REVIEW

Orexin/hypocretin receptor signalling: a functional perspective

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Multiple homeostatic systems are regulated by orexin (hypocretin) peptides and their two known GPCRs. Activation of orexin receptors promotes waking and is essential for expression of normal sleep and waking behaviour, with the sleep disorder narcolepsy resulting from the absence of orexin signalling. Orexin receptors also influence systems regulating appetite/metabolism, stress and reward, and are found in several peripheral tissues. Nevertheless, much remains unknown about the signalling pathways and targets engaged by native receptors. In this review, we integrate knowledge about the orexin receptor signalling capabilities obtained from studies in expression systems and various native cell types (as presented in Kukkonen and Leonard, this issue of *British Journal of Pharmacology*) with knowledge of orexin signalling in different tissues. The tissues reviewed include the CNS, the gastrointestinal tract, the pituitary gland, pancreas, adrenal gland, adipose tissue and the male reproductive system. We also summarize the findings in different native and recombinant cell lines, especially focusing on the different cascades in CHO cells, which is the most investigated cell line. This reveals that while a substantial gap exists between what is known about orexin receptor signalling and effectors in recombinant systems and native systems, mounting evidence suggests that orexin receptor signalling is more diverse than originally thought. Moreover, rather than being restricted to orexin receptor 'overexpressing' cells, this signalling diversity may be utilized by native receptors in a site-specific manner.

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Abbreviations

2-AG, 2-arachidonoylglycerol; AA, arachidonic acid; (s)AHP, (slow) afterhyperpolarization; AR42J, a rat pancreatic acinar tumour cell line; BAPTA, 1,2-bis(*O*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; Caco-2, HT29-D4, LoVo and SW480, human colon carcinoma cell lines, cPLA$_2$, cytosolic (Ca^{2+}-sensitive) phospholipase A$_2$; DGL, DAG lipase; H-current, a non-selective cation current mediated by hyperpolarization-activated, cyclic nucleotide-gated (HCN) channels; HSD3B2, 3β-hydroxysteroid dehydrogenase; IMR-32 and SK-N-MC, human neuroblastoma cell lines; IP$_3$, inositol-1,4,5-trisphosphate; I_{sAHP}, sAHP current; Kir channels, inward rectifier K$^+$ channels; MEF, mouse embryonic fibroblast; 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; PC12, a rat pheochromocytoma cell line; PIP, phosphatidylinositol-4-phosphate; PIP$_2$, phosphatidylinositol-4,5-bisphosphate; TRP (channel), transient receptor potential (channel); VGCC, voltage-gated Ca^{2+} channel

Introduction

The landmark discoveries of the orexin (hypocretin) peptides (orexin-A and orexin-B) and their two known GPCRs (OX$_1$ and OX$_2$ receptors) in 1998 (de Lecea *et al.*, 1998; Sakurai *et al.*, 1998) set the stage for a new understanding of the brain's arousal system and the sleep disorder narcolepsy – a disorder characterized by excessive sleepiness, sleep attacks

and the sudden loss of postural muscle tone (cataplexy) (for review, Siegel and Boehmer, 2006). Beyond arousal, orexins are implicated in the regulation of appetite, metabolism, stress, reward and autonomic function (for review, see Carter *et al.*, 2009; Sharf *et al.*, 2010; Girault *et al.*, 2012; Nattie and Li, 2012), suggesting that these peptides work globally to organize and adjust homeostatic responses (see Li and de Lecea, 2013). In spite of these advances, many questions remain concerning how the orexin system operates. One critical question relates to the nature of the signalling cascades and effectors utilized by native orexin receptors. The answer to this question is important both from a functional perspective and from the perspective of developing new pharmacotherapeutics focused on these receptors (see Winrow and Renger, 2013) or their signalling pathways. As indicated in our sister review (Kukkonen and Leonard, 2013), the signalling of orexin receptors is multifaceted and complex. However, the widest spectrum of responses is seen in recombinant cell lines, like CHO. Do these diverse responses result from receptor overexpression or can native orexin receptors also couple to so many signal cascades? And if so, are all the cascades utilized in all tissues or is there specificity perhaps based on specific tissue needs? This review summarizes what is known about native orexin receptor signalling in the brain and peripheral tissues and aims at answering these questions. We also try to correlate orexin responses with particular orexin receptor subtypes. As orexin receptor identification by antibodies or by use of a reputedly selective agonist is unreliable (reviewed in Kukkonen, 2012; 2013), we have focused on studies utilizing mRNA detection, selective receptor antagonists and receptor knockout data.

CNS

The CNS is the most important orexin target tissue as orexin peptides are synthesized in the hypothalamus by a small group of neurons (de Lecea *et al.*, 1998; Sakurai *et al.*, 1998) with axons that are widely distributed in the CNS (Peyron *et al.*, 1998; Nambu *et al.*, 1999; van den Pol, 1999). Consequently, CNS neurons are the most extensively investigated native cells for orexin responses. However, CNS neurons are not a single cell type but represent an unknown number of different subtypes, with multiple subtypes often interdigitated within the same structures. This, of course, makes biochemical and pharmacological analysis difficult or impossible, and therefore, we have focused primarily on single cell electrophysiology and Ca^{2+} imaging approaches *in vitro,* mainly in brain slices, which have cellular resolution.

Based on *in situ* hybridization, both known orexin receptors are distributed throughout the CNS, each having a different, yet overlapping, pattern (Trivedi *et al.*, 1998; Marcus *et al.*, 2001). Moreover, several single-cell RT-PCR studies indicate that, at least in some places, mRNA for both receptors can be co-expressed (Eriksson *et al.*, 2001; Brown *et al.*, 2002; Korotkova *et al.*, 2003; van den Top *et al.*, 2003). By comparing the published distribution of orexin receptor mRNA with the results from *in vitro* recordings, it is clear that there is excellent correspondence between receptor mRNA expression and functional receptors, as determined by the presence of postsynaptic orexin responses (see Supporting Information Table S1). It is also clear that while the response to orexin application has been tested for many cell groups, there are regions expressing modest to high levels of mRNA that have not yet been studied (e.g. taenia tecta, intergeniculate leaflet and numerous hypothalamic nuclei; compare Supporting Information Table S1 to Marcus *et al.*, 2001, table 1). There are also regions where mRNA was not noted but where neurons have functional receptors (e.g. thalamic parafascicular nucleus, area postrema and sublaterodorsal nucleus) where more detailed *in situ* analysis is needed (see Mieda *et al.*, 2011). Finally, there are a couple of regions where neurons show direct postsynaptic responses to applied orexin but have mRNA levels appearing no higher than background (e.g. substantia nigra pars reticulata, medial dorsal nucleus of the thalamus), suggesting that functional receptors may be present in neurons having low mRNA levels or in regions with low densities of expressing neurons. Thus, it is possible that functionally important groups of orexin-responding neurons have not yet been detected.

Orexin receptor actions in the CNS

So far, for neurons that respond to exogenous orexin-A or orexin-B with a change in membrane potential, orexins produce a slow and long-lasting depolarization that can be large enough to initiate firing, or – if the neuron is already firing – to increase its firing rate. Thus, orexins are universally considered excitatory neuropeptides because their receptors couple to effectors that produce depolarization.

Although there are a few cases where the effectors underlying these depolarizations have been identified at a molecular level (Kukkonen and Leonard, 2013), in most cases, depolarization has been attributed to three categories of action: (i) closure of K^+ channels active at rest (Figure 1A; Horvath *et al.*, 1999; Ivanov and Aston-Jones, 2000; Brown *et al.*, 2001; Hwang *et al.*, 2001; Bayer *et al.*, 2002; Grabauskas and Moises, 2003; Hoang *et al.*, 2003; 2004; van den Top *et al.*, 2003; Yang and Ferguson, 2003; Yang *et al.*, 2003; Bayer *et al.*, 2004; Wu *et al.*, 2004; Murai and Akaike, 2005; Bisetti *et al.*, 2006; Govindaiah and Cox, 2006; Huang *et al.*, 2006; Kolaj *et al.*, 2008; Doroshenko and Renaud, 2009; Zhang *et al.*, 2010; 2011); (ii) activation of an electrogenic sodium-calcium exchanger (NCX) (Figure 1B; Eriksson *et al.*, 2001; Wu *et al.*, 2002; 2004; Burdakov *et al.*, 2003; Acuna-Goycolea and van den Pol, 2009; Zhang *et al.*, 2011); and (iii) the activation of non-selective cation channels (NSCCs) (Figure 1C; Eriksson *et al.*, 2001; Hwang *et al.*, 2001; Brown *et al.*, 2002; Burlet *et al.*, 2002; Liu *et al.*, 2002; Wu *et al.*, 2002; 2004; Yang and Ferguson, 2002; Yang and Ferguson, 2003; Huang *et al.*, 2006).

As noted in Kukkonen and Leonard (2013), closure of K^+ channels active at rest has been relatively straightforward to demonstrate following application of orexin. For example, the membrane depolarization produced by orexin in layer 6b neurons (Bayer *et al.*, 2004) results from a reduction in membrane current that has a reversal potential that shifts in a Nernstian manner with the K^+ equilibrium potential (Figure 1A). In contrast, the distinction between currents arising from the NCX and NSCC are more problematic, especially in the absence of detailed biophysical analyses because these currents can have similar reversal potentials, are difficult to isolate from other membrane currents, are lacking
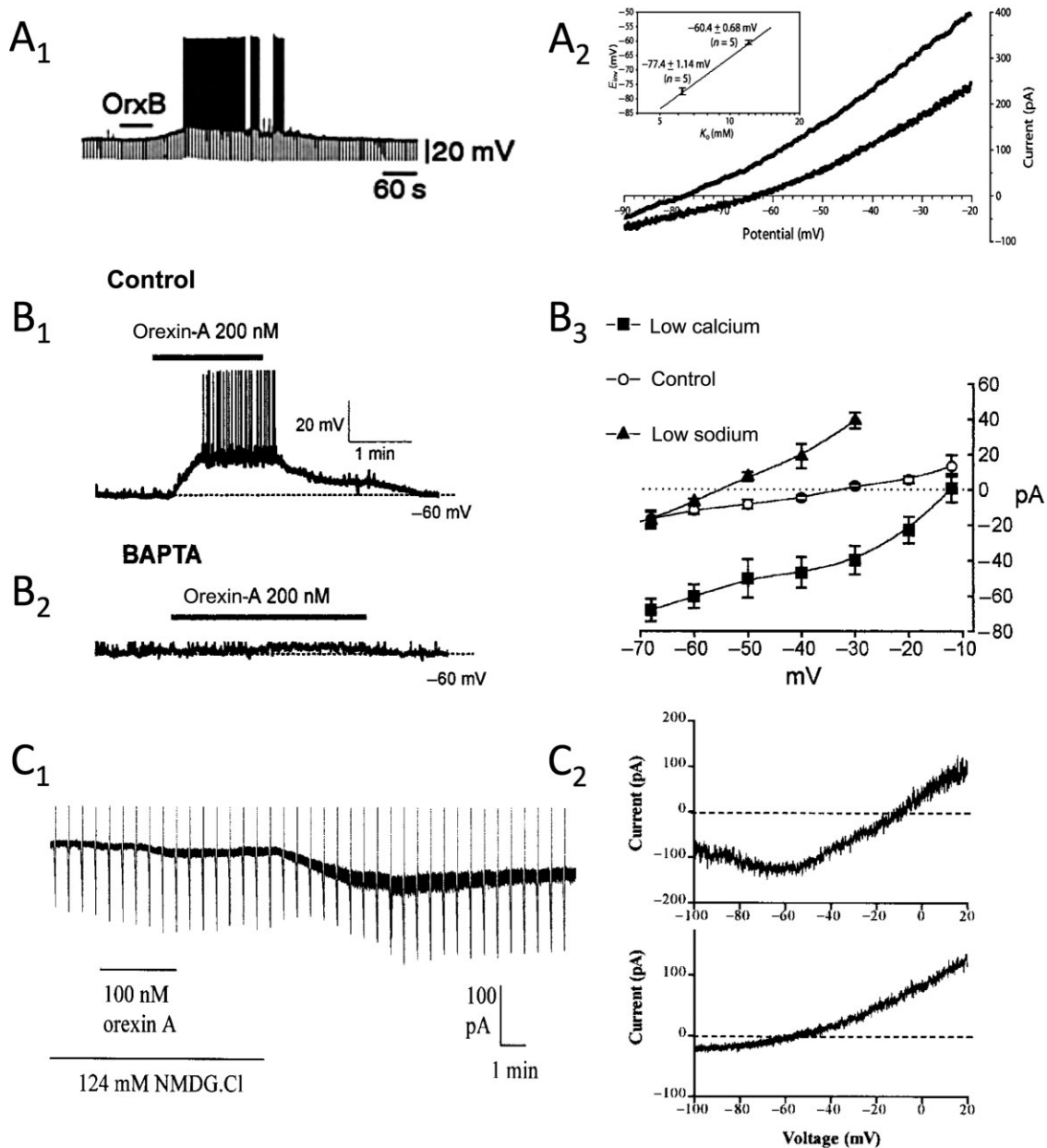
Figure 1

