THE REGULATION OF OREXIN RECEPTOR FUNCTION BY DYNEIN LIGHT CHAINS

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ABSTRACT

Orexins (OX-A, OX-B) are involved in the regulation of sleep, feeding and reward. The action of these peptides is governed by Orexin Receptors 1 and 2 (OX1R, OX2R). In aim to understand the mechanisms involved upon activation of these receptors, we have identified the dynein light chains 1 and 3 (Dynlt1/3) as novel partners. We hypothesize that Dynlt1/3 are important for orexin receptor intracellular regulation. After identification of a strong interaction between OX1R and Dynlt1 and the importance of the OX1R C-terminal domain residues, the functional implication of this novel interaction was assessed. Ligand-induced internalization of OX1R was not altered by modification of Dynlt1/3 expression, yet its transit in early endosomes was accelerated by Dynlt1 over-expression. In conclusion, these data suggest that Dynlt1 promotes the exit of OX1R from early endosomes following ligand-induced internalization, in association with an accelerated signal termination as measured by the phosphorylation levels of ERK1/2.
Les orexines (OX-A, OX-B) sont impliquées dans le sommeil, l’alimentation et la récompense. Leur action est médiée par les récepteurs aux orexines 1 et 2 (OX1R, OX2R). Pour comprendre les mécanismes découlant de leur activation, nous avons identifié les chaînes légères de la dynéine 1 et 3 (Dynlt1/3) comme partenaires de ces récepteurs. Notre hypothèse est que les Dynlt1/3 sont importantes pour réguler les récepteurs. Après avoir identifié une forte interaction entre OX1R et Dynlt1 et l’importance du domaine C-terminal d’OX1R, l’importance fonctionnelle de cette nouvelle interaction a été caractérisée. Le départ d’OX1R de la membrane n’est pas affecté par une modification de l’expression des Dynlt1/3, mais sa transition dans les endosomes a été accélérée par la surexpression de Dynlt1. En conclusion, nos données suggèrent que Dynlt1 favorise la sortie d’OX1R des endosomes après l'internalisation, accélérant la fin de signalisation du récepteur (mesurée par les niveaux de phosphorylation d’ERK1/2).
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<th>Full Form</th>
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<tr>
<td>3-AT</td>
<td>3-Amino-1,2,4-Triazole</td>
</tr>
<tr>
<td>a.a.</td>
<td>amino acid</td>
</tr>
<tr>
<td>Ab</td>
<td>antibody</td>
</tr>
<tr>
<td>AD</td>
<td>activating domain</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CB1</td>
<td>cannabinoid receptor 1</td>
</tr>
<tr>
<td>CHO</td>
<td>chinese hamster ovary</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>co-IP</td>
<td>co-immunoprecipitation</td>
</tr>
<tr>
<td>CTD</td>
<td>C-terminal domain</td>
</tr>
<tr>
<td>DAG</td>
<td>diacylglycerol</td>
</tr>
<tr>
<td>DBD</td>
<td>DNA-binding domain</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
</tr>
<tr>
<td>DMH</td>
<td>dorsomedial hypothalamus</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>Dyncl2</td>
<td>cytoplasmic dynein 1 intermediate chain 2</td>
</tr>
<tr>
<td>Dynlt</td>
<td>dynein light chain Tctex type 1</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EEA1</td>
<td>early endosome antigen 1</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-Linked ImmunoSorbent Assay</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular-signal-regulated kinase</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>G418</td>
<td>geneticin</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>--------------------------------------------</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein coupled receptor</td>
</tr>
<tr>
<td>GRK</td>
<td>G-protein coupled receptor kinases</td>
</tr>
<tr>
<td>GST</td>
<td>glutathione S-transferase</td>
</tr>
<tr>
<td>HC</td>
<td>heavy chain</td>
</tr>
<tr>
<td>HEK</td>
<td>human embryonic kidney</td>
</tr>
<tr>
<td>IC</td>
<td>intermediate chain</td>
</tr>
<tr>
<td>ICV</td>
<td>intracerebroventricular</td>
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<tr>
<td>IP3</td>
<td>phosphotidyl-inositol</td>
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<tr>
<td>IPTG</td>
<td>Isopropyl-β-D-1-thiogalactoside</td>
</tr>
<tr>
<td>KO</td>
<td>knock out</td>
</tr>
<tr>
<td>LB</td>
<td>luria broth</td>
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<td>locus coeruleus</td>
</tr>
<tr>
<td>LC</td>
<td>light chain</td>
</tr>
<tr>
<td>LH</td>
<td>lateral hypothalamus</td>
</tr>
<tr>
<td>LIC</td>
<td>light intermediate chain</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MCH</td>
<td>melanin-concentrating hormone</td>
</tr>
<tr>
<td>ONPG</td>
<td>2-nitrophenol β-D-galactopyranoside</td>
</tr>
<tr>
<td>OPD</td>
<td>O-phenylenediamine</td>
</tr>
<tr>
<td>OX1R</td>
<td>orexin 1 receptor</td>
</tr>
<tr>
<td>OX2R</td>
<td>orexin 2 receptor</td>
</tr>
<tr>
<td>OX-A</td>
<td>orexin A</td>
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<td>orexin B</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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CONTRIBUTION TO STUDIES

Experimental works presented in Figure 1.6, Figure 1.8 and Figure 1.9 were done by Dr. David Duguay, with his consent to present in this thesis (to be published).
1. INTRODUCTION

Orexins are neuropeptides involved in the regulation of appetite, sleep-wake cycles, energy homeostasis, addiction and other processes. The function of these peptides is governed by two G-protein coupled receptors (GPCRs), Orexin Receptors 1 and 2 (OX1R, OX2R) (Sakurai et al., 1998), through activation of intracellular signalization. It is well-known that upon receptor activation there is a robust calcium influx (shown in several cell types), which is upstream and central to orexin signaling cascades (Kukkonen et al., 2002). The cell bodies of orexin-producing neurons are exclusive to the hypothalamus but with vast projections throughout the brain. Accordingly, orexins are well positioned to influence various systems and receive many inputs as well. Notably, the loss of orexin peptides (Nishino et al., 2000) or mutations of the orexin receptors (Lin et al., 1999) have been shown to cause the sleep disorder, narcolepsy. This disease is mainly characterized by excessive daytime sleepiness and cataplexy. In particular, it is our interest to understand the intracellular mechanisms that are involved upon activation of these receptors, as little is known about their response to orexins, their targeting and recycling. Moreover, it is fundamental to treatment of orexin-related disorders to obtain a better understanding of what these receptors do.
1.1 THE OREXIN SYSTEM

1.1.1 The orexin peptides

Orexin A and B (OX-A/ OX-B) (Sakurai et al., 1998), also named hypocretin 1 and 2 (de Lecea et al., 1998), are secreted peptide products of the precursor, prepro-orexin. Prepro-orexin gene is composed of 2 exons separated by an intron, coding for the 131 (human) amino acid (a.a.) precursor; it contains prohormone convertase cleavage sites, which eventually, through proteolytic cleavage, leads to the two peptides (Sakurai et al., 1999) (FIGURE 1.1).

The orexins show similarity in their C-terminus (several residues are identical) yet OX-A is 33 a.a. in length, with cysteine residues allowing for disulphide bond formation and thus the ability to fold on itself, while OX-B is 28 a.a. (Sakurai et al., 1998) (FIGURE 1.2). Moreover, OX-A is completely conserved while OX-B has minor a.a. (single residue) substitutions across mammalian species so far identified (Ohno & Sakurai, 2008). As well, both peptides are highly conserved across other species: fish, xenopus and chicken. Although the levels of OX-B are higher than OX-A in several areas of the central nervous system (CNS) (Date et al., 2000; Mondal et al., 1999), OX-A appears to be more biologically active. Indeed, OX-A is more stable in cerebrospinal fluid and blood and also has a high lipid solubility and thus can cross the blood brain barrier, in contrast to OX-B (Kastin et al., 1999).
1.1.2 *The orexin-producing neurons*

The orexin peptides are found in neuronal cell bodies which are exclusive to the perifornical area (PFA), dorsomedial hypothalamus (DMH) and lateral and posterior hypothalamus (de Lecea et al., 1998; Peyron et al., 1998; van den Pol et al., 1999; Zhang et al., 2001). Interestingly, only one other peptide, melanin-concentrating hormone (MCH), is known to be expressed exclusively in the lateral hypothalamus. Although the orexin-producing neurons are intermingled with neurons producing MCH, there is no evidence of overlap between these two neuronal populations (Broberger et al., 1998). Crosstalk between these two distinct neuronal populations allows modulation of similar physiological function, notably, arousal and goal-oriented behaviors (Adamantidis & de Lecea, 2008). However, orexin-producing neurons also release the inhibitory dynorphin peptide (Chou et al., 2001) and glutamate (Abrahamson & Moore, 2001) and thus their co-release could nullify or potentiate the actions of orexins, respectively. These excitatory neurons project widely throughout the brain, densely innervating noradrenergic (locus coeruleus; LC), histaminergic (tuberomammillary nucleus; TMN), serotoninergic (raphe nuclei) and dopaminergic (ventral tegmental area; VTA) nuclei as well as the paraventricular nucleus (PVN) and arcuate nucleus (Peyron et al., 1998) (FIGURE 1.3). Thus, it has been speculated that orexins have many physiological functions.
1.1.3 The orexin receptors

