *et al.*, 2009). This cascade may contribute to differentiation towards steroid-producing cells (Ramanjaneya *et al.*, 2008; Ramanjaneya *et al.*, 2009; Wenzel *et al.*, 2009; see *Adrenal gland* in Leonard and Kukkonen, 2013). Plancentally produced orexins were recently identified to pivotally contribute to the prenatal development of brown adipose tissue in mice via OX_1 receptors (Sellayah *et al.*, 2011; see *White and brown adipose tissue* in Leonard and Kukkonen, 2013). In contrast, differentiation of 3T3-L1 cells or rat primary adipocytes to mature adipocytes was not stimulated by orexin-A despite the presence of other orexin-mediated responses (Skrzypski *et al.*, 2012; see *Phosphoinositide-3-kinase* and *White and brown adipose tissue* in Leonard and Kukkonen, 2013).

In the CNS, there is also evidence that the absence of one or both orexin receptors leads to cellular phenotypic differences compared with wild types in markers of cholinergic transmission (Kilduff *et al.*, 1986; Kalogiannis *et al.*, 2010). Moreover, there is new evidence that the numbers of histaminergic neurons in human narcoleptic brains and orexin peptide null mice are increased [Dr. T. E. Scammell, pers. comm.]. Whether these changes depend directly on the loss of orexin receptor signalling or other receptor properties or whether they are mediated by compensatory alterations is unknown.

Orexins and endocannabinoids

In recombinant CHO cells expressing human OX_1 receptors, ERK phosphorylation is observed following orexin receptor stimulation at intermediate potency ($EC_{50} \approx 10–30$ nM) (Hilairet *et al.*, 2003; Ammoun *et al.*, 2006a). When human CB_1 cannabinoid receptors (also a GPCR) were co-expressed in these cells, the potency of orexin-A increased by 100-fold in a manner dependent on CB_1 receptor activity (i.e. inhibited by a CB_1 antagonist) (Hilairet *et al.*, 2003). In contrast, CB_1 signalling to ERK was not affected by OX_1 co-expression, and neither was OX_1 signalling to PLC by CB_1 co-expression. The result was interpreted to mean OX_1-CB_1 heterodimerization (Figure 6A). Further studies in recombinant HEK-293 cells elaborated this view, and indeed, FRET and BRET techniques and co-immunoprecipitation have supported it (Ellis *et al.*, 2006; Ward *et al.*, 2011a; 2011b). However, we have recently shown that OX_1 receptor stimulation in CHO cells (as well as in similarly recombinant neuro-2a and HEK-293 cells) induces production of the endocannabinoid 2-AG via the PLC–DGL cascade (see *Phospholipase C*) (Turunen *et al.*,

2012). Furthermore, we have been able to show that blocking of the 2-AG production fully abolishes the CB_1 receptor-mediated potentiation of OX_1 signalling to ERK (Jäntti *et al.*, 2013). We therefore believe that OX_1 receptor stimulation produces DAG via the PLC pathway, DAG is hydrolyzed by DGL to 2-AG, and 2-AG stimulates CB_1 receptors, which, by being able to more strongly couple to $G_{i/o}$ proteins, co-stimulate ERK activation (Figure 6B). This does not necessarily exclude receptor heterodimerization, but 2-AG production seems to be the key to the process. A currently unresolved question is whether orexin receptors have a special propensity to stimulate 2-AG release in addition to the likely activation of PLCβ. Orexin receptors mediate receptor-operated Ca^{2+} influx and that could be an important factor in either stimulating DGL or boosting the activity of some PLC species.

Endocannabinoid signalling has great importance for modulating synaptic transmission in the brain. Endocannabinoids are produced postsynaptically and mediate retrograde inhibitory actions on presynaptic terminals via CB_1 receptors (reviewed in Kano *et al.*, 2009). The gross response does not, however, need to be feedback inhibition as endocannabinoids also can inhibit the release of GABA from inhibitory terminals leading to postsynaptic disinhibition. Endocannabinoid responses in the brain are usually analyzed utilizing pharmacological inhibitors of the receptors or endocannabinoid generation or breakdown. Two studies nicely illustrate endocannabinoid actions in orexin signalling. In the dorsal raphé nucleus, orexin receptor signalling inhibits glutamate release onto serotonergic neurons. This appears mediated by orexin-induced production of 2-AG, which then acts on presynaptic CB_1 receptors on glutamatergic terminals to inhibit glutamate release (Haj-Dahmane and Shen, 2005). The analgesic effects of orexin on the ventrolateral periaqueductal gray matter also takes place by endocannabinoid production and release, followed by action on presynaptic CB_1 receptors on GABAergic terminals (Ho *et al.*, 2011). In both cases, the endocannabinoid involved is 2-AG; involvement of 2-AG is easier to determine than that of the other important endocannabinoid, *N*-arachidonoylethanolamine (anandamide) as there are good inhibitors for 2-AG generation.

Conclusions

A wide variety of signals originate from orexin receptors. Orexins are able to trigger responses in many tissues in addition to the CNS, and different responses are observed at different sites. What determines the choice of specific signal cascades is unknown. To explain this, it is most logical to suggest that GPCRs would participate in preformed signal complexes with their effectors and the necessary elements of the signalling cascades. There is strong circumstantial evidence for such complexes (see, e.g. Kleuss, 1995; Brown, 2010), but very little direct molecular proof for any GPCR.

For orexin receptors, it is, in most cases, difficult to pinpoint signal cascades carrying the responses observed due to the limited set of tools available and sometimes even lack of analytical approach. It is also currently difficult to suggest the physiological significance of responses to exogenous orexins observed in sites other than CNS as the production of orexin peptides outside the CNS is not firmly proven.

We further explore these issues in the context of tissue responses to orexins in the sister review (Leonard and Kukkonen, 2013). Final conclusions concerning orexin receptor signalling are presented there.

Acknowledgements

This study was supported by the Academy of Finland (J. P. K.), the Magnus Ehrnrooth Foundation (J. P. K.), the Liv & Hälsa Foundation (J. P. K.) and grants NS027881 and HL064150 from the NIH of the USPHS (C. S. L.).

Conflicts of interest

None.

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