Orexin-mediated depolarizations in the CNS. (A) Orexins close leak-like K^+ channels. The orexin-B-mediated (100 nM) depolarization in layer 6b neocortical pyramidal neurons, which causes firing, is associated with an increase in membrane resistance (note the larger negative-going voltage deflections produced by constant current pulses during the depolarization (A_1). The *I-V* curve for the orexin-activated current reverses near the predicted K^+ equilibrium potential in an extracellular $[K^+]$ of 6.25 mM (upper *I-V* curve) and 12 mM (lower *I-V* curve) (A_2). The inset shows the mean reversal potential measured at each extracellular $[K^+]$. (B) Orexins stimulate electrogenic NCX. Orexin-A produces a suprathreshold depolarization in type C arcuate nucleus neurons of the hypothalamus (B_1). The depolarizing current is insensitive to lowering extracellular $[Ca^{2+}]$ (not shown) but is completely blocked by strong buffering of intracellular Ca^{2+} (BAPTA, 10 mM), consistent with it being triggered by the release of Ca^{2+} from intracellular stores (B_2). The reversal potential of the orexin-activated exchanger current shifts with changes of Na^+ and Ca^{2+} gradients (B_3). (C) Orexins activate a 'noisy' non-selective cation current that is not reduced by low extracellular $[Ca^{2+}]$ or strong buffering of intracellular $[Ca^{2+}]$. The orexin-A-activated current in dorsal raphé neurons was initiated, but was almost absent in low extracellular $[Na^+]$ (26 mM; NMDG). The current was reinstated by switching back to normal extracellular $[Na^+]$ (150 mM; C_1). The reversal potential of this cation current is near 0 mV in normal extracellular $[Na^+]$ (C_2, upper subfigure) but shifts to near −60 mV in low extracellular $[Na^+]$ (C_2, lower subfigure). Subfigures (A), (B) and (C) are adapted from Bayer *et al.* (2004); Burdakov *et al.* (2003); and Brown *et al.* (2002), respectively and are reproduced with permission.

highly selective antagonists (Clapham, 2003; Török, 2007; Numata *et al.*, 2011) and can be interdependent (Rosker *et al.*, 2004; Louhivuori *et al.*, 2010). Further complicating the matter, simultaneous closure of K^+ channels and activation of one or both of the other effectors appears to underlie orexin actions in some neurons. Thus, we feel that identification of either NCX or NSCCs as targets for orexin receptor activation in most neurons should be considered provisional. Nevertheless, in some neurons, distinction between a NCX and a NSCC underlying the orexin-mediated depolarization appears warranted (see Supporting Information Table S1; Figure 1B,C). For example, in type C GABA cells of the arcuate nucleus (Burdakov *et al.*, 2003), the membrane depolarization produced by orexin is mediated by a current that reverses near −30 mV under control conditions and is strongly reduced both by the NCX antagonist KB-R7943 and by buffering intracellular Ca^{2+} to very low levels with 10 mM BAPTA [1,2-bis(*O*-aminophenoxy)ethane-*N*,*N*,*N'*,*N'*-tetraacetic acid], suggesting that elevation of intracellular Ca^{2+} triggers an inward NCX current in these neurons. It was also shown that lowering extracellular Na^+ shifts the reversal potential of this current to more negative values, while lowering extracellular Ca^{2+} shifts the reversal potential to more positive values – changes that at least qualitatively fit expectations for an NCX current (Luo and Rudy, 1994). In contrast, the orexin-mediated depolarization in dorsal raphé neurons appears mainly mediated by NSCCs as it is not blocked by strong buffering of intracellular Ca^{2+} with BAPTA (Liu *et al.*, 2002) or low extracellular Ca^{2+} (Brown *et al.*, 2002; Liu *et al.*, 2002; Kohlmeier *et al.*, 2008), is not effectively blocked by KB-R7943 (Liu *et al.*, 2002; Kohlmeier *et al.*, 2008) or by intracellular (Liu *et al.*, 2002) and extracellular Cs^+ (Kohlmeier *et al.*, 2008), and has a reversal potential – in low extracellular Ca^{2+} – that shifts from near 0 mV in normal extracellular Na^+ to near −50 mV in 26 mM extracellular Na^+ (Brown *et al.*, 2002).

A hallmark orexin action in expression systems is the elevation of intracellular Ca^{2+}. Both OX_1 and OX_2 receptors release Ca^{2+} from intracellular stores and, at lower orexin concentrations, a primary Ca^{2+} influx is evoked (Kukkonen and Leonard, 2013). The effect of orexin on intracellular Ca^{2+} levels has been examined in only a few types of central neurons (van den Pol *et al.*, 1998; 2001; van den Pol, 1999; Uramura *et al.*, 2001; Lambe and Aghajanian, 2003; Kohlmeier *et al.*, 2004; 2008; Muroya *et al.*, 2004; Ishibashi *et al.*, 2005; Tsujino *et al.*, 2005). Interestingly, there are only a few examples where orexin actions may depend on release from intracellular stores (Korotkova *et al.*, 2002; Burdakov *et al.*, 2003; Muroya *et al.*, 2004). In one study, chelation of intracellular Ca^{2+} with BAPTA, but not reduction of extracellular Ca^{2+}, prevented orexin depolarizations (Burdakov *et al.*, 2003), while in another, thapsigargin reduced orexin-mediated firing (Korotkova *et al.*, 2002). In the third study, direct Ca^{2+} measurements showed that part of the orexin-induced Ca^{2+} elevation was prevented by pretreatment with thapsigargin (Muroya *et al.*, 2004), but the noisy responses make firm conclusions difficult. In other neurons, the orexin-mediated Ca^{2+} elevation is mediated by depolarization and the activation of voltage-gated Ca^{2+} channels (VGCCs) (van den Pol *et al.*, 1998; Uramura *et al.*, 2001; Kohlmeier *et al.*, 2004; 2008; Ishibashi *et al.*, 2005) as it was blocked by low-

ering extracellular Ca^{2+} and, in some cases, was shown to require depolarization, was insensitive to store depletion and/or was sensitive to blockers of VGCCs. Orexin also produces an enhancement of Ca^{2+} influx mediated by VGCCs in some neurons (Kohlmeier *et al.*, 2008; see also Kukkonen and Leonard, 2013). Nevertheless, evidence indicates that orexin receptors can activate PKC (see below) in CNS neurons, which likely requires PLC activity and thus may coincide with inositol-1,4,5-trisphosphate (IP_3)-triggered Ca^{2+} release. It is therefore noteworthy that Ca^{2+} imaging studies in central neurons have been limited to somata and proximal dendrites and space-averaged Ca^{2+} signals, as orexin receptor-mediated release of Ca^{2+} from intracellular stores may occur in a very localized manner and/or in neuronal compartments not yet examined such as the dendrites or synaptic terminals. Methods having high spatial resolution, like 2-photon imaging, should be utilized to address these possibilities.

In addition to mediating depolarization, there is emerging evidence that native orexin receptors alter the integrative properties of their target neurons by modulating postsynaptic ion channels and thereby strongly influence how synaptic inputs from other sources get converted into firing. Orexin-A (400 nM), acting through OX_1 receptors and PKC, reduces the H-current (mediated by hyperpolarization-activated, cyclic nucleotide-gated HCN channels) and produces a negative shift in its voltage dependence in layer 5 prefrontal pyramidal neurons (Li *et al.*, 2010). The prevalence of this action remains to be established but it could profoundly alter excitability and synaptic integration, as these channels are strongly expressed in dendrites (Magee, 1999). Orexins have also been reported to slow spike repolarization (Figure 2A) in solitary tract neurons (Yang and Ferguson, 2003; Yang *et al.*, 2003) and reduce the post-spike afterhyperpolarization (AHP; Figure 2B) in noradrenergic locus coeruleus neurons (Horvath *et al.*, 1999; Murai and Akaike, 2005) consistent with inhibition of a repolarizing K^+ current mediated by voltage-dependent K^+ channels or large-conductance Ca^{2+}-activated K^+ channels, which have recently been shown to be inhibited by orexin in smooth muscle (Squecco *et al.*, 2011). Orexins also inhibit the long-lasting slow AHP that builds up following multiple spikes in paraventricular thalamic neurons (Figure 2C) (Zhang *et al.*, 2010). These cells show robust low-threshold Ca^{2+} spiking, which drives rhythmic bursting, in part, via a Ca^{2+}-dependent K^+ current, termed the slow AHP current (I_{sAHP}). This current, along with a Na^+-dependent K^+ current, produces strong spike-frequency adaptation in these cells when firing is elicited by prolonged depolarizing current pulses. Orexins inhibit both these currents via PKC, thereby enhancing the excitability of these neurons to sustained depolarizing input (Zhang *et al.*, 2009; 2010). I_{sAHP} is found in many neurons and, although multiple channels appear to mediate this current, it is well known to be suppressed by neurotransmitters via GPCRs that couple to G_s and G_q and it may be regulated by phosphatidylinositol-4,5-bisphosphate (PIP_2) (for review, see Andrade *et al.*, 2012).