Orexins A and B bind with different affinities to two receptors: orexin receptors 1 and 2 (also called hypocretin receptors 1 and 2 (de Lecea et al., 1998)) (FIGURE 1.1). OX1R responds selectively to OX-A (IC50=20 nM), while OX2R responds to both OX-A (IC50=40 nM) and OX-B (IC50=40 nM) (Sakurai et al., 1998). The orexin receptors have 7 hydrophobic membrane-spanning domains and belong to the superfamily of GPCRs (FIGURE 1.4). The human receptors share 64% amino acid identity, where OX2R (444 a.a.) is slightly longer than OX1R (425 a.a.), and both are highly conserved across species (Sakurai et al., 1998) (FIGURE 1.4). GPCRs are the largest group of cell surface receptors whose response to stimuli causes them to undergo a conformational change and initiate a variety of physiological changes (Neer, 1995). Upon agonist binding, the heterotrimeric guanine nucleotide binding proteins (G proteins) are activated, through exchange of GDP to GTP, which causes dissociation of the Gα and βγ subunits and subsequent signal transduction (Ferguson, 2001). GPCRs are highly regulated, often at the third intracellular loop and C-terminus, by phosphorylation events and protein partner binding (Ritter & Hall, 2009).

1.1.4 Orexin receptor distribution

The orexin receptors are expressed centrally as well as peripherally. In the CNS, the distribution of the receptors is consistent with the vast projections of the orexin-producing neurons (Carter et al., 2009a). In some regions, both receptor mRNAs are present but remarkably, the distribution of receptor mRNAs in
various nuclei is distinct and complementary (**FIGURE 1.3**). In the cerebral cortex, different layers express one receptor over the other and in the hypothalamus, both receptors are present but OX1R is anterior and ventromedial, whereas OX2R is lateral (Marcus et al., 2001). Both receptors are found in the VTA and raphe nucleus, but solely OX1R mRNA is enriched in LC neurons, whereas only OX2R mRNA is expressed in the TMN (Marcus et al., 2001). So does this differential distribution reflect their involvement in regulating distinct physiological events and behaviours? Moreover, are both receptors necessary for modulation of these systems or is the presence of one receptor sufficient?

### 1.1.5 Orexin receptor mediated-signaling

Both receptors are coupled to the Gq subclass of the Ga subunit, whereas there is evidence that OX2R is also coupled to pertussis toxin-sensitive Gi/Go and Gs (Hoang et al., 2003; Zhu et al., 2003). In neurons, Gq activation is excitatory, which leads to non-selective cation channel opening and subsequent depolarization, while Gi/o is inhibitory, which leads to activation of inward-rectifier potassium channels and thus hyperpolarization, depending on the target neuron (Zhu et al., 2003).

For both receptors, Gq activation triggers the phospholipase C (PLC) cascade, generating phosphotidyl-inositol (IP3) and diacylglycerol (DAG) which further elevates intracellular calcium or induces protein kinase C (PKC) activity, respectively (Johansson et al., 2007; Lund et al., 2000). In addition, orexin receptor activation independently affects cAMP levels, through Gi/o, Gs or PKCδ.
This signaling ultimately causes activation of the ERK (extracellular-signal-regulated kinase) MAPK (mitogen-activated protein kinase) pathway (Ammoun et al., 2006). ERK proteins become active by being phosphorylated and can then activate transcription factors or other downstream protein kinases, resulting in proliferation, differentiation and cell cycle regulation (Pouyssegur et al., 2002). Both receptors are capable of activating ERK, but OX2R with less potency compared to OX1R. OX2R-mediated ERK MAPK activation is transient and appears to occur via multiple signaling cascades, mediated by three G protein subunits (Gq, Gs and Gi) in HEK293-OX2R cells (Tang et al., 2008). OX1R is able to activate ERK phosphorylation in Chinese hamster ovary (CHO)-OX1R (Kukkonen et al., 2002), which seems to be largely mediated by PKCβ (and receptor-operated calcium channels) and not cAMP, IP3 or cytoplasmic calcium elevation itself (Ammoun et al., 2006).

OX1R signaling has been examined in only a few studies and yet different responses with regards to OX1R-mediated cAMP alteration have been reported (Holmqvist et al., 2005; Zhu et al, 2003). These cell-type specific variations on orexin receptor signaling are thought to be due to the different adenylyl cyclase isoforms expressed in different cells (Ammoun et al., 2006). Yet, regardless of the expression system (recombinant orexin receptor expression or native neurons), the biphasic calcium response (increase in intracellular calcium levels) that occurs upon stimulation of the OX1R, is always present. This intracellular increase in calcium occurs through two mechanisms; calcium influx via receptor-operated calcium channels (in part mediated by PKC) (Larsson et al., 2005; van den Pol et
al., 1998), which amplifies a PLC-mediated mobilization of calcium from thapsigargin-sensitive intracellular stores (Kukkonen et al., 2002; Sakurai et al., 1998; Smart et al., 1999). More specifically, stimulation of OX1R causes release of calcium from different sources depending on the concentration of OX-A used (Johansson et al., 2007).

β-arrestins 1 and 2 are well known to be recruited to the serine and threonine sites that have been phosphorylated by GPCR kinases (GRK) on GPCRs and thus regulate receptor desensitization and internalization (Ferguson, 2001). Specifically, β-arrestin 1 and 2 have been shown to traffic with the OX1R upon internalization (Evans et al., 2001) and promote its internalization via clathrin-coated pits by binding to phosphorylated sites at the extreme carboxy end of the receptor (Milasta et al., 2005). On the other hand, β-arrestins have also been implicated in G-protein-independent activation of ERK MAPK which can likely occur at the plasma membrane, as well as in endosomes if the GPCR remains associated with β-arrestins in endosomes (Oakley et al., 1999). β-arrestins have been shown to interact directly with proteins involved in signal transduction and thus link the associated agonist-occupied GPCR to signal transduction. For example, β-arrestins interact with: c-Src family of tyrosine kinases, upon activation of the β2-adrenergic receptor (Luttrell et al., 1999); ERK, in the case of protease-activated receptor 2 activation (DeFea et al., 2000); as well as JNK3 MAPK signaling cascade components (McDonald et al., 2000). A specific C-terminal mutant of OX1R, that hindered the interaction with β-arrestin-2, also lead to a less sustained ERK MAPK pathway activation when compared to wild type
OX1R, suggesting that β-arrestin-2 acts as a scaffold for ERK signaling in this system as well (Milasta et al., 2005).

1.1.6 The roles of orexins

In the CNS, the physiological functions of orexins are numerous, which can be expected due to the large innervations of orexin neurons throughout the entire CNS as well as the abundant afferents that modulate their activity. Most notably, they have been implicated in energy homeostasis, sleep-wake regulation, and motivation and reward. Moreover, different subsets of orexin neurons, classified according to their localization (lateral hypothalamus (LH), PFA or DMH) in the lateral hypothalamus were suggested to have different functions (see below) (Estabrooke et al., 2001; Harris et al., 2005; Harris & Aston-Jones, 2006).

Suitable to their hypothalamic localization, orexins were initially implicated in the stimulation of appetite, where ICV injection of OX-A in freely moving rats at the beginning of the light phase increased feeding (Sakurai et al., 1998; Sakurai T., 1999). Moreover, mice lacking orexins (prepro-orexin knock-out (KO) or orexin neuron-ablated) ate less than wild type controls (Hara et al., 2001). The appetite-stimulating effects of orexins are in part due to projections to the arcuate nucleus and thus modulation of neuropeptide Y activity, as measured by induction of c-Fos (a marker of neuronal activity) in these neurons, after OX-A administration (Yamanaka et al., 2000). Despite this evidence, further investigations suggested that the feeding-stimulation effect is also likely an indirect effect of orexin’s role in arousal states.
Orexins are not only necessary to maintain wakefulness (Saper et al., 2005) but can provoke it by increasing the probability of sleep (slow wave and rapid eye movement) to wake transitions (Adamantidis et al., 2007) throughout the entire light/dark cycle (Carter et al., 2009b). Thus, the feeding effect of orexins may be secondary to their wake-promoting effect. Indeed, mice lacking orexins (orexin neuron-ablated) were unable to respond to food-deprivation by increasing wakefulness and activity, as compared to wild type littermates who demonstrated increased vigilance in search for food (Yamanaka et al., 2003). Specifically, orexin neurons located in the PFA and DMH (not the LH) showed diurnal changes in c-Fos activation which is consistent with a role in the production and maintenance of arousal (Estabrooke et al., 2001). Likewise, electrophysiological studies in freely moving rats, across the sleep-wake cycle and unit recording of orexin cells demonstrated that these neurons are maximally active during active wake (when muscle tone is important) in association with movement (Mileykovskiy et al., 2005). Also, orexin neurons have reduced activity during quiet wake, slow wave or REM sleep (when muscle tone is low or absent) but increased activity just before wake (Mileykovskiy et al., 2005). Amongst the densest orexin neuron innervations are the wake promoting nuclei: dorsal raphe, LC and TMN. These neurons have similar firing rates as the orexin neurons; they fire rapidly during wake and slowly in non-REM sleep and hardly during REM sleep (Siegel, 2004). In addition, orexins regulate REM sleep through direct and indirect projections to brainstem nuclei; latero-dorsal tegmental and pedunculopontine tegmental nucleus, to either excite or inhibit cholinergic tone, respectively (Ohno & Sakurai, 2008; Takahashi et al., 2002). Finally, a role of
orexin in regulating wake states is made clear by their involvement in narcolepsy (see section 1.1.7, below).