Recently, we found a functionally opposite action of orexin-A on serotonergic dorsal raphé neurons. These neurons have a large, medium-duration post-spike AHP that contributes to their characteristic pacemaker firing and strong spike-frequency adaptation (Aghajanian and Vandermaelen, 1982), which is almost entirely mediated by
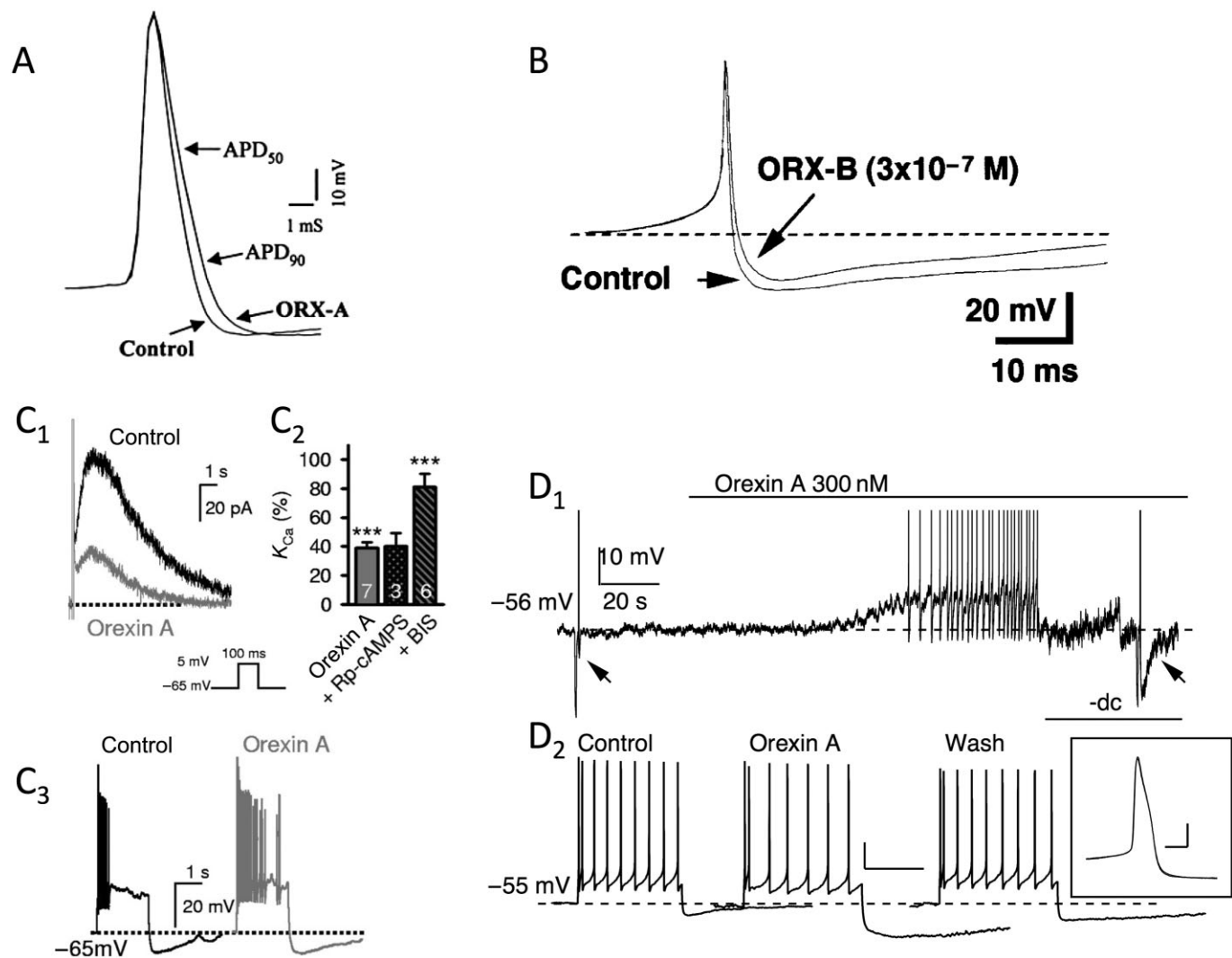
Figure 2

In addition to producing depolarization, orexins also modulate the integrative properties of neurons in the CNS. (A) Orexin-A (ORX-A, 10 nM) slowed action potential repolarization and broadened the spike without altering the magnitude of AHP in nucleus of the solitary tract neurons. The duration of the action potential at both 50% (APD_{50}) and 90% (APD_{90}) recovery was longer following orexin. This was associated with a reduction in non-inactivating voltage-dependent outward currents (not shown). (B) In dissociated locus coeruleus neurons, orexin-A and orexin-B (ORX-B) reduced the magnitude and duration of the AHP. This too was associated with a reduction in a non-inactivating, voltage-dependent outward current (not shown), which may be different from that reduced in (A). Reprinted from Murai and Akaike (2005), with permission from Elsevier. (C) In paraventricular thalamic neurons, orexins reduce an I_{sAHP} and decrease spike-frequency adaptation. These neurons have a classical I_{sAHP}, which is mediated mainly by a Ca^{2+}-dependent K^+ current and a Na^+-dependent K^+ current, which is activated with stronger stimuli. I_{sAHP} was strongly reduced by orexin-A (200 nM; C_1) and by stimulating either the PKA and PKC pathways (not shown). The orexin-A-mediated reduction was blocked by an inhibitor of PKC but not an inhibitor of PKA(C_2). The reduction of this current by orexins (and PKA and PKC activators) decreases spike-frequency adaptation and increases the number of spikes fired for the same input current in these cells (C_3). (D) Orexin-A (300 nM) depolarizes serotonergic dorsal raphé neurons and also *enhances* a late AHP. Orexin-A (300 nM) produced a suprathreshold depolarization (D_1). The late AHP was evoked (5 spikes at 20 Hz) before orexin application (left arrow) and during orexin (right arrow) after returning to the same baseline membrane potential by current injection (-dc). Orexin made the late AHP larger (198 ± 19 % of control, $n = 20$) and longer (429 ± 52 % of control half recovery time, $n = 20$). This orexin-enhanced AHP slowed steady-state firing in response to 100 pA (2 s) injected current pulses (D_2) without changing the shape of the action potential (spikes superimposed from before and after orexin; D_2 inset). Subfigures (A), (B) and (C) are adapted from Yang and Ferguson (2003), Murai and Akaike (2005), Zhang *et al.* (2010) and are reproduced with permission. Subfigure (D) was adapted from Ishibashi M, Gumenchuk I, Leonard CS (unpubl. data).

small-conductance Ca^{2+}-activated K^+ channels (see Rouchet *et al.*, 2008). Orexin-A *enhances* this K^+ current and *induces* a longer-lasting, unidentified Ca^{2+}-dependent late AHP current that appears distinct from I_{sAHP}. Together, these orexin-

enhanced outward currents increase spike-frequency adaptation by slowing down steady-state firing and producing a delayed *decrease* in excitability (Ishibashi *et al.*, 2012). Collectively, and in contrast to a simple depolarizing action, these

modulatory actions imply that orexin receptor activation can finely tune how target neurons will respond to other inputs. Future studies aimed at understanding the mechanisms, prevalence and impact of this emerging class of actions could provide important insights into how orexins normally regulate information processing in the brain and how this is perturbed in the absence of orexins in narcolepsy.

In addition to postsynaptic actions, orexins have been recognized since the earliest studies in hypothalamic cultures to release neurotransmitters independently of tetrodotoxin-sensitive impulses (van den Pol *et al*., 1998), suggesting that orexin receptors are located at, or near, presynaptic terminals of some glutamate- and GABA-releasing neurons. This ability to release glutamate and/or GABA by increasing miniature postsynaptic current frequency has been observed in the CNS (van den Pol *et al*., 1998; Li *et al*., 2002; Smith *et al*., 2002; Davis *et al*., 2003; Acuna-Goycolea and van den Pol, 2009), although the underlying signalling mechanisms have not been explored. In some structures, orexin preferentially releases GABA (Davis *et al*., 2003) or glutamate (Smith *et al*., 2002; Acuna-Goycolea and van den Pol, 2009), typically in conjunction with postsynaptic actions. Presumably, this allows the orexins to amplify or attenuate information transmission arising from particular afferents.

Another mechanism by which orexins can influence the release of neurotransmitter from presynaptic terminals is by retrograde paracrine signalling via the endocannabinoid 2-AG (2-arachidonoylglycerol) (see *Orexins and endocannabinoids* in Kukkonen and Leonard, 2013). This has so far been investigated in only a few nuclei: postsynaptic orexin action is supposed to lead to production of 2-AG, which upon exit to the synaptic space stimulates presynaptic CB_1 receptors, leading to a decrease in transmitter release, glutamate in the dorsal raphé nucleus (Haj-Dahmane and Shen, 2005) and GABA in the ventrolateral periaqueductal grey matter (Ho *et al*., 2011). The mechanism of stimulation of production of 2-AG and its substrate DAG is not clear, but if it is related to the ability of orexin receptors to activate PLC and/or induce Ca^{2+} influx, as expected (see *Orexins and endocannabinoids* in Kukkonen and Leonard, 2013), then the same mechanism is likely operational at other central orexin targets too.

In addition to dynamically adjusting the pre- and postsynaptic properties of their targets, orexins also regulate synaptic plasticity in the hippocampus (Selbach *et al*., 2004; 2010) and ventral tegmental area (for review, see Baimel and Borgland, 2012). Remarkably, in the ventral tegmental area, this plasticity is critical to behavioural sensitization resulting from cocaine administration (Borgland *et al*., 2006) and involves an initial but transient increase in the number of postsynaptic NMDA-type glutamate receptors. While it is yet unclear whether regulation of NMDA (or AMPA receptors; Shin *et al*., 2009) is a prevalent orexin function, it has recently been shown that orexins potentiate the NMDA receptor-driven release of noradrenaline from the somata and dendrites of locus coeruleus neurons (Chen *et al*., 2008b). These effects on NMDA receptors were sensitive to OX_1 and PKC inhibitors (Borgland *et al*., 2006; Chen *et al*., 2008b), which hints at involvement of the PLC–PKC pathway. In the VTA, a similar potentiation of NMDA receptor-mediated synaptic currents was produced by orexin-B in the presence of the OX_1 antagonist SB-334867 (Borgland *et al*., 2008). This effect was sensitive to the OX_2 antagonist JNJ-10397049 and PKC inhibitors, suggesting that both OX_1 and OX_2 induce this type of regulation by a common signalling pathway. It will be important to determine if glutamate receptors are regulated by orexins in other neurons.

Do orexin receptor subtypes show effector preferences in the CNS?

The tools available to identify which receptor underlies orexin action on central neurons are quite limited. Expression analysis, which could exclude the presence of one subtype, is hampered by receptor antibodies that are unreliable (Kukkonen, 2012; 2013). Hence, assessment of receptor mRNA and pharmacological analysis utilizing concentration–response relations and receptor subtype-selective antagonists have mainly been used. For orexin agonists, it is generally believed – based on the originally reported affinities and potencies (Sakurai *et al*., 1998) – that an equal potency of orexin-A and orexin-B indicates OX_2, while a 10-fold greater potency of orexin-A indicates OX_1. The synthetic ligand [Ala11, D-Leu15]-orexin-B, has been reported to be OX_2-selective. However, agonist potency profiles alone are unreliable due to possible *biased agonism/agonist trafficking of receptor responses* or other processes (discussed in Kenakin, 1997; 2011; Kukkonen, 2012). Such issues have been identified for orexin-A, orexin-B and [Ala11, D-Leu15]-orexin-B (Putula *et al*., 2011), and therefore, agonist-based determinations of orexin receptor subtypes without additional supporting evidence should be considered provisional. In a few cases, receptor knockout mice have also been used to assess whether one or the other receptor is necessary to mediate an orexin action (see below), but this approach is also not without potential problems (Routtenberg, 1996). The use of orexin receptor subtype-selective antagonists requires a pharmacological analysis to show that inhibition occurs with the expected potency, since the available antagonists are not absolutely selective. In the best case, selective antagonists for both subtypes would be used.

Based on the results from expression systems and receptor sequence analysis, there is no reason that both receptors would not be able to couple to any of the effector families described (Kukkonen and Leonard, 2013), although this issue has not been thoroughly studied. The evidence indicates that both native orexin receptors in the CNS inhibit K^+ currents and activate NSCCs. For example, OX_2 receptor expression is enriched in layer 6 of the neocortex in rat (Trivedi *et al*., 1998; Marcus *et al*., 2001). Layer 6b neocortical neurons are depolarized by reduction of a resting K^+ current and show equivalent responses to 30 nM orexin-A and orexin-B, consistent with OX_2 receptors mediating this action (Bayer *et al*., 2004). In contrast, rat layer 2/3 prefrontal cortex primarily expresses OX_1 mRNA (Trivedi *et al*., 1998; Marcus *et al*., 2001). These pyramidal neurons are depolarized by both reduction of a resting K^+ current and activation of an NSCC, and the entire response to 400 nM orexin-A is blocked by 1 μM of the OX_1 antagonist SB-334867 while no response is triggered by the 'selective' OX_2 agonist [Ala11, D-Leu15]-orexin-B (Yan *et al*., 2012), consistent with OX_1 receptors reducing a resting K^+ current.

Similarly in rat dorsal raphé nucleus, mRNA for both orexin receptors are co-distributed (Trivedi *et al*., 1998;

Marcus *et al.*, 2001) and can be recovered from the same cells (Brown *et al.*, 2002). Orexin-A and orexin-B activate a NSCC with a similar potency in rat (Brown *et al.*, 2002; Liu *et al.*, 2002; Soffin *et al.*, 2004) but recordings from slices made from OX_1 and OX_2 knockout mice indicate that either receptor is sufficient to mediate the NSCC current (Leonard *et al.*, 2011).

In contrast, reports of NCX activation by orexin are associated with responses thought to be mediated by OX_2 receptors (Eriksson *et al.*, 2001; Wu *et al.*, 2002; 2004; Burdakov *et al.*, 2003). For example, in the tuberomammilary nucleus, NCX is reported to drive the orexin-mediated depolarization and this appears entirely mediated by OX_2 as only OX_2 mRNA is detected by *in situ* hybridization in rat (Marcus *et al.*, 2001), orexin-evoked Ca^{2+} transients are absent in the OX_2 knockout mice (Willie *et al.*, 2003), and orexin-stimulated firing can be rescued by re-expressing OX_2 in tuberomammillary nucleus neurons of conditional OX_2 knockout mice (Mochizuki *et al.*, 2011). In the lateral vestibular nucleus, the orexin-mediated depolarization is suggested to be mediated by both the closure of K^+ channels and the activation of NCX (Zhang *et al.*, 2011). The depolarization appeared partly mediated by either orexin receptor subtype, although it was not resolved whether OX_2 activation was necessary for the NCX component of the depolarization. Future studies aimed at identifying both the receptors and evoked currents at the molecular level (see *Orexin receptor actions in the CNS*) will be needed to determine if the correlation of OX_2 and NCX is of functional importance, and if so, how it might be mediated.

Finally, while it is likely that orexins mediate the same major physiological responses in the CNS of different species, the expression of individual receptor subtypes may vary between species, and thus the use of techniques with resolving power for the function of receptor subtypes (e.g. subtype-selective antagonists) is required. Moreover, mouse shows alternative splicing of the OX_2 gene with two functional receptor proteins as a result (Chen and Randeva, 2004; Chen *et al.*, 2006), which may complicate the analysis.

Molecular signalling mechanisms in the CNS

Much less is known about the signalling cascades engaged by orexin receptors in the brain than in expression systems. Direct studies suggest orexin receptors couple to (at least) $G_{i/o}$ proteins in rat brain stem and to $G_{i/o}$, G_s and G_q in rat hypothalamus (see Kukkonen and Leonard, 2013 for details). Other evidence is indirect. A number of studies have implicated the PLC–PKC pathway based on the effectiveness of PKC inhibitors in blocking the orexin-mediated depolarization (Yang *et al.*, 2003; Muroya *et al.*, 2004; Xia *et al.*, 2005), elevation of intracellular Ca^{2+} (Uramura *et al.*, 2001; Kohlmeier *et al.*, 2004; 2008; Muroya *et al.*, 2004), inhibition of the sAHP current (Zhang *et al.*, 2010), enhancement of NMDA receptor currents (Borgland *et al.*, 2006; Chen *et al.*, 2008b) and suppression of H-current (Li *et al.*, 2010); in addition, sensitivity to the PLC inhibitor U73122 is found in some studies (Muroya *et al.*, 2004; Borgland *et al.*, 2006). However, it has also been reported that the orexin-mediated depolarization of dorsal raphé neurons, which is mainly mediated by a NSCC, was not blocked by inhibitors of PKC, PKA or the ERK cascade (Brown *et al.*, 2002). In these same neurons, a PKC inhibitor blocked

the orexin-enhanced Ca^{2+} transients, but the orexin-mediated activation of the NSCC current was still detectable in some of the same neurons, even though fewer neurons showed this response (Kohlmeier *et al.*, 2008), suggesting the presence of PKC-dependent and PKC-independent processes. Yet, in other neurons, the orexin-mediated depolarizations are blocked by inhibitors of PKA, but not PKC (Korotkova *et al.*, 2002; van den Top *et al.*, 2003; Nakamura *et al.*, 2010). Analyses of the signal coupling are hampered by multiple signalling mechanisms of these receptors and possibly even multiple similar channel types in the selected cell population or even in single cells, together with the shortage of good tools (Kukkonen and Leonard, 2013). The situation is further complicated by potential signalling pathway cross-coupling and convergence onto common effectors. For example, somatostatin and met-enkephalin activate inward rectifier K^+ 3.1/3.2 (Kir3.1/3.2, a.k.a. GIRK) channels in rat locus coeruleus neurons via pertussis toxin-sensitive G-proteins, while substance P suppresses the induced activity of these channels via a pertussis toxin-insensitive mechanism (Velimirovic *et al.*, 1995). Orexin is able to both induce the rapid activity and inhibit the sustained activity of the channels via pertussis toxin-sensitive and -insensitive mechanisms respectively (Hoang *et al.*, 2003).

Thus, in the CNS, there is evidence that orexin receptors couple to multiple effectors including those leading to Ca^{2+} and Na^+ influx – for which, in most cases, we do not know the channel type or activation mechanism. Moreover, it is evident that that in some central neurons, PLC–PKC and AC–PKA pathways are engaged as has been elaborated in expression systems and other tissues. Given the relative paucity of orexin receptor signalling studies in the CNS, it is plausible that the diverse signalling pathways described in other systems may also be found in the CNS as these studies develop.

How these signalling pathways function in the CNS may depend on the nature of the orexin signals to be detected. Orexins may operate as paracrine signals that act over substantial diffusion distances at very low concentrations or they may operate as more localized synaptic signals at high concentrations. Evidence for the former mode is suggested by the accumulation of orexin in the cerebrospinal fluid over the entire active periods (Zeitzer *et al.*, 2004) and the ability of either intracerebroventricular orexin delivery or ectopic re-expression of the pre-propeptide to rescue orexin-deficient genetic models (Mieda *et al.*, 2004; Liu *et al.*, 2011). Evidence for the latter is suggested by the ability to record orexin-mediated synaptic potentials following electrical stimulation (Yamuy *et al.*, 2004) and rapid arousal responses following optogenetic *in vivo* stimulation (Adamantidis *et al.*, 2007) of orexin neurons.

Orexin receptor modulation of central circuits

Orexin receptor signalling influences multiple homeostatic systems, including those regulating arousal, appetite, metabolism, reward, stress and autonomic function (for review, see Carter *et al.*, 2009; Sharf *et al.*, 2010; Girault *et al.*, 2012; Nattie and Li, 2012). Perhaps most strikingly, orexin signalling is essential for the normal regulation of sleep and waking as its disruption results in narcolepsy with cataplexy – a sleep disorder characterized by excessive sleepiness, sleep attacks, sudden loss of postural muscle tone in response to

strong emotions (cataplexy), and state instability and fragmentation produced by shorter and more frequent bouts of waking and sleep (for review, see Siegel and Boehmer, 2006). Overall, the circuits controlling these systems are not known precisely and undoubtedly involve overlapping populations of neurons in many cases (for review, see Brown *et al.*, 2012). Thus, our understanding of the role played by orexin receptor signalling at particular nodes in these circuits is currently quite incomplete.

In the control of arousal and behavioural state, orexin actions have been identified at many cell groups thought to be important for maintaining arousal including the orexin neurons themselves (Yamanaka *et al.*, 2010) and all tested ascending cholinergic systems (Eggermann *et al.*, 2001; Burlet *et al.*, 2002; Wu *et al.*, 2002; 2004; Hoang *et al.*, 2004; Kohlmeier *et al.*, 2004; 2008; Arrigoni *et al.*, 2010; Leonard *et al.*, 2011) and monoaminergic systems (Hagan *et al.*, 1999; Horvath *et al.*, 1999; Ivanov and Aston-Jones, 2000; Nakamura *et al.*, 2000; Bayer *et al.*, 2001; Brown *et al.*, 2001; 2002; Eriksson *et al.*, 2001; Uramura *et al.*, 2001; Liu *et al.*, 2002; van den Pol *et al.*, 2002; Yamanaka *et al.*, 2002; Hoang *et al.*, 2003; Willie *et al.*, 2003; Kohlmeier *et al.*, 2004; 2008; Korotkova *et al.*, 2003; Soffin *et al.*, 2004; Haj-Dahmane and Shen, 2005; Murai and Akaike, 2005; Borgland *et al.*, 2006; Leonard *et al.*, 2011; Mochizuki *et al.*, 2011; Ishibashi *et al.*, 2012) which form part of the ascending reticular activating system and project to the thalamus and/or cortex (for review, see Jones, 2005). Moreover, orexins excite neurons in the sublaterodorsal region (Brown *et al.*, 2006; 2008), which is an area containing neurons with multiple transmitter phenotypes, and recently recognized as critical in regulating rapid eye movement sleep expression and muscle tone during sleep (Boissard *et al.*, 2002; Lu *et al.*, 2006). At the level of thalamocortical systems, orexin actions appear highly suited to promote arousal and suppress sleep because orexins excite midline, intralaminar and reticular thalamic nuclei, which are part of the 'non-specific' projection system, without exciting 'specific' thalamocortical neurons (Bayer *et al.*, 2002; Govindaiah and Cox, 2006). Except for prefrontal cortex, orexin actions in the neocortex appear restricted to deep layer (6b) neurons, which make intracortical connections to layer 1 where they can interact with midline and intralaminar thalamic inputs (Bayer *et al.*, 2004). Thus, orexins can promote neocortical arousal through both non-specific thalamocortical pathways and the numerous pre-cortical afferent systems that are directly activated by orexins.