More recently, food- and drug-associated reward have been linked to increased orexin activity and thus to modulation by the orexin system (Boutrel et al., 2005; Harris et al., 2005; Sharf et al., 2010; Zheng et al., 2007). The dense projections of orexin neurons to the VTA and nucleus accumbens (part of the ventral striatum) allows for influence on dopamine neurons in these areas, which are involved in reward and motivated behaviors such as drug addiction. The first evidence for orexin influence was using a conditioned place preference model with food, morphine (injected) or cocaine (injected) in rats (Harris et al., 2005). C-Fos activation in orexin neurons correlated with the intensity of reward seeking only in the LH and the conditioning to morphine was extinguished when an orexin antagonist was administered (intraperitoneal) (Harris et al., 2005).

### 1.1.7 Narcolepsy

The most studied physiological role of orexins has been in sleep-wake regulation due to its central importance in the sleep disorder, narcolepsy. In humans, narcolepsy is sporadic, affecting 1 in 2000 individuals, with typical onset at adolescence. Narcolepsy is initially characterized by chronic, uncontrollable sleepiness in periods of usual wake and intrusions into wakefulness of REM-like sleep phenomena. These sleep attacks are often rapidly followed by cataplexy attacks, occurring in 85% of cases: sudden loss of muscle tone in wake triggered by strong emotions (laughter, surprise or anger) (Bourgin et al., 2008). Secondary
symptoms of narcolepsy include: hypnagogic hallucinations at sleep onset, sleep paralysis (at sleep-wake transitions) and shortened sleep latency (Dauvilliers et al., 2007).

The first discovery implicating the orexin system in this disease was the identification of a mutation in the OX2R gene in the canine model, where its manifestation is autosomal recessive (Lin et al., 1999). Subsequent generation of mice lacking prepro-orexin manifested a phenotype similar to human narcolepsy, which included cataplexy and fragmentation of vigilance states (Chemelli et al., 1999). Later, orexins were associated with the human pathophysiology of narcolepsy. Levels of OX-A in cerebrospinal fluid were low in patients who had narcolepsy with cataplexy (Nishino et al., 2000). The absence of both prepro-orexin mRNA (Peyron et al., 2000) and prepro-orexin peptide (Thannickal et al., 2000) in narcoleptic patient brains radically changed the field of sleep research. This rapidly lead to the hypothesis of narcolepsy being a disease involving a specific neurodegeneration of orexin neurons and the possibility of being of autoimmune nature due to its association with the HLA subtype (Lin et al., 2001).

Subsequent generation of animal models with disruption of the orexin system confirmed their importance in narcolepsy. The closest animal model to human narcolepsy is that where a toxic gene (ataxin-3) is selectively expressed in orexin-producing neurons and leads to the gradual loss of orexin neurons (Hara et al., 2001). These animals show a phenotype similar to prepro-orexin KO, and OX-A administration rescues the narcoleptic phenotype (Mieda et al., 2004). OX1R/OX2R KO mice behave like the prepro-orexin KO, but interestingly, OX2R
KO exhibit a less severe narcolepsy phenotype and OX1R KO have only mild sleep-wake disturbances and no behavioural abnormalities (Ohno & Sakurai, 2008). Thus, although both receptors are important in the sleep pathology, they seem to have distinct functions in other aspects of the disease. Support towards this notion separates vigilance and muscle tone in narcolepsy, according to orexin receptor expression patterns in the LC (OX1R) and TMN (OX2R). TMN neurons still fire in cataplexy (similar to quiet wake) and thus their activity correlates with alertness, unlike the LC and dorsal raphe neurons which are inactive in cataplexy (similar to REM sleep), whose activity correlates more with muscle tone (Sutcliffe & de Lecea, 2004).

Thus far, treatment for narcolepsy has been purely symptomatic. Treatment for sleepiness in narcolepsy is achieved by targeting catecholamine tone (Modafinil) or using amphetamine-like stimulants, whereas cataplexy symptoms are treated with tricyclic antidepressants (Nunez et al., 2009). It is also quite important to note that orexin’s modulation of reward (see section 1.1.6) may explain why narcoleptic patients on amphetamines, which are potentially addictive, do not show any signs of addiction to these drugs (Guilleminault et al., 1974). Although no drugs target the orexin system yet, orexin receptor antagonists are already being developed to target insomnia and to prevent drug relapse, in an aim to suppress the orexin system (Brisbare-Roch et al., 2007). Thus, the paucity of information on orexin receptor biology is surprising considering the ideal pharmacotherapy would be targeted to the orexin system and hence receptor activity. Moreover, since orexins are involved in a multitude of systems, it is
important to specifically target only one facet without disrupting others. Hence, it is imperative to study orexin receptor regulation to realize better treatment for orexin-related disorders.
FIGURE 1.1 THE OREXIN SYSTEM

Prepro-orexin is cleaved at prohormone-convertase sites with basic amino acid residues (KR, RR) to produce the mature peptides orexins A and B. Filled circles: Identical amino acids between orexins A and B. Orexin A possesses two intramolecular disulfide bridges and can interact with OX1R and OX2R, whereas orexin B is linear and has high affinity only for OX2R. OX1R is coupled to Gq/11, while OX2R is coupled to either Gq/11 or Gi/o G proteins. OX1R, orexin 1 receptor; OX2R, orexin 2 receptor.¹

FIGURE 1.2 COMPARISON OF HUMAN OREXIN-A AND OREXIN-B STRUCTURES

Upper panel: amino acid sequences of human orexin-A and orexin-B deduced from nucleic acid sequence. Green circles indicate amino acid identity. The two disulfide bonds in orexin-A are shown in yellow. Lower panel: schematic view of three-dimensional structures of human orexin-B, determined by two-dimensional 1H-NMR and dynamic simulated annealing calculations and orexin-A, constructed by homology using the Modeller 6.0 program.

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FIGURE 1.3 PROJECTIONS OF OREXIN-PRODUCING NEURONS AND EXPRESSION OF OREXIN RECEPTORS

Orexin neurons project widely throughout the brain and orexin receptors co-exist in some nuclei but also have a complementary distribution. LC- locus coeruleus, TMN- tuberomammillary nucleus, VTA-ventral tegmental area, PPT- pedunculopontine tegmental area, LDT-laterodorsal tegmental area, VLPO- ventrolateral preoptic nucleus. Hcrt- hypocretin (orexin), Hcrt-1- hypocretin 1 receptor (OX1R- orexin 1 receptor), Hcrt-2- hypocretin 2 receptor (OX2R- orexin 2 receptor)³.

Amino acid sequences of OX1R and OX2R indicate homology and existence of the insertion of single residues or clusters of residues\(^4\)

1.2 THE REGULATION OF OREXIN RECEPTORS

Despite the numerous roles of orexins, little is known about the regulation of their receptors. Indeed, as mentioned above, GPCRs are highly regulated proteins, often at the third intracellular loop and cytoplasmic carboxy-terminus (Ritter & Hall, 2009). Such events and partners regulating the orexin receptor’s life cycle, from membrane targeting to its degradation, remain to be defined. Thus, it can be anticipated that each step along the life cycle of orexin receptors can be regulated by protein partners (FIGURE 1.5).

A protein partner could be involved in:

1) The proper transport of orexin receptors from the ER to the golgi, where the receptor will subsequently be packaged in vesicles and targeted to the plasma membrane; 2) The signaling cascades initiated once the receptor is activated; 3) The de-sensitization (dissociation from G-proteins) and subsequent internalization of the receptor; 4) The re-sensitization of the receptor and thus its recycling back to the plasma membrane for another round of signaling; 5) The degradation of the receptor (sorting to lysosomes) to terminate its life cycle.