Consistent with the idea that thalamocortical actions of orexin are critical for waking, orexin action at these sites appears mediated mainly by OX_2 receptors (Bayer *et al.*, 2002; 2004). OX_2 receptors are particularly important for normal waking as the defect in canine narcolepsy is a null OX_2 receptor (Lin *et al.*, 1999) and mice lacking OX_2 receptors have a narcolepsy phenotype with shorter wake bouts and sleep attacks (Willie *et al.*, 2003), unlike OX_1 null mice that do not have a narcolepsy phenotype (Mieda, 2012). Moreover, in the absence of OX_2, the ability of intracerebroventricularly infused orexin to promote arousal and suppress sleep through OX_1 receptors is impaired (Mieda, 2012). However, recent findings utilizing a conditional OX_2 receptor knockout mouse indicate that re-expression of OX_2 in the tuberal region of the hypothalamus, including the histaminergic tuberomammillary nucleus, is sufficient to reinstate normal wake bout durations although fragmentation of sleep was not rescued (Mochizuki *et al.*, 2011). This indicates, however, that thalamocortical orexin actions are not necessary for maintaining normal wake bouts. Given the apparent redundancies in arousal systems, orexin actions in thalamocortical systems may still be sufficient to stabilize normal wake bouts and/or it may be required for other aspects of waking (e.g. vigilance) yet to be determined.

Beyond the control of arousal and behavioural state, orexins influence numerous other systems as noted earlier. Several lines of evidence indicate orexins act on the neural substrates regulating motivated behaviour and reward. For example, OX_1 receptors depolarize ventral tegmental area GABA and dopamine neurons (Nakamura *et al.*, 2000; Uramura *et al.*, 2001; Korotkova *et al.*, 2003), and actions of orexin here appear necessary for behavioural sensitization to cocaine administration by up-regulating synaptic NMDA receptors (see above). Orexins depolarize neurons in many of the structures that are efferent targets of the ventral tegmental area system including neurons in the shell region of the nucleus accumbens via OX_2 (Mukai *et al.*, 2009), the bed nucleus of the stria terminals (Lungwitz *et al.*, 2012) and neurons that provide input to the nucleus accumbens, including the central nucleus of the amygdala via OX_2 receptors (Bisetti *et al.*, 2006), paraventricular thalamic nucleus (Ishibashi *et al.*, 2005; Huang *et al.*, 2006; Kolaj *et al.*, 2007; Doroshenko and Renaud, 2009; Zhang *et al.*, 2009; 2010) and prefrontal cortex (Li *et al.*, 2010; Yan *et al.*, 2012). Orexins also depolarize neurons in structures that provide afferent input to the ventral tegmental area including laterodorsal tegmentum (Burlet *et al.*, 2002; Kohlmeier *et al.*, 2004; 2008; Leonard *et al.*, 2011; Ishibashi *et al.*, 2012; Takahashi *et al.*, 2002) and pedunculopontine tegmentum (Kim *et al.*, 2009a; 2009b) via OX_1 receptors.

Orexins stimulate feeding (Sakurai *et al.*, 1998) and orexin neurons respond to signals of metabolic status (Yamanaka *et al.*, 2003; Burdakov *et al.*, 2006; Karnani *et al.*, 2011). They provide strong output to the arcuate nucleus where they excite orexigenic neuropeptide Y/agouti-related protein (NPY/AgRP) neurons and produce rhythmic bursting (van den Top *et al.*, 2004) via OX_2 receptors and activation of NCX, likely stimulated by Ca^{2+} release from intracellular stores (Burdakov *et al.*, 2003; Muroya *et al.*, 2004). Orexins also can excite the anorexigenic preproopiomelanocortin (POMC) arcuate neurons, many of which project into the median eminence, by apparent activation of NCX (Acuna-Goycolea and van den Pol, 2009). This result contradicts a previous study that concluded orexins indirectly decrease firing in POMC arcuate neurons by suppressing spontaneous excitatory and increasing spontaneous inhibitory synaptic currents (Ma *et al.*, 2007). As noted by the authors, the orexin-mediated depolarization reversed near −40 mV and in the prior study, orexin actions were studied at depolarized membrane potentials suggesting that the depolarizing action might have been missed. While future studies should resolve the basis for these differences, these studies serve to remind us that the pathways and effectors engaged by orexin receptors may well be sensitive to the internal state of the cell, yielding different actions under different conditions. NCX might serve such a purpose as its activity is tied to metabolic

factors as well as signalling pathways (DiPolo and Beauge, 2006; Török, 2007).

Orexins strongly activate the autonomic nervous system and regulate visceral sensory-motor control (Hwang *et al.*, 2001; Follwell and Ferguson, 2002; Smith *et al.*, 2002; Yang and Ferguson, 2002; 2003; Davis *et al.*, 2003; Grabauskas and Moises, 2003; van den Top *et al.*, 2003; Yang *et al.*, 2003; Dergacheva *et al.*, 2005; 2012; Huang *et al.*, 2010). Finally, orexins also can also influence the somatic sensory-motor systems, including superficial dorsal horn neurons of the spinal cord (van den Pol, 1999; Grudt *et al.*, 2002) and vent-rolateral periaqueductal grey neurons (Ho *et al.*, 2011), both of which regulate pain transmission, and descending vestibulospinal neurons, which can powerfully influence posture and motor performance (Zhang *et al.*, 2011).

In conclusion, native orexin receptors throughout the CNS drive membrane depolarization, increase intracellular Ca^{2+} and increase transmitter release from presynaptic terminals. Emerging evidence indicates that these receptors also strongly modulate cell excitability through multiple means and can regulate synaptic plasticity. Multiple effectors and signalling mechanisms are involved but our limited knowledge mostly precludes molecular identification of the signalling pathways and effectors mediating these actions. Future approaches should make use of the recently developed pharmacological tools to inhibit signal cascades [e.g. (phosphor)lipases and kinases] and orexin receptor subtypes. In addition, better resolution of the membrane currents regulated by orexins is necessary for their identification. This will require better electrophysiological isolation to enable their study over wider range of voltages, more extensive ion substitution experiments, development and use of better pharmacological blockers and ultimately utilization of knockout or knockdown approaches.

Orexin responses outside the CNS

Gastrointestinal tract

OX_1 and OX_2 mRNA was detected in rat and guinea pig myenteric plexi (Kirchgessner and Liu, 1999). Orexin-A (30 nM) excited guinea pig ileal submucosal neurons, and an increase in input resistance was seen. Afterhyperpolarization was also reduced. Both responses suggest inhibition of K^+ channels (Kirchgessner and Liu, 1999). However, orexin-A also reduced the amplitude of the evoked excitatory postsynaptic potentials. No synaptic/action potential block was applied in these studies. In another study, orexin-A depolarized guinea pig ileal myenteric neurons; reduced input resistance, current reversal around the equilibrium potential of K^+ and resistance to synaptic block suggest postsynaptic inhibition of K^+ channels (Katayama *et al.*, 2003). Orexin also enhanced evoked cholinergic potentials but did not affect direct acetylcholine responses, suggesting presynaptic facilitation. Acetylcholine overflow and contraction was seen in an orexin-A-stimulated guinea pig ileal preparation; results with tetrodotoxin, atropine and SB-334867 suggest that orexin-A acts on neuronal OX_1 receptors increasing acetylcholine overflow, which depolarizes the muscle via muscarinic receptors (Matsuo *et al.*, 2002).

Mouse duodenum was also contracted by orexin-A (300 nM) (Squecco *et al.*, 2011). The contraction was partially inhibited by the L-type VGCC blocker nifedipine (1 µM), nearly fully by 10 µM 2-aminoethoxydiphenyl borate [inhibitor of IP_3 receptors, orai1 and some transient receptor potential (TRP) channels] and fully by 50 µM Ni^{2+} and 1 mM tetraethylammonium chloride. Multiple channels were affected by orexin-A: activity of voltage-gated Na^+ channels as well as VGCCs of T- and L-type was enhanced while BK-type K^+ channels were inhibited. In addition, activation of receptor- and store-operated Ca^{2+} channels was suggested (Squecco *et al.*, 2011). The results demonstrate the problems orexin researchers are facing from the multiple signal pathways utilized by orexin receptors and the limitations of the pharmacological inhibitors.

STC-1 mouse gastric endocrine carcinoid tumour cells, apparently isolated from genetically oncogene-harbouring mice, express mRNA for both OX_1 and OX_2 receptors (Larsson *et al.*, 2003). Orexin-A potently stimulated cholecystokinin secretion from these cells, putatively by inducing depolarization and activation of L-type VGCCs. No obvious Ca^{2+} or cAMP response to orexin-A was seen, but the former may be hidden under the strong spontaneous Ca^{2+} oscillations.

OX_1 receptors have been suggested to be expressed in primary human colorectal tumours and also in many cell lines established from primary tumours but not in the normal colon epithelium (Voisin *et al.*, 2011; see *Cell lines natively expressing orexin receptors* below and *Cell death* in Kukkonen and Leonard, 2013).

Pituitary gland

It is somewhat unclear which parts of the pituitary express which orexin receptors in which species (see Kukkonen, 2013). In rat corticotropes, orexin-A inhibits adrenocorticotropin release in response to corticotropin-releasing hormone (Samson and Taylor, 2001). An inhibitor of PKC blocks the orexin response, while pertussis toxin does not; the target of PKC is unknown. In isolated sheep somatotropes, orexin-A, instead, enhances L-type VGCC current in a PKC-dependent manner and augments growth hormone release (Chen and Xu, 2003). Whether the latter is mediated by the PKC pathway was not assessed.

Adrenal gland

Orexin receptor activation of G-proteins has been investigated by Randeva and co-workers in adrenal cortex utilizing the GTP-azidoanilide labelling methods (see *Orexin receptor coupling partners: heterotrimeric G-proteins* in Kukkonen and Leonard, 2013). In human and rat cells, coupling to G_s, G_i and G_q is seen (Karteris *et al.*, 2001; 2005; Randeva *et al.*, 2001). Whether mainly OX_1 or OX_2 receptors are expressed in man is unsettled (Karteris *et al.*, 2001; Randeva *et al.*, 2001; Mazzocchi *et al.*, 2001b), while rat has been reported to express mRNA for both OX_1 and OX_2 (Karteris *et al.*, 2005). Both human and rat cells respond to orexin by increasing glucocorticoid (cortisol and corticosterone, respectively) synthesis/release via the AC–PKA pathway (Malendowicz *et al.*, 1999; Mazzocchi *et al.*, 2001b). This could be mediated by G_s, but this has not been verified, and PLC is also activated by orexins in these cells (Randeva *et al.*, 2001; Karteris *et al.*, 2005).