1.2.1 Interactors of orexin receptors

Known partners of OX1R which have been shown to modulate its function are: cannabinoid receptor 1 (CB1) and β-arrestins 1 and 2.

CB1 receptor is one of two receptors through which cannabinoids exert their actions and are highly expressed throughout the brain, including the lateral
hypothalamic area (Tsou et al., 1999). Their various roles include conditioned
drug seeking as well as stimulation of appetite (Mackie K., 2005). Indeed,
considering the overlap in distribution of both receptors as well as their similar
roles, it was confirmed that these two receptors co-localized at the membrane in
CHO cells (Hilairet et al., 2003). In addition, CB1 potentiated orexin-induced
ERK phosphorylation through association with OX1R, which was blocked by
addition of a CB1 antagonist (Hilairet et al., 2003). Although it was initially
believed that GPCRs function as monomers, the body of evidence for the
functional implication of GPCR homodimerization and heterodimerization has
increased in recent years, and is now known as a very common mode of GPCR
life cycle regulation (Terrillon & Bouvier, 2004). Actually, the heterodimerization
of CB1/OX1R was shown by fluorescence resonance energy transfer in HEK293
cells (Ellis et al., 2006). The CB1 localization phenotype predominated in
conditions where they heterodimerize; OX1R had altered cellular distribution in
basal conditions, from being at the plasma membrane to being constitutively
recycled (Ellis et al., 2006). In the presence of specific antagonists for each
receptor (which had no affinity for the other), the partner receptor had an altered
distribution (Ellis et al., 2006). Thus, these studies confirm the importance of
finding protein partners of orexin receptors, as in the case of CB1, they can alter
the localization of the receptor and hence its response to agonist.

In many cases, β-arrestins have been involved in GPCR de-sensitization through
physical disruption of the GPCR/heterotrimeric G-protein coupling. Subsequently,
β-arrestins may act as adaptor proteins for clathrin-dependent endocytosis, to
allow for efficient GPCR internalization (Ferguson, 2001). The sustainability of the interaction determines how quickly β-arrestin segregates from the GPCR (Oakley et al., 1999). β-arrestin 1 and 2 were first shown to be involved in OX1R internalization by observing the co-internalization of β-arrestin-GFP fusions with OX-A when it was added in CHO cells stably expressing the receptor (Evans et al., 2001). The specific site of interaction between the two proteins was further explored by mutating a series of aliphatic hydroxy a.a. at the extreme carboxy end of OX1R (Milasta et al., 2005); as it has been shown for other GPCRs (e.g. thyrotropin-releasing hormone receptor) that the C-terminus is required for β-arrestin association and internalization (Groarke et al., 2001). Specifically, a cluster of 3 threonines and 1 serine at the carboxy end (a.a. 418 to 422) of the human OX1R is important for sustained interactions with β-arrestin-2 in HEK293 cells (Milasta et al., 2005). Surprisingly, in HEK293 cells, the internalization of OX1R was maintained despite the disrupted interaction with β-arrestin-2, whereas in β-arrestin 1/2 KO mouse embryonic fibroblasts, the internalization of this mutated OX1R was disrupted and thus β-arrestin-dependent (Milasta et al., 2005). In turn, the weakened interaction between β-arrestin-2 and the point mutated-OX1R caused a less maintained activation of the ERK pathway (see section 1.1.5, above) (Milasta et al., 2005).

### 1.2.2 Novel protein partners of orexin receptors

In order to find potential protein partners whose interaction with orexin receptors could regulate their function and thus have implications in any of the
physiological roles of orexins mentioned above, previous lab members performed a yeast two-hybrid screen. This screen was done using a mouse brain cDNA library (clones fused to GAL4 activating domain (AD)) with the C-terminus of mouse OX1R as bait (fused to the GAL4 DNA-binding domain (DBD)). The C-terminus (a.a. 361 to 416) was precisely chosen as this assay requires soluble proteins. Moreover, the C-terminus of GPCRs is most often the site of regulation by protein partners. Successful transformation and subsequent interaction of these proteins in yeast allows the transcription of reporter genes (*HIS3* or *lacZ*) due to the AD and DBD of the GAL4 protein being within close proximity to one another, and able to bind DNA and initiate transcription. Among the 14 novel putative partners isolated, Dynlt3 (previously called rp3), one of the two members of the Dynlt family of light chains, was chosen as the major focus. Interestingly, it has been shown that the other member of the Dynlt family of light chains, Dynlt1 (Dynlt3’s close homolog, previously called tctex1), can interact and regulate the function of other GPCRs (Sugai et al., 2003; Tai et al., 1999). So, its interaction with OX1R was studied and since there exists even less information on OX2R, interaction with this receptor was pursued as well (FIGURE 1.6).

1.2.3 *Dynein light chains*

Dynein is a multi-subunit motor protein (others include the kinesins and myosins). More specifically, cytoplasmic dynein 1 is a minus-end directed microtubule motor protein, which walks along microtubules primarily in the retrograde fashion (Paschal et al., 1987) (FIGURE 1.7). Its roles include: axonal transport, cell
migration, transport of organelles and mitosis. This large protein is composed of 2 heavy chains (HC; ~530 kDa), 2 intermediate chains (IC; ~70–80 kDa), 4 light intermediate chains (LIC; ~50–60 kDa) as well as a variable number of light chains (LC; ~8–22 kDa) (FIGURE 1.7).

The C-terminal region of the HCs contains the ATPase and motor activities, which allow movement along microtubules. While the LICs and ICs directly associate with the N-terminal region of the HCs, the LCs (which include the Dynlt, Dynll and Dynlrb families) do not and achieve binding to the dynein complex through the other subunits (King, 2000). The organization of these subunits allows for cargo-binding.

There are several ways in which dynein can achieve cargo transport: indirectly through dynactin, which binds the cargo and then links it to dynein through the IC subunit, or through direct dynein subunit-cargo interactions. Dynactin, another multi-subunit protein, is required for many dynein functions (Schroer, 2004) where the p150<sup>glued</sup> dynactin subunit binds to the IC and can regulate IC-cargo interactions (Vaughan & Vallée, 1995; Vaughan et al., 2001). Of interest, although few IC or LIC have been shown to bridge cargo (through direct binding) to the dynein complex for transport, the LCs have many known binding partners (Williams et al., 2007). Specifically, Dynlt1, but not its close homolog Dynlt3, has been shown to interact with receptors, viral proteins and ion channels (Vallée et al., 2003). The direct binding of Dynlt1 to its partner occurs through very precise residues in the latter protein: a proximal consensus motif: R/K-R/K-X-X-R/K and a distal motif needed for effective Dynlt1 binding: V-S-K/H-T/S-X-V/T-
T/S-N/Q-V (Mok et al., 2001). Interestingly, Dynl1’s role in bridging cargo to the dynein motor complex was shown with two GPCRs: Rhodopsin and parathyroid hormone receptor (PTHR). Dynl1 was shown to bind directly to the C-terminal tail of the GPCR to either regulate apical inner segment delivery (intracellular membrane targeting) of post-golgi vesicles containing Rhodopsin (Tai et al., 1999) or suggested to play a role in PTHR internalization following agonist binding (Sugai et al., 2003).

Interestingly, Dynl1/3 are ubiquitously expressed proteins (DiBella et al., 2001), whereas the IC isoforms are tissue and cell-type specific (Myers et al., 2007). Therefore, dynein achieves its specificity for cargo through the different interactions that can occur between its subunits; the LCs, ICs and LICs, which is partially dependent on the tissue/cell (Lo et al., 2007).

In addition to its dynein-dependent function, Dynl1 can also be found in a soluble form and thus not associated with the dynein complex. Its dynein-independent roles include: activator of G-protein signaling by binding to the Gβγ subunit (Takesono et al., 1999), regulator of neurite outgrowth by modulating actin dynamics and Rac1 activity (Chuang et al., 2005; Sachdev et al., 2007) and functional repressor of LFC (a Rho specific exchange factor) (Meiri et al., 2009).

1.2.4 Characterization of orexin receptor/dynein light chain interactions

After the initial yeast two-hybrid screen, other yeast two-hybrid assays were done (by Dr. David Duguay) to verify the interaction between both orexin receptors and both Dynl family members. Among the four interactions tested, surprisingly, the
strongest was that of OX1R C-terminal domain (CTD) and Dynlt1; even stronger than the initial interaction identified between OX1R CTD and Dynlt3 (FIGURE 1.6). In addition, Dynlt1 did weakly interact with OX2R CTD, but no association was detected between Dynlt3 and OX2R CTD (FIGURE 1.6).

Subsequent yeast two-hybrid assays (also done by Dr. David Duguay) were used to verify which residues in the dynein light chains were important for orexin receptor binding. Thus, different N-terminal and C-terminal deletion mutants of Dynlt1 or Dynlt3 were fused to the GAL4 AD and used in the assay with the orexin receptors fused to the GAL4 DBD. It was concluded that the carboxy-terminal region of Dynlt1 is important for the interaction with OX1R CTD as all C-terminal deletion mutants abolished the interaction (data not shown). For Dynlt3, the amino-terminus may be inhibitory to its association with OX1R CTD as its removal strongly increased the strength of the interaction and although not as striking, the same phenomena was seen with the Dynlt1 N-terminal deletion mutant and OX2R CTD (data not shown).

Next, the interaction was confirmed using full length OX1R, in a mammalian system (HEK293 cells), by means of co-immunoprecipitation (co-IP) experiments with transfected Dynlt1 and OX1R constructs (done by Dr. David Duguay, FIGURE 1.8). Indeed, these results corroborate that the C-terminal domain of OX1R is necessary for interaction with Dynlt1 (FIGURE 1.8). It was also found that the N-terminus and C-terminus of Dynlt1 are necessary for a sustained interaction with OX1R following stimulation of cells with OX-A (data not shown).
As a first step towards identifying the functional implications of the orexin receptor-Dynlt1/3 interactions, our lab (done by Dr. David Duguay) studied orexin-induced signaling. ERK proteins are rapidly phosphorylated upon OX1R activation, which is clearly dependent on the carboxy-terminal domain as its deletion abolishes this signaling event (data not shown). In the presence of Dynlt1 (over-expression in HEK293 cells), a less sustained ERK response is observed, whereas when *Dynlt1* is down-regulated (using siRNA), the ERK proteins are phosphorylated for a longer period of time (**FIGURE 1.9**). Thus, Dynlt1 affects a response elicited by OX-A and mediated by OX1R, that is, activation of the ERK pathway.
FIGURE 1.5 POSSIBLE STEPS IN OREXIN RECEPTOR LIFE CYCLE REGULATED BY PROTEIN PARTNERS

Schematic model of the regulation of orexin receptors, illustrating the different steps that could be affected by protein partners: 1) Processing and transport through the secretory pathway and targeting to the plasma membrane; 2) Regulation of downstream signaling pathways (upper insert); 3) Ligand-induced internalization; 4) Recycling back to the plasma membrane; 5) Degradation. OXR, orexin receptor; OX-A, orexin A; ER, endoplasmic reticulum; PLC, phospholipase C; PKC, protein kinase C; AC, adenylate cyclase; Y, Tyr358 in OX1R; IP3, inositol triphosphate; DAG, diacylglycerol; PIP2, phosphatidylinositol bisphosphate.
FIGURE 1.6 IDENTIFICATION OF THE DYNEIN LIGHT CHAINS AS NOVEL INTERACTORS OF OREXIN RECEPTORS

Yeast two-hybrid assays showing that Dynlt1 and 3 interact with OX1R CTD but only Dynlt1 interacts with OX2R CTD as per ONPG liquid β-galactosidase assays in Y187 yeast strain. DBD, GAL4 DNA-binding domain; AD, GAL4 activating domain; OX1R CTD, C-terminal domain of orexin 1 receptor; OX2R CTD, C-terminal domain of orexin 2 receptor; Dynlt, dynein light chain tctex type 1. *: p<0.05, ***: p<0.001 when compared to control (DBD/AD-Dynlt1 or BDB/AD-Dynlt3).5

5 Data collected by Dr. David Duguay.
Cytoplasmic dynein is a 1.2 MDa multisubunit protein complex composed of two copies each of six polypeptides. The C-terminal regions of two identical heavy chains contain the motor domains, microtubule-binding sites, and the ATP-binding and hydrolysis sites. The heavy chains contain binding sites for the light intermediate chain and intermediate chain at their N-terminus. Cytoplasmic dynein contains three distinct families of light chains—Tctex1 (Dynlt1/3), Roadblock (Dynrb1/2) and LC8 (Dynll1/2). The dynein cargo-binding domain is composed of the intermediate chains, light intermediate chains and light chains\(^6\).

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FIGURE 1.8 INTERACTION OF OREXIN 1 RECEPTOR AND DYNLT1 IN MAMMALIAN CELLS

HEK293 cells were transfected with the indicated constructs (V5-OX1R WT, V5 tagged full length OX1R or V5-OX1R Δ364-416, V5 tagged OX1R lacking the C-terminus and Myc tagged Dynlt1) and whole-cell lysates were subjected to immunoprecipitation. Anti-Myc western blots show that removing the CTD of OX1R abolishes the interaction of OX1R with Dynlt17.

7 Data collected by Dr. David Duguay.
FIGURE 1.9
MODULATION
OF AN OREXIN
1 RECEPTOR-
MEDIATED
SIGNALLING
EVENT
Anti-phospho-
ERK1/2 and anti-
ERK1/2 western
blots of protein
extracts from
HEK293 cells
transfected with
V5-tagged OX1R
analyzed by SDS-
PAGE. A) Co-
expression of
Dynlt1 led to a
less sustained
ERK1/2
activation in
response to OX-A
100 nM. B)
Down-regulation
of Dynlt1 using
10nM siRNA
(88% down-regulation, unaffected by control siRNA) led to a sustained activation
of the ERK1/2 pathway in response to OX-A 100 nM. ***: p<0.001 vs data
without Dynlt1 transfected8.

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8 Data collected by Dr. David Duguay.
1.3 RATIONALE

There is huge interest in the orexin system and in creating therapies for sleep-, appetite- and addiction-related disorders. Thus, understanding how the orexin receptors are regulated is imperative. In view of the fact that there is lack of information on the regulation of orexin receptors, our lab has identified novel protein partners, the dynein light chains Dynlt1/3, using the yeast two-hybrid approach.

Exploring this novel interaction was promising since Dynlt1 has already been shown to directly associate with other GPCR CTDs and regulate their function (Sugai et al., 2003; Tai et al., 1999). Yet, Dynlt3 was unique in that it has not been previously associated with GPCRs. Moreover, towards identifying the functional implication of the interaction as well as understanding how Dynlt1 could be affecting the orexin-induced ERK pathway activation (see FIGURE 1.9 above), further studies were needed.

The hypothesis of this project is that the dynein light chains act to regulate the orexin receptors at the intracellular level. To test this hypothesis, we have addressed the following objectives:

**AIM 1: Characterize the interaction between the dynein light chains and orexin receptors.**

The specificity of the interaction between Dynlt1 and OX1R was partly established by identification of the C-terminal end of Dynlt1 as being crucial for
the interaction (see section 1.2.4, above). To ensure specificity of both proteins towards each other, as well as to obtain a better understanding of which regions of the orexin receptors were involved in the interaction with the dynein light chains, C-terminal domain mutants of the receptors were created. Subsequently, in vitro pulldown assays were used to verify if other proteins were mediating the interaction between Dynlt1 and OX1R or if the interaction was direct.

**AIM 2: Study the effect of dynein light chains on orexin 1 receptor internalization.**

It was previously shown in our lab that Dynlt1 modulates the orexin-induced ERK pathway activation through OX1R (see section 1.2.4, above). Thus, surface ELISAs were used to identify if this was a consequence of upstream changes in receptor fate. By studying the amount of orexin receptor at the membrane, alterations (induced by Dynlt1) in receptor internalization or targeting of the receptor to the membrane following its synthesis can be detected.

**AIM 3: Investigate the effect of Dynlt1 on orexin 1 receptor intracellular localization.**

Again, changes in receptor location could subsequently affect orexin-induced signaling events. Early endosomes are the first compartment in which OX1R is located once internalized into clathrin-coated pits, after stimulation with OX-A. Thus, to study if the role of Dynlt1 was on the localization of OX1R, single cells were visualized after stimulation.
2. EXPERIMENTAL PROCEDURES

2.1 MATERIALS

For cell culture and transfections: Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), Lipofectamine 2000, OPTIMEM I (31985070) and Geneticin (G418, # 11811-023) were purchased from Invitrogen; L-glutamine (D-2902) and poly-L-lysine (P-9155) were obtained from Sigma; Orexin A (OX-A) peptide (human, bovine, rat, mouse) was obtained from NeoMPS (SC1337).

For RNA interference: siRNA was ordered from Ambion; TRIzol® was ordered from Invitrogen; high capacity cDNA reverse transcriptase kit (4374966), TaqMan Universal PCR Master Mix (4369510), human Dynein Light Chain 1 (Hs00831821_s1) and 3 (Hs00359622_m1) qPCR probe and GAPDH qPCR probe were all from Applied Biosystems.

For yeast two-hybrid assays: AH109 yeast strain and Y187 yeast strain were obtained from Clontech; 2-Nitrophenol β-D-galactopyranoside (ONPG) (N-1127), 3-Amino-1,2,4-Triazole (3-AT) (A-8056) and all yeast amino acids were obtained from Sigma; yeast nitrogen base without amino acids (SD medium) (CADF0919-15) was purchased from VWR; yeast extract (BP1422500) was ordered from Fisher.