H295R human adrenocortical carcinoma cells express both OX_1 and OX_2 receptor mRNA, although OX_2 is more highly expressed (Ramanjaneya *et al.*, 2008; Wenzel *et al.*, 2009). Orexin receptor activation stimulates cortisol release and expression of proteins involved in steroidogenesis like steroidogenic acute regulatory protein (StAR; mRNA and protein), different cytochrome P450 (CYP) species (mRNA) and 3β-hydroxysteroid dehydrogenase (HSD3B2; mRNA) (Ramanjaneya *et al.*, 2008; Wenzel *et al.*, 2009). Ca^{2+} is elevated at high orexin concentrations, but the MAPKs, ERK and p38 are phosphorylated in the low nanomolar range (Ramanjaneya *et al.*, 2009; Wenzel *et al.*, 2009). PKC phosphorylation is responsible for the ERK activation, and inhibition of either one inhibits most of the HSD3B2 mRNA induction (Ramanjaneya *et al.*, 2009; Wenzel *et al.*, 2009). The results are complicated and partly contradictory, and it remains unclear whether ERK activation is responsible for all the steroidogenic actions (only HSD3B2 mRNA induction was assessed). The result concerning the PKC–ERK cascade would appear contradictory to the results with the primary adrenal cortical cells. This could be explained by a distorted cancer phenotype of the cell line; however, it is also conceivable that the PKC–ERK response seen in H295R cells relates to cell differentiation rather than regular stimulation of steroid biosynthesis.

Many studies find no response to orexins with respect to aldosterone or catecholamine release, but recently, amperometric recordings showed catecholamine release via OX_1 receptors in cultured rat chromaffin cells (Chen *et al.*, 2008a). Primary human pheochromocytomas (OX_2 mRNA) also release catecholamines upon orexin stimulation (Mazzocchi *et al.*, 2001a). PLC, but not AC, is stimulated in these cells, and catecholamine release is suggested to be mediated by the PLC–PKC pathway. Aldosterone release from cultured porcine chromaffin cells is stimulated only at high orexin concentrations (Nanmoku *et al.*, 2002). The signal pathways are unclear.

Endocrine and exocrine pancreas

Whether (exogenous) orexinergic stimulation is capable of regulating release of pancreatic glucagon and insulin or other processes in endocrine pancreas is under debate. Some reports show expression of orexin receptor mRNA in endocrine pancreas and even responses in isolated tissue fragment, but the signal cascades have not been analysed. InR1-G9 cells are glucagon-secreting cells subcloned from Syrian golden hamster insulinoma cells. It is unclear, which orexin receptors subtype these cells express (as this was only determined by antibodies), but the cells functionally responded to orexin-A in nanomolar range by phosphorylation of phosphoinositide-dependent kinase 1 (PDK1) and PKB, which suggests activation of the PI3K pathway (Göncz *et al.*, 2008). Orexin-A stimulated phosphorylation of the PKB-target protein, transcription factor Forkhead box protein O1 (FOXO1), and inhibition of PI3K or knockdown of FOXO1 reversed orexin-A-mediated decrease of proglucagon mRNA (Göncz *et al.*, 2008). GPCRs are known to utilize several pathways for activation of class Ia and class Ib PI3Ks; it is thus far unclear how orexin receptors do it in these cells. There are a few other indications of PI3K activation by orexin receptors (Ammoun *et al.*, 2006a; *White and brown adipose tissue*). In the

paper (Göncz *et al.*, 2008), the authors use rat insulinoma Ins-1 cells as a positive control for OX_1 receptors; however, there are no published reports indicating the presence of OX_1 receptors in Ins-1 cells.

There are no reports of orexin signalling in the exocrine pancreas except for the rat pancreatic acinar tumour AR42J cells, which express OX_2 receptors. Orexins rather weakly stimulated Ca^{2+} mobilization in these cells but with high potency (subnanomolar) (Harris *et al.*, 2002). Amylase secretion was stimulated 100-fold less potently (Harris *et al.*, 2002) and cell death, in the long run, by a further 10-fold lower potency (Voisin *et al.*, 2006). Causal relationships between these responses have, unfortunately, not been determined.

Male reproductive system

OX_1 and OX_2 receptor mRNA has been detected in different sites in the male reproductive tract of man and rat (Jöhren *et al.*, 2001; Barreiro *et al.*, 2004; Karteris *et al.*, 2004). Orexin-A (possibly via OX_1 receptors) stimulates testosterone production and decreases mRNA for anti-Müllerian hormone in rat testis via unknown pathways (Barreiro *et al.*, 2004; 2005). In human male testicular membranes, orexin-A stimulates PLC (Karteris *et al.*, 2004).

White and brown adipose tissue

Human primary white adipocytes of subcutaneous and omental adipose tissue express mRNA for both OX_1 and OX_2 receptors (Digby *et al.*, 2006). Orexin responses (orexin-A and orexin-B, 100 nM) were investigated. In subcutaneous fat, PPARγ2 mRNA was increased, while in omental fat, hormone-sensitive lipase mRNA was decreased (Digby *et al.*, 2006). In differentiated mouse white adipocyte-like 3T3-L1 cells (OX_1 and OX_2 mRNA), orexin-A stimulated PI3K and, in PI3K-dependent manner, glucose uptake and triglyceride synthesis (Skrzypski *et al.*, 2011). Orexin-A stimulated ERK phosphorylation, increased PPARγ2 mRNA and stimulated adiponectin release, while siRNA against PPARγ (γ2?) blocked orexin-A-induced increase in triglyceride content and adiponectin release. In contrast, baseline lipolysis was inhibited. Orexin-A has been reported to stimulate proliferation (but not differentiation) of undifferentiated 3T3-L1 cells (and also of another fibroblast cell line, NIH3T3); however, the concentration–response relationship and the response magnitude is very different in the two studies (Zwirska-Korczala *et al.*, 2007; Skrzypski *et al.*, 2012). Orexin-A also activated ERK but not PKB (Skrzypski *et al.*, 2012). This appears contradictory to the finding that PI3K would be stimulated in differentiated cells; however, the authors have not assessed the PKB in undifferentiated cells or phosphatidylinositol-3,4,5-trisphosphate in differentiated cells. ERK activity was suggested to be protective against cell death by serum starvation as the protective effect of orexin-A was blocked by MAPK/ERK kinase 1 (MEK1) inhibition. Glucose uptake and PPARγ2 expression was increased in rat primary pre-adipocytes by orexin-A, but differentiation to adipocytes was not stimulated (Skrzypski *et al.*, 2011; 2012).

Plancentally produced orexins were recently identified as pivotally contributing to the prenatal development of brown adipose tissue in mice (Sellayah *et al.*, 2011). Orexin-A, via OX_1 receptors, triggered the differentiation programme in

mouse primary preadipocytes and mouse embryonic fibro-blasts (MEFs), C3H10T1/2 mouse mesenchymal stem cells and HIB1b mouse preadipocytes; differentiation was evaluated by morphological and mRNA markers. The signal pathways inducing differentiation are unclear because signal transduction was mainly assessed in already differentiated C3H10T1/2 cells. In these cells and in mouse primary brown pre-adipocytes, orexin stimulation activated p38 and the transcription factor SMAD1/5. Upon more detailed investigations in C3H10T1/2 cells, orexin-A was seen to induce expression of mRNA for both bone morphogenic protein 7 (BMP7) and BMPR1A receptor during differentiation. The signal cascade, based on pharmacological inhibitors, was suggested to proceed from OX_1 receptors partly via BMP7/BMPR1A and partly via p38 MAPK. Even BMP7 signalling was suggested to partially require p38 (Sellayah *et al.*, 2011).

Haematopoietic cells
Despite some reports, firm evidence is lacking for the expression of orexin receptors in hematopoietic cells or their precursors. See discussion in Kukkonen (2013).

Cell lines natively expressing orexin receptors
A number of cancer cell lines were identified to express orexin receptor mRNA by RT-PCR. The cell lines expressing OX_1 mRNA include human neuroblastoma SK-N-MC and human colon carcinoma cells HT29-D4, Caco-2, SW480 and LoVo (Rouet-Benzineb *et al.*, 2004) (please note that not all the cell lines have been examined for expression of OX_2). OX_2 receptor mRNA is expressed in rat pancreatic acinar tumour line AR42J (Harris *et al.*, 2002). These cell lines show decreased cell division and enhanced cell death upon orexin exposure (Rouet-Benzineb *et al.*, 2004; Voisin *et al.*, 2006); however, it has not been examined whether the former results from a decrease in proliferation or from the proceeding cell death. There also may be heterogeneity in these cell lines (as in most cell lines); we, for instance, have not been able to measure orexin responses in our SK-N-MC cells (Kukkonen *et al.*, unpubl. data).

H295R human adrenocortical carcinoma cells and pancreatic tumour cells are addressed under the sections *Adrenal gland* and *Endocrine and exocrine pancreas*. Also, some other cell lines have been reported to respond to orexin, but they have found no wider use, or are problematic, for instance, by responding to orexins only at high concentrations.
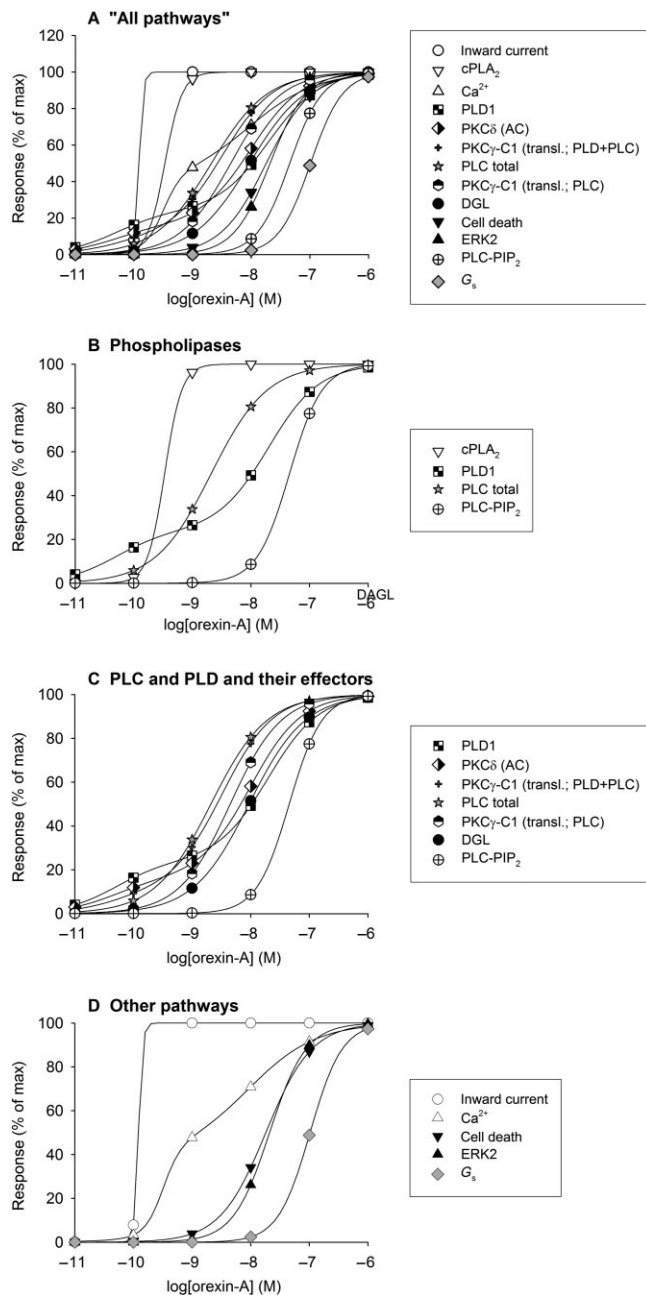
In summary, many peripheral tissues express orexin receptors and respond (sometimes strongly) to exogenous orexins. However, a major question remaining is whether orexin (or another ligand) can reach these receptors under physiological (or pathological) conditions (see the *Conclusions* section). While this seems to be so for brown adipose tissue, the question remains open for other tissues. For possible orexin system-targeting therapy (see, e.g. *Cell death* in Kukkonen and Leonard, 2013, and the *Conclusions* section), such physiological access would, however, be less significant.