For in vitro pulldown assays: LB broth (L-3022), LB agar (L-2897), the horseradish peroxidase-coupled goat anti-mouse Ab (A9044) and the monoclonal anti-HA Ab (H9658) were purchased from Sigma; glutathione sepharose 4B (17-
0756-01) was obtained from GE Healthcare; Isopropyl-β-D-1-thio-galactoside (IPTG) (BP1755-1) was ordered from Fisher; T7 Quick Coupled Transcription/Translation system (#L1170) was purchased from Promega.

For ELISAs: rabbit anti-V5 antibody (AB9116) was obtained from Abcam; goat anti-rabbit horse radish peroxidase (A9169) was purchased from Sigma; O-phenylenediamine (OPD) was purchased from Pierce; 37% formaldehyde (BP531-25) was obtained from Fisher.

For immunocytochemistry: paraformaldehyde (P6148) and anti-FLAG M2 monoclonal Ab (F3165) was purchased from Sigma; triton X-100 (BP151) and bovine serum albumin (BSA) (BP1600-100) were obtained from Fisher; marker for early endosomes (anti-EEA1) (610456) was ordered from BD Biosciences; donkey anti-mouse ALEXA 568 (A100037) was obtained from Invitrogen; Vectashield was purchased from Vector Labs (H1000).

2.2 MOLECULAR CONSTRUCTS

2.2.1 OX1R constructs

pGBKT7 constructs: the pGBKT7 plasmid expresses proteins fused to the DBD of the yeast transcription factor GAL4 (it also contains the T7 promoter and a c-Myc epitope tag) (Clontech). The coding sequence for mouse OX1R CTD (amino acids 361 to 416) was amplified by PCR (using mouse brain cDNA as a template) and inserted in-frame with the DBD of the GAL4 protein, in pGBKT7 plasmid.
All pGBKT7-OX1R CTD deletion mutant constructs (Δ 10 CTD, mouse OX1R with amino acids 407-416 deleted; Δ 20 CTD, mouse OX1R with amino acids 397-416 deleted; Δ 30 CTD, mouse OX1R with amino acids 387-416 deleted) were subcloned from the corresponding pGEX4T1-OX1R deletion mutant CTD constructs by digestion with EcoRI (5’) and XhoI (3’). Similarly, OX1R T409, 412A CTD mutant construct (mouse OX1R with 2 Threonines mutated to 2 Alanines at residues 409 and 412) was subcloned from the corresponding pGEX4T1-OX1R point mutant CTD construct by digestion with EcoRI (5’) and XhoI (3’). The various OX1R CTD fragments were then inserted between EcoRI and Sall sites of pGBKT7 plasmid.

GST fusions: for the GST-OX1R CTD fusion, mouse OX1R CTD fragment was subcloned from the pGBKT7-OX1R CTD construct by digestion with EcoRI (5’) and XhoI (3’) and inserted, in frame with glutathione S-transferase, in the multiple cloning site of pGEX4T1 (GE Healthcare). For the GST-OX1R 30 a.a. CTD construct (amino acids 387 to 416), mouse OX1R CTD was used as a template with oligonucleotides created to amplify only the last 30 a.a. of the CTD and then inserted between EcoRI and XhoI sites, in frame with glutathione S-transferase, in pGEX4T1.

pSG5 constructs: pSG5-V5-His6 plasmid is a SV40 promoter plasmid (Stratagene) which has been engineered to contain a V5 and 6 HIS tag sequences. pSG5-V5-His6-mOX1R construct was created by PCR amplification with oligonucleotides targeted to the coding sequence of mouse OX1R (NM_198959) and inserted between EcoRI and XhoI sites of pSG5-V5-His6.
pEGFP fusion: pEGFP-C1-OX1R was created by PCR amplification of the coding sequence of mouse OX1R, using a reverse oligonucleotide designed to remove the STOP codon as well as the last base pair from mouse OX1R, to create a protein in frame with EGFP. PEGFP-N1-OX1R fusion was then prepared by subcloning from pEGFP-C1-OX1R by digestion with EcoRI (5’) and XhoI (3’) and the insert was cloned before EGFP in pEGFP-N1 plasmid (Clontech).

2.2.2 Dynlt1 constructs

pGAD construct: pGADT7-HA-Dynlt1 construct was created by PCR amplification using oligonucleotides targeted to the coding sequence of mouse dynein light chain Tctex type 1 (NM_009342). The digested insert was then cloned into pGADT7-HA (Clontech) between EcoRI and XhoI sites, in frame with the AD of the yeast transcription factor GAL4.

pCS2+MTK construct: pCS2+MTK plasmid is a CMV promoter plasmid derived from pCS2 with 5 MYC tag sequences at the 5’ end of the multiple cloning site. pCS2+MTK-Dynlt1 was constructed by subcloning from pGADT7-HA-Dynlt1 construct and then insertion of Dynlt1 between EcoRI and XhoI sites in pCS2+MTK plasmid.

pCS2-FLAG construct: The pCS2+MTK-Dynlt1 construct was used to create pCS2-FLAG-Dynlt1. A FLAG tag was generated using complementary oligonucleotides containing the FLAG sequence (ATG GAC TAC AAA GAC GAT GAC GAT AAA) and BamHI/EcoRI cohesive ends and annealed by mixing equimolar concentrations of each oligo and incubating at 90°C for 5 min,
following slow cooling to room temperature. The MYC tag was excised from pCS2+MTK-Dynlt1 using BamHI and EcoRI, and the newly synthesized FLAG tag was inserted.

2.2.3 Other constructs

OX2R constructs: the coding sequence of mouse OX2R CTD (amino acids 367 to 460) was amplified by PCR and inserted, in-frame with the DBD of the GAL4 protein, in pGBK7 plasmid. Deletion mutants of OX2R CTD (Δ extra a.a., mouse OX2R with amino acids 433-460 deleted; Δ extra + 10 a.a., mouse OX2R with amino acids 423-460 deleted; Δ extra + 20 a.a., mouse OX2R with amino acids 413-460 deleted; Δ extra + 30 a.a., mouse OX2R with amino acids 403-460 deleted) were created by inserting a stop codon at the desired position. Subsequently, these fragments were inserted between EcoRI and SalI sites of pGBK7 plasmid, in frame with the DBD of the GAL4 protein.

Dynlt3 constructs: the pGAD-HA-Dynlt3 construct was created by PCR amplification using oligonucleotides targeted to the coding sequence of mouse dynein light chain Tctex type 3 (NM_025975). The digested fragment was inserted, in frame with the AD of the GAL4 protein, into pGADT7-HA plasmid between EcoRI and XhoI sites.

Dyncti2 construct: pGEX4T1-Dyncti2 fusion was constructed using mouse testis cDNA as a template and specific oligonucleotides targeted to a region of the coding sequence of mouse cytoplasmic dynein 1 intermediate chain 2 (NM_010064) where Dynlt1 is known to bind (Williams et al., 2006). The
Dyncl1i2 fragment was then inserted, in frame with glutathione S-transferase, between EcoRI and XhoI sites in pGEX4T1 plasmid.

2.3 CELL CULTURE

2.3.1 Transfections

Human Embryonic Kidney (HEK) 293 cells were maintained in DMEM, supplemented with 10% FBS and 1% L-glutamine at 37°C with 5% CO₂. Transient transfections in 24-well culture dishes were done at 70%-80% confluency using Lipofectamine 2000 reagent for 5 h according to manufacturer recommendations.

Generation of stable pEGFP-N1-OX1R HEK293 cells was done as follows. HEK293 cells were maintained in 6-well plates and transfected at subconfluency for 5 h with a mixture of pEGFP-N1-OX1R and a plasmid encoding neomycin resistance using Lipofectamine 2000 as per manufacturer’s instructions. After 48 h, the cells were diluted 1:10 using medium containing 0.8 mg/mL G418 to initiate selection of stably transfected cells. Resistant clones were screened using a LEICA fluorescent microscope to monitor GFP fluorescence and stability of transfection was ensured by the presence of 0.4 mg/mL G418.
2.3.2 RNA interference

The oligonucleotides targeting the human Dynein Light Chain TCTEX type 1 (Dynlt1, ref # 139627) and Dynein Light Chain TCTEX type 3 (Dynlt3, ref # 17984) genes were purchased from Ambion. The negative control SiRNA #1 (cat # AM4611) was also from Ambion. HEK293 cells at 60-70% confluency were transfected, 24 h after seeding, with 0.25 µg of empty pSG5 or pSG5-V5-OX1R in combination with 10 nM (or 20 nM) siRNA targeted towards Dynlt1 and/or Dynlt3 using Lipofectamine 2000 reagent according to manufacturer instructions. To ensure successful inhibition of targeted RNAs, RNA was extracted from cells lysed with TRIzol® and then quantified and verified for purity by spectrophotometer (Biorad) and agarose gel. RNA was diluted to 50 ng/µL to use in the reverse transcriptase PCR to generate cDNA. The cDNA was then diluted to 5 ng/µL to use in a Quantitative PCR (TaqMan Universal PCR mix) with probes against Dynlt1, Dynlt3 and GAPDH (endogenous control). The efficiency of reducing Dynlt1 RNA levels was 80% for experiments with only Dynlt1 siRNA, whereas it was 75% when used in combination with Dynlt3 siRNA. The efficiency of reducing Dynlt3 RNA levels, using a siRNA targeted to Dynlt3, was 80%.
2.4 ASSAYS

2.4.1 Yeast two-hybrid assay

Screening of proteins interacting with the C-terminal domain of OX1R was performed in two strains of *Saccharomyces cerevisiae*. AH109 (MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4Δ, gal80Δ, LYS2:: GAL1UAS-GAL1TATA-HIS3, GAL2UAS-GAL2TATA-ADE2, URA3:: MEL1UAS-MEL1TATA-lacZ, MEL1) was used for HIS3 reporter activity as assessed by histidine selection test in yeast two-hybrid assays. The strongest interactions should have the highest levels of HIS3. Briefly, yeast were co-transformed with either wild type or C-terminal mutants of OX1R fused to the GAL4 DBD in pGBKT7 expression vector (contains *TRP1*) and Dynl1 fused to the GAL4 AD in pGADT7 expression vector (contains *LEU2*) using a lithium acetate-mediated transformation according to Clontech yeast two-hybrid protocols. Successful co-transformations were determined by growth on SD plates lacking L-tryptophan (W) and L-leucine (L) streaked with yeast cells re-suspended in sterile water. For the histidine selection test, simultaneous streaks on SD plates lacking L, W, and histidine (H), demonstrated protein-protein interactions. To eliminate false positives arising from “leaky” HIS3 expression and to evaluate the strength of interactions, varying amounts of 3-amino-1,2,4-triazole (3-AT; 1-10 mM), a competitive inhibitor of the product of the HIS3 gene, was added to positive interactions. Thus only the strongest interactions remained at the highest 3-AT concentrations. The plates were incubated at 30°C for 5 days and then observed for growth.
Saccharomyces cerevisiae Y187 (MATα, ura3-52, his3-200, ade2-101, trp1-901, leu2-3, 112, gal4Δ, met–, gal80Δ, MEL1, URA3::GAL1UAS-GAL1TATA-lacZ) was used for lacZ reporter activity as assessed by β-galactosidase in yeast two-hybrid assays as per Clontech manual. Yeast cells were co-transformed as described above. For the β-gal assay, cells were lysed using a freeze/thaw cycle in liquid nitrogen and then incubated with the substrate of β-galactosidase, ONPG, at 30°C for up to 24 h. Reactions were stopped with 1 M sodium carbonate when a yellow color appeared, centrifuged and read at 420 nM with a spectrophotometer (Biorad). β-galactosidase Miller units were calculated as follows: 1000 x (OD420)/ (t x V x OD600), where t=minutes of incubation, with ONPG, V= 0.1mL x concentration factor of cells, OD600= measure of culture cell density. Positive controls included the known p53/large T-antigen interaction. The pGBKT7- and pGADT7- encoded fusions between the GAL4 DNA BD and AD and murine p53 and large T-antigen, respectively. Negative controls included empty vectors, lacking proteins fused to either of the GAL4 domains.

2.4.2 In vitro pulldown assay

GST fusions: All GST fusion proteins (GST, GST-dync1i2, GST-OX1R CTD, GST-OX1R 30 a.a., CTD) were expressed in Escherichia coli and prepared as previously described (CSH protocols, 2007). Single colonies were grown overnight in LB, with proper antibiotic selection, at 37°C at 250rpm. Cultures were then inoculated into a larger flask and grown (3 h) until mid-log phase (OD600 0.5-1.0) at 37°C (an aliquot was kept before induction). Protein expression
was induced by addition of 0.4 mM IPTG to the cultures for 3 h at 30°C with shaking (an aliquot was kept before solubilization). Cells were collected by centrifugation and lysed in ice cold GST pulldown lysis buffer (1% Nonidet P-40, 20 mM Tris-Cl, .5 mM EDTA, 20%, glycerol, .2 M KCl, 1 M DTT and 100 mM PMSF) using a sonicator on ice, in 15 second bursts alternating with 30 seconds of rest for 3 cycles. After centrifugation, the supernatant of the GST-fusion was kept and purified on glutathione resin. Subsequent SDS-PAGE and Coomassie blue stain of all aliquots was done to verify production and solubility of GST-fusions as well as the affinity of the GST-fusions to the column.

In vitro transcription/translation: pGAD-HA-Dynlt1 was synthesized via a T₇ Quick Coupled Transcription/Translation system (Promega #L1170) as defined in the manual using 0.5 µg plasmid DNA with appropriate reagents at 30°C for 75 min. An aliquot (1:10 dilution) of the product was subjected to SDS-PAGE and subsequent anti-HA (1:5000) immunoblot to ensure in vitro synthesis.

Pulldown assay: 200 µL of either GST-fusions was mixed with 20 µL of HA-Dynlt1 and incubated at 4°C for 2 h with end-over-end mixing (+ 500 µL of GST pulldown lysis buffer to allow mixing). Glutathione-sepharose beads (kept in 25% ethanol) were washed 3X with PBS 1X and re-suspended in GST pulldown lysis buffer to create a 50/50 bead slurry. Then, 40 µL of slurry was added to the incubating proteins for 1 h at 4°C. The samples were centrifuged at 5000 rpm for 1 min and the beads washed 4X with ice-cold lysis buffer (same as above but with .5 M KCl). Samples were boiled off the beads by adding an equal volume (20 µL) of SDS gel-loading buffer to the beads and incubating at 100°C for 5 min.
Analysis was achieved through SDS-PAGE and subsequent anti-HA (1:5000) immunoblot for the presence of HA-Dynlt1. Negative controls included all GST-fusions with the empty vector containing Dynlt1 (pGADT7), glutathione beads alone and GST + glutathione beads. The positive control was that of GST-Dync1i2 which is a known to interact with Dynlt1 (Williams et al., 2006).

2.4.3 Cell surface ELISA

ELISAs were performed as previously described (Roy et al., 2007). Briefly, HEK293 cells were seeded into poly-L-lysine (0.1 mg/mL) coated 24-well plates at 120,000 cells/well and transfected with 0.25 µg of empty pSG5-V5 or pSG5-V5-OX1R vectors with 0.125 µg empty pCS2-Myc (control) or pCS2-Myc-Dynlt1 vectors. To maintain 0.5 µg total DNA (to ensure similar transcription and translation activity) the empty pSG5-V5 vector was used. After 48 hrs, cells at confluency were stimulated with 100 nM OX-A peptide from 0 to 30 min at 37°C to allow endocytosis and then placed on ice to stop all activity (0 min indicates cells without OX-A). On ice, cells were washed twice with PBS 1X and fixed for 15 min in 2% formaldehyde in PBS 1X. Subsequently, un-permeabilized cells were blocked with 10% FBS in PBS 1X for 30 min at room temperature, and then incubated with rabbit anti-V5 (1:1000) for 1 h at room temperature to allow detection of the extracellular V5 epitope linked to OX1R. A goat anti-rabbit HRP-linked antibody (1:1000) was added to the cells for 1 h at room temperature. These complexes were incubated with 200 µL OPD (1 mg/mL) in citrate phosphate buffer (0.5 M each), pH 5.0 for 9 min to allow colorimetric assessment.
of the amount of receptor at the membrane. The reactions were stopped by addition of an equal volume (200 µL) of 4N sulphuric acid and then 200 µL was transferred to a 96-well plate for absorbance reading at 490 nM by a benchplate microplate reader system (Biotek Instruments, 2005). Negative controls included empty wells, wells without OX1R (transfected with V5-tagged pSG5) and transfected HEK293 cells incubated without primary antibody. Corrected OD values refer to background-subtracted data at 490 nM (background is the value for HEK293 cells transfected with empty V5-tagged pSG5 vector). For siRNA experiments, additional wells were transfected as above, but instead of cell fixation, RNA was extracted with TRIzol® reagent and stored at -80°C until processing.