Recombinant cells

Recombinant cell lines have found much use in signalling research for orexin receptors as native orexin receptor-

expressing cells have been difficult to isolate in suitable quantities, and native cell lines are scarce. Recombinant cells allow for genetic and physiological methods of analysis that are difficult to apply to native cells. However, recombinant cell lines are unrelated to, or only distantly related to, native orexin receptor-expressing cells. Therefore, results may be difficult to extrapolate to native situations. Nevertheless, remarkably similar responses have been seen: orexins, for instance, regulate NSCCs in both neurons and recombinant neuronal and non-neuronal cells (see *Orexin receptor actions in the CNS*, and *Ca²⁺ and Depolarization and synaptic functions* in Kukkonen and Leonard, 2013). Heterologous receptor expression platforms are often also criticized for causing artificial signal coupling not present in native cells. While being a justified concern, there is no clear evidence for this with orexin receptors. Moreover, the orexin receptor levels are unknown in native cells, and receptor levels could also be high at postsynaptic regions. Moreover, *human* orexin receptor signalling can be studied in recombinant expression systems in contrast to studies on native cells, which have focused mainly on rodent cells. Thus, it is possible that species-specific differences in signalling and regulation are yet to be discovered.

CHO cells (based on the CHO-K1 clone) are the cells most used for orexin receptor studies. These were also the first stable recombinant cell lines to be produced, probably simultaneously, in several laboratories (Sakurai *et al.*, 1998; Smart *et al.*, 1999; Lund *et al.*, 2000; Ammoun *et al.*, 2003). The human OX_1 receptor has been most extensively investigated; the fact that the same cell lines have been used in a number of studies allows comparison of the potency of activation of different signal cascades, which are summarized in Figure 3. As discussed in Kukkonen and Leonard (2013), multiple signalling cascades are activated in these cells. G-proteins of the families G_q, G_i and G_s are suggested to be activated (Lund *et al.*, 2000; Holmqvist *et al.*, 2005), but there is mainly indirect evidence for this (see *Orexin receptor coupling partners: heterotrimeric G-proteins* in Kukkonen and Leonard, 2013). Downstream, a potent activation of Ca^{2+} influx/inward current and the phospholipases cytosolic phospholipase A_2 (cPLA$_2$), PLC (hydrolysing phosphoinositides PI and/or PIP) and phospholipase D1 (PLD1) is seen (Lund *et al.*, 2000; Kukkonen and Åkerman, 2001; Larsson *et al.*, 2005; Ammoun *et al.*, 2006a; Johansson *et al.*, 2007; 2008; Turunen *et al.*, 2010; 2012; reviewed in Kukkonen and Leonard, 2013) (Figure 3). Fairly potently activated signal cascades go to ERK, cell death (p38 MAPK) and diacylglycerol lipase (DGL) (via the PLC pathway) (Ammoun *et al.*, 2006a,b; Turunen *et al.*, 2012; reviewed in Kukkonen and Leonard, 2013), while hydrolysis of PIP$_2$ (and generation of IP$_3$) and activation of G_s require relatively high orexin concentrations (Holmqvist *et al.*, 2005; Johansson *et al.*, 2008; reviewed in Kukkonen and Leonard, 2013) (Figure 3). We have assessed some of the signal cascades via their downstream effectors, like PLC and/or PLD signalling via DGL activity, translocation of protein kinase Cγ-C1 domain to the plasma membrane and the novel PKC (nPKC)-dependent component of AC activation (Figure 3). The signal cascades are dependent on each other, as determined utilizing a pharmacological analysis. The cPLA$_2$ cascade contributes to the Ca^{2+} influx, nPKC is required for PLD1 activation, both PLD1 and PLC cascades

A "All pathways"

Legend:
- ○ Inward current
- ▽ cPLA₂
- △ Ca²⁺
- ◨ PLD1
- ◇ PKCδ (AC)
- ✦ PKCγ-C1 (transl.; PLD+PLC)
- ★ PLC total
- ⊛ PKCγ-C1 (transl.; PLC)
- ● DGL
- ▼ Cell death
- ▲ ERK2
- ⊕ PLC-PIP₂
- ◆ G$_s$

B Phospholipases

Legend:
- ▽ cPLA₂
- ◨ PLD1
- ★ PLC total
- ⊕ PLC-PIP₂

C PLC and PLD and their effectors

Legend:
- ◨ PLD1
- ◇ PKCδ (AC)
- ✦ PKCγ-C1 (transl.; PLD+PLC)
- ★ PLC total
- ⊛ PKCγ-C1 (transl.; PLC)
- ● DGL
- ⊕ PLC-PIP₂

D Other pathways

Legend:
- ○ Inward current
- △ Ca²⁺
- ▼ Cell death
- ▲ ERK2
- ◆ G$_s$

Figure 3

Summary of concentration–response relations for different responses produced by orexin-A at the human OX_1 receptor. This illustrates the different potencies with which orexin-A stimulates different signalling cascades under conditions of equal receptor expression levels in the same clone of CHO cells. Thus, cell background and receptor expression levels are equal across cases so potencies are directly comparable. The results are expressed as % of maximal response for each output. The responses measured are: 'inward current' (whole-cell patch-clamp recordings), 'cPLA₂' (pyrrophenone-sensitive and tetrahydrolipstatin-insensitive AA release chromatographically measured in ^{14}C-AA-labelled cells), 'Ca²⁺' (cytosolic Ca²⁺ elevation measured with fura-2 parallel to the patch-clamp recordings), 'PLD1' (PLD transphosphatidylation assay in ^{14}C-oleic acid-labelled cells with chromatographic separation; PLD1 defined by pharmacological PLD1 and PLD2 inhibitors), 'PKCδ (AC)' [nPKC (likely PKCδ)-dependent AC stimulation measured chromatographically in ^{3}H-adenine-labelled cells], 'PKCγ-C1 (transl.; PLD + PLC)' (DAG generation by both PLC and PLD measured by translocation of the fusion protein of C1-domain of PKCγ and green fluorescent protein to the plasma membrane), 'PLC total' (total inositol phosphate release from ^{3}H-inositol-labelled cells determined by chromatography), 'PKCγ-C1 (transl.; PLC)' (DAG generation by PLC alone measured by translocation of the fusion protein of C1-domain of PKCγ and green fluorescent protein to the plasma membrane in cells expressing dominant-negative PLD), 'DGL' (tetrahydrolipstatin-sensitive and pyrrophenone-insensitive 2-AG release chromatographically measured in ^{14}C-AA-labelled cells), 'cell death' (assessed by staining and counting of viable cells), 'ERK2' [ERK2 phosphorylation assessed by Western blotting against the phosphorylated (active) ERK1/2 species], 'PLC-PIP₂' (IP₃ release assessed by a IP₃-binding protein kit and by translocation of the PH-domain of PLCδ1 from the plasma membrane upon PLC activity towards PIP₂), and 'G$_s$' (Gα$_s$-dependent AC stimulation measured chromatographically in ^{3}H-adenine-labelled cells). For the sake of clarity, only curve-fitting results and not the original data points are presented. The curves can be identified with the symbols added on the traces. The measurements originate from the following studies: Holmqvist *et al.* (2005); Larsson *et al.* (2005); Ammoun *et al.* (2006a,b); Johansson *et al.* (2008); Jäntti *et al.* (2012); Turunen *et al.* (2012).

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but this seems to be independent of PLC activity (Jäntti *et al.*, 2012). OX₂ signalling cascades have been more seldom assessed. OX₂ receptor stimulation equally well elevates Ca²⁺ and activates ERK in CHO cells (Sakurai *et al.*, 1998; Smart *et al.*, 1999; Ammoun *et al.*, 2003; 2006a; Putula *et al.*, 2011), and also activates PLC (Tang *et al.*, 2008; Kukkonen *et al.*, unpubl. data). Some of the putative relationships of the signal cascades are presented in Figure 4.

CHO-S cells, which represent a suspension-adapted clone of CHO-K1, have been used in the studies of Laburthe and co-workers on cell death (see *Cell death* in Kukkonen and Leonard, 2013). In these cells, OX₁ receptors elevate Ca²⁺, activate PLC and induce cell death (Voisin *et al.*, 2008; El Firar *et al.*, 2009). The authors have also shown that both rat orexin receptor subtypes induce apoptosis of these cells (Voisin *et al.*, 2006).

HEK-293 cells are the next most used cell line. Stable clones, including tetracyclin-inducible ones, have been established in several laboratories (Ellis *et al.*, 2006; Magga *et al.*, 2006; Tang *et al.*, 2008; Ward *et al.*, 2011), and the cells have also been utilized for transient orexin receptor expression.
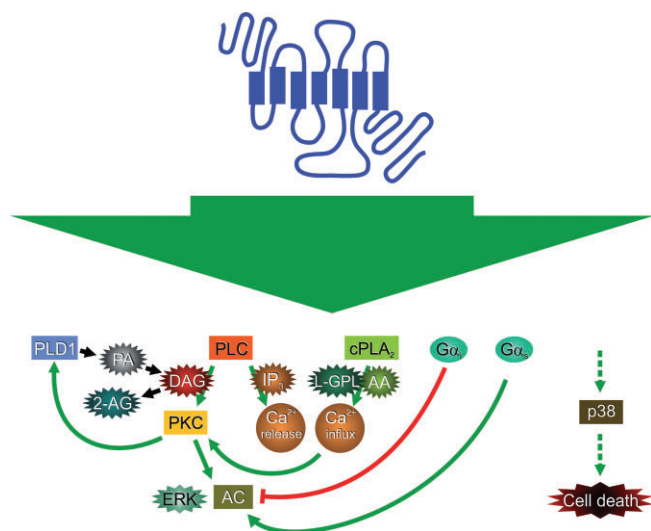
contribute to the PKC translocation/activation, PLC cascade produces the DAG required for DGL, and IP₃ induces Ca²⁺ release (Ekholm *et al.*, 2007; Johansson *et al.*, 2008; Jäntti *et al.*, 2012; Turunen *et al.*, 2012) (Figure 3). PLC, PLD and cPLA₂ cascades should be linked via PIP₂ as well: PLD should stimulate PIP₂ generation, and PIP₂ is required for PLD activity and it may also enhance cPLA₂α activity (reviewed in Ghosh *et al.*, 2006; Kukkonen, 2011). The ERK cascade can be partially inhibited by inhibiting many signal cascades (Ammoun *et al.*, 2006a). The most perplexing findings relate to Ca²⁺ influx and nPKC. Ca²⁺ influx seems to amplify or gate many of the pathways via an unknown mechanism, but it is also itself regulated by one of these pathways, cPLA₂ (Turunen *et al.*, 2010; 2012; see *Ca²⁺* in Kukkonen and Leonard, 2013). Some nPKC (possibly PKCδ) gates PLD1 activation by orexin,

Figure 4

Schematic summary of some of the relationships of human OX_1 receptor signalling cascades in CHO cells as described in Figure 3. References are as for Figure 3. While G_q may underlie PLC activation (and possibly other responses), this has not been explicitly assessed and is thus not indicated in the figure. L-GPL, lyso-glycerophospholipid; PA, phosphatidic acid.

Human OX_1 receptors are known to connect to Ca^{2+} elevation (Magga *et al.*, 2006). Transient expression has shown the same for human OX_2 receptors, and both receptors couple to PLC as well (Tang *et al.*, 2008; Putula *et al.*, 2011; Putula and Kukkonen, 2012). Ca^{2+} signalling of OX_1 receptors utilizes both influx and release; the mechanisms of the Ca^{2+} influx-dependent oscillations are described under *Ca^{2+}* in Kukkonen and Leonard (2013). We have seen release of arachidonic acid (AA) and 2-AG from stably and transiently OX_1-expressing cells upon OX_1 receptor stimulation (Peltonen *et al.*, 2009; Turunen *et al.*, 2012); the origins of these have not been determined, but it is reasonable to assume that at least 2-AG results from PLC activity. Transiently expressed OX_1 receptors have been shown to engage β-arrestin in their signalling to ERK in HEK-293 (see *Orexin receptor coupling partners: interaction with other proteins* in Kukkonen and Leonard, 2013). OX_2 receptors also activate AC and ERK and p38 MAPK cascades; both PKA and PKC are suggested to contribute to the ERK but not to the p38 cascade (Tang *et al.*, 2008).

As an attempt to create a more neuronal model for orexin signalling studies, we established stable human orexin receptor-expressing clones of neuro-2a mouse neuroblastoma and PC12 rat pheochromocytoma cells (Holmqvist *et al.*, 2002). Unfortunately, the cells have remained rather unexplored. The original study showed that both receptor subtypes couple to Ca^{2+} signalling (likely both influx and release as in CHO and HEK-293 cells) and PLC in these cell lines (Holmqvist *et al.*, 2002). Ca^{2+} release was shown to require IP_3 while the receptor-operated Ca^{2+} influx was independent of IP_3 in neuro-2a-OX_1 cells, similar to CHO cells (Ekholm *et al.*, 2007). Also, ERK is activated upon OX_1 receptor stimulation in these cells (Ekholm *et al.*, 2007). In neuro-2a-OX_1 cells, but not in PC12-OX_1 cells, 2-AG and AA release is seen upon

orexin stimulation; the source, like in HEK-293 cells, is undetermined (Turunen *et al.*, 2012).

Stable MEF-hOX_1 cells have been used in studies of cell death to assess the role of $G_{q/11}$ in the response. In these cells, orexin-A (1 μM) induces cell death and stimulates PLC, but the responses are absent when MEF cells from the $G_{q/11}$-knockout are used (Voisin *et al.*, 2008).

Also, some other cell lines have been constructed, but they have found no wider use, or show some problems. Transient expression of orexin receptors utilizing liposome-based transfection or transduction with mammalinized baculovirus has also been reported in IMR-32, CHO and MEF cells (Näsman *et al.*, 2006; Louhivuori *et al.*, 2010; Jäntti *et al.*, 2013) (and also for HEK-293 cells as described earlier). Differentiated IMR-32 cells show Ca^{2+} transients related to the action of NSCCs and NCX (see *Ca^{2+}* in Kukkonen and Leonard, 2013). MEF and HEK-293 cells have been utilized for translocation and β-arrestin studies (see *Orexin receptor coupling partners: interaction with other proteins* in Kukkonen and Leonard, 2013). Transiently transfected HEK-293 cells have also been utilized for the investigation of orexin receptor coupling to Kir3.1/3.2 channels; orexin receptors inhibit these channels, which may mimic their action in the CNS (Hoang *et al.*, 2003) (see *Central nervous system* and *Depolarization and synaptic functions* in Kukkonen and Leonard, 2013).

Conclusions

In these two reviews (the present study and Kukkonen and Leonard, 2013), we have gathered and analysed current knowledge about orexin receptor signalling. It is obvious that orexin receptors possess the ability to signal in multiple ways. The mechanisms that determine which pathway is used in which cell type and situation are unknown. The analysis of signal cascades and target proteins is not easily performed in native cells; this is especially difficult in neurons, and as a consequence, relatively little molecular data are available. Thus, most of the signalling analysis has been performed in recombinant cell lines, mainly CHO cells, and with respect to some specific responses, in certain native cell lines. We believe that the responses seen in recombinant cell lines indeed represent physiological signalling mechanisms of orexin receptors, but where these different pathways are utilized remain mostly obscure as signalling pathways in native cells have been underexplored. The information obtained in cell lines could be utilized to formulate specific tests for native cells especially by utilizing novel pharmacological inhibitors that would be expected to reveal important new information. Thus far, the information flow in this direction seems limited, and traditional, less conclusive approaches with inhibitors having less selective or even doubtful pharmacological profiles (e.g. D609, U73122, KB-R7943) are still often used without adequate controls. However, it must be noted that the pharmacological tools for this type of research are still very limited, which thus calls for even more judicious use. More information is constantly generated and novel tools developed, which will hopefully allow, for instance, distinction of TRP channel isoforms.

One major question relating to orexin receptor signalling is its physiological importance. In CNS neurons, orexin receptors act at all levels of the neuroaxis to effect depolarization. Emerging evidence suggests that orexin receptor action is more subtle than this simple excitation, and that they dynamically adjust presynaptic activity and postsynaptic responsiveness. Orexin receptors also can regulate the expression of synaptic plasticity and potentially the expression of target neuron phenotypes. Nevertheless, linking the action mediated by orexin receptors at particular sites to overall system or circuit functions remains quite challenging, although the use of receptor/peptide deletion models and orexin receptor antagonists has been fruitful is some cases. The major effect of orexin peptide/receptor dysfunction is dysregulation of sleep-arousal behaviours, but other phenotypes, while perhaps less obvious, have (Kayaba *et al.*, 2003) and will be discovered, considering the widespread influence of the peptide-receptor system. Being harder to uncover does not mean they are less important, but that they may involve processes protected by redundancy in regulatory systems or developmental compensation. For the peripheral systems, no clear phenotypes have yet been described, except for the developmental failure of brown adipocytes. While we find orexin receptor mRNA expression and functional responses in many peripheral tissues, preproorexin expression is found at very few sites – preproorexin mRNA expression has been reported in the gastrointestinal tract, testis, adrenal gland, pancreas and kidney (Sakurai *et al.*, 1998; Kirchgessner and Liu, 1999; Jöhren *et al.*, 2001; Nakabayashi *et al.*, 2003; Karteris *et al.*, 2004) – while there are probably no circulating orexins. Would then the peripheral orexin receptors and the responses they show to exogenous orexin stimulation have any physiological functions? One possibility, assuming there is a reason that these receptors are expressed, is that there are natural ligands for these receptors other than preproorexin-derived peptides. Based mainly on a single study (Nakabayashi *et al.*, 2003), it seems that preproorexin expression might be more widespread in peripheral organs of man than rodents; could there be species-specific functions? If the peripheral orexin receptors have physiological significance in man, these may be manifested in side effects of orexin receptor antagonist therapy (Winrow and Renger, 2013).

The orexin system is an obvious therapeutic target for disorders of sleep and wakefulness. Both the native ligands (peptide processing, degradation) and the receptors (agonists, antagonists, allosteric modulation) could be targeted for drug development. Many orexin receptor antagonists (mainly either OX_2-selective or non-selective) are under development or testing (Roecker and Coleman, 2008) and the first one of these dual receptor antagonists, suvorexant (MK-4305), is likely to be released in the US market soon (Winrow and Renger, 2013). Synthetic orexin receptor agonists, in contrast, have not been reported on except for one patent (Yanagisawa, 2010), although they would likely be useful in increasing/stabilizing wakefulness and as a treatment for excessive sleepiness (e.g. in narcolepsy) and for killing cancer cells (*Cell death* in Kukkonen and Leonard, 2013). For the receptor agonists, we might also expect other responses, like increased metabolic rate and peripheral responses; whether these were therapeutically useful for

other indications or detrimental as side effects remains to be shown.

An interesting feature of the multiple orexin receptor responses is their relation to the agonist concentration. This is most clearly seen in the recombinant OX_1 receptor-expressing CHO cells, in which different responses are triggered with distinct potencies spanning three orders of magnitude (Figure 3). Similar findings have also been reported in native OX_2-expressing AR42J cells (see *Endocrine and exocrine pancreas*). This could, most simply, be explained by response amplification differences obtained via mutual affinities and efficacies of the specific signal transduction cascade components and/or thresholds, but it may also require other processes like receptor dimerization or trafficking. Would such orexin peptide concentration dependence of the response also be seen in physiological context? This would open up interesting possibilities of completely different responses mediated by synaptic and extrasynaptic orexin receptors. Another aspect of orexin responses are the roles played by the functional orexin peptides orexin-A and orexin-B. The original studies in recombinant CHO cells presented an often-cited selectivity of OX_1 receptor between these peptides. While seen also in many other recombinant cell types with respect to Ca^{2+} elevation or PLC activation, this does not hold true for other responses (Kukkonen, 2013). We would thus like to suggest that orexin peptides display *biased agonism* (Kenakin, 2011), that is, that different agonists, like the endogenous orexin peptides, may preferentially activate different receptor responses. This could be further complicated by each receptor subtype having a different bias, and homo-oligomerization among orexin receptor 'monomers' or hetero-oligomerization with other GPCRs could affect these response profiles as well. Finally, if orexin-A and orexin-B were released together, their time courses of action could differ according to their relative stabilities (Yoshida *et al.*, 2003). In native cells, there are some dramatic examples of orexin-B being more potent or efficacious than orexin-A. As this has not observed in any recombinant receptor expression system (with exception of Tang *et al.*, 2008), it raises the question of whether it is brought about by *biased agonism* (via receptor monomers or oligomers), or if there are one or more additional receptors for orexin peptides or if some other pharmacodynamic and pharmacokinetic properties of these peptides are involved?

In conclusion, studies with both recombinant and native cells support the view that orexin receptors are able to regulate many physiologically important molecular signalling cascades, but that not all cells utilize all possible cascades and that these cascades in native cells are not necessarily activated with the same efficacy as in recombinant systems. While it is clear that orexin signalling is diverse in the brain, future studies are needed to narrow the gap between what is known there and in recombinant systems. Advances in this area should benefit from the application of available pharmacological and molecular biological tools and knowledge of signalling patterns in recombinant systems. Finally, what regulates how orexin receptors (and other GPCRs) select a particular signalling pathway from all possible pathways at different sites is not known and is expected to be an important topic for future investigations.

Acknowledgements

Conflicts of interest

None.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

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Table S1 Postsynaptic orexin actions in the CNS.