2.5 IMMUNOCYTOCHEMISTRY

2.5.1 Immunofluorescence

Cells were imaged using a Zeiss Axio Observer.Z1 inverted microscope for transmitted light and epifluorescence with ApoTome attachment (Carl Zeiss, Inc.). Images were taken with a 40x objective lens with exposure times of 80-100 ms in the red channel (ALEXA568), 500-700 ms in the green channel (GFP) and variable in the blue channel (DAPI). Stable OX1R-GFP HEK293 cells were seeded at 150 000 cells/well in a 24-well culture dish with poly-L-lysine (0.1 mg/mL) coated glass coverslips the day before transfection. Either Flag-tagged Dynlt1 or empty vector was transfected as described above. 48 h after
transfection, cells were stimulated with 100 nM OX-A as above and then fixed for 10 min at room temperature with 4% paraformaldehyde. Cells were permeabilized using 0.5% Triton-X for 30 min, then blocked with 3% BSA in PBS 1X for 30 min, followed by incubation with anti-EEA1 (1:500) for 1 h at room temperature. Finally, ALEXA 568 (1:200) was added for 1 h at room temperature, followed by a 2 min DAPI staining (1:20 000) and coverslips mounted on slides using Vectashield. Negative controls included unlabelled cells (for autofluorescence) as well as cells labelled with no primary antibody.

Additional wells were transfected as above to verify Dynlt1 transfection efficiency. Briefly, cells were fixed in 100% -20°C methanol for 6 min at room temperature, followed by 30 min blocking in 3% BSA in PBS 1X. Sequentially, anti-FLAG Ab (1:400) and ALEXA 568 (1:400) were added for 1 h at room temperature, followed by DAPI staining. Coverslips were mounted using Vectashield. Negative controls included cells transfected with empty vector containing the Myc tag, as well as labelling without primary antibody of cells transfected with pCS2-FLAG-Dynlt1.

2.5.2 Co-localization analysis

Images taken in black and white were opened in Openlab Software (Perkin Elmer) and assigned their respective colors (green for OX1R and red for early endosomes). Colored images were then contrast-enhanced and randomly cropped in Openlab Software to include 1-3 cells (with specific dimensions of $\Delta_x 450000 \times \Delta_y 450000$ to ensure uniformity). Images corresponding to OX1R-GFP (green) and
endosomes (red) were subsequently co-localized, using the whole image as a region of interest according to guidelines in the Openlab co-localization module (TN348). Thresholds were established in each experiment by using basal conditions (0 min) as the reference for no co-localization. Mander’s co-localization co-efficient was generated from the scatterplots of green (OX1R-GFP) and red (early endosome) pixel fluorescence and used to assess the degree of co-localizing green and red pixels relative to the total number of green pixels over threshold (Mandars et al., 1993).

\[
\text{Mander’s co-localization coefficient (OX1R):} \\
\frac{\text{co-localized green and red pixels}}{\text{total number of green pixels over threshold}}
\]

This allowed an estimation of the amount of OX1R located in early endosomes.

2.6 STATISTICAL ANALYSIS

Data from β-galactosidase assays (yeast two-hybrid) were analyzed by one-way ANOVA followed by Bonferroni post tests, using Graphpad software. The effect of over-expressing or down-regulating Dynlt1 (and Dynlt3) was assessed by two-way ANOVA followed by Tukey’s post tests where applicable, using Datasim software. Likewise, unpaired Student’s t-tests was done when comparing the effect of Dynlt1 at one time point. p < 0.05 (*) was considered significant. Data are expressed as means ± SEM.
3. RESULTS

3.1 AIM 1: CHARACTERIZE THE INTERACTION BETWEEN THE DYNEIN LIGHT CHAINS AND OREXIN RECEPTORS.

To obtain a better understanding of the novel interaction identified between the dynein light chains and orexin receptors, specific residues in the CTD of orexin receptors were investigated. Since a Dynlt1 sequence motif has been identified for several Dynlt1 binding partners, which includes a proximal and distal part, its presence in orexin receptors was verified and confirmed (FIGURE 3.1). Interestingly, although the third intracellular loop and the C-terminal tail of OX1R and OX2R show the weakest amino acid identity compared to other domains (several inserted residues), the amino acids (proximal and distal motifs) important for Dynlt1 binding are present in both.

3.1.1 Orexin 2 receptor C-terminal mutants alter the interaction with Dynlt1

Since we observed a weak interaction between OX2R CTD and Dynlt1, further yeast-two hybrid assays were used to verify which residues in OX2R CTD were important. OX2R CTD contains 27 extra a.a. (“ex” in FIGURE 3.2A) compared to OX1R CTD, and thus these residues (A434 to V460) were deleted (FIGURE 3.1). In addition, 30 a.a. were further removed, 10 a.a. at a time from the extreme CTD (FIGURE 3.2A). All mouse OX2R CTD constructs were fused to the GAL4 DBD in the pGBK7 expression plasmid and Dynlt1 fused to the GAL4 AD in the pGAD7 expression plasmid.
OX2R CTD and Dynlt1 interact weakly, but interestingly, removal of the extra a.a. present in OX2R CTD (that are not there in OX1R CTD) strengthened the interaction with Dynlt1. This was assessed using the histidine selection test (HIS3 reporter, FIGURE 3.3, lower panel) and confirmed with the β-galactosidase assay (lacZ reporter, FIGURE 3.3, upper panel). Thus, the extra a.a. in OX2R CTD may be inhibitory to the interaction, perhaps due to hindrance and the selectivity of Dynlt1 towards OX1R likely lies in the fact that it does not contain these extra residues. Moreover, further removal of 10 a.a. (V423 to V460) in OX2R CTD, which included the putative Dynlt1-binding residues, abolished the effect (strengthened interaction) seen upon removal of only those residues specific to OX2R. This indicates the importance of the putative Dynlt1-binding residues in mediating this interaction.

3.1.2 Orexin 1 receptor C-terminal mutants disrupt the interaction with the dynein light chains

In view of the strong interaction observed between OX1R CTD and Dynlt1 in the yeast two-hybrid system (FIGURE 1.6), we were interested in identifying where exactly Dynlt1 is binding to OX1R CTD. To define amino acid sequences responsible for the interaction with Dynlt1, the CTD of OX1R was divided into smaller segments (FIGURE 3.2B) and subjected again to yeast two-hybrid assays (FIGURE 3.4). In addition, point mutants were created, where threonine residues were replaced with alanine residues (T409A, T412A) (FIGURE 3.2B, bottom), in the putative Dynlt1-binding site of OX1R CTD (FIGURE 3.1). Again, mouse OX1R CTD constructs were fused to the GAL4 DBD in the pGBK7 expression vector.
plasmid and Dynlt1 fused to the GAL4 AD in the pGADT7 expression plasmid. Once co-transformed in yeast, interactions were assessed by activation of HIS3 or lacZ.

The interaction between Dynlt1 and OX1R CTD was greatly reduced when the last 10 a.a. of OX1R CTD was deleted, similar to what occurred when only the two threonines were mutated in the last 10 a.a. of the carboxy-terminal region (FIGURE 3.4). These amino acid residues are crucial for the interaction and provide strong support as the core Dynlt1-binding site on OX1R CTD. These results were more striking for the β-galactosidase assay (FIGURE 3.4, upper panel; black bars) than for the histidine selection test (FIGURE 3.4, lower panel; left). Furthermore, removal of 20 or more a.a. of OX1R CTD completely abolished the interaction with Dynlt1, implying that Dynlt1 must have a binding site between 10 and 20 a.a. as well. For Dynlt3, removing as little as 10 a.a. or mutating only 2 residues in OX1R CTD resulted in basal activity and thus complete loss of interaction (FIGURE 3.4, upper panel; white bars, lower panel; right). Therefore, an intact CTD of OX1R is needed to detect the weak interaction with Dynlt3.

3.1.3 Orexin 1 receptor C-terminal domain does not interact with Dynlt1 in an in vitro pulldown assay

Since the strongest interaction occurred between OX1R and Dynlt1 and we subsequently observed the biggest effects in mutational studies, this interaction was the focus for further investigations. To determine if the interaction between
OX1R CTD and Dynlt1 was a direct one, we used an in vitro pulldown assay. The OX1R CTD was cloned in the pGEX4T1 expression plasmid to generate a GST-OX1R CTD fusion which was expressed in *Escherichia coli*. Meanwhile, Dynlt1 was synthesized via *in vitro* transcription/translation (Promega’s T7 Quick Coupled Transcription/Translation System) from the same pGADT7-based construct described above, which encodes Dynlt1 tagged with HA epitope. The two proteins were allowed to interact in vitro and proteins bound to glutathione-sepharose beads were separated by SDS-PAGE and subsequently immunoblotted with anti-HA antibody (for Dynlt1).

The in vitro pulldown assay requires soluble GST-fusion proteins in order to function. OX1R, being a transmembrane protein, is not soluble and thus only its CTD (a cytoplasmic portion of 56 a.a.) was used. This should not hinder the interaction, as we have seen from yeast two-hybrid that this is sufficient to see an interaction with Dynlt1. Even with this small portion of the receptor, solubility issues (i.e. fusion protein packaged in inclusion bodies) were encountered when comparing OX1R CTD GST-fusion to positive controls (GST-Dync1i2) in the assay (**FIGURE 3.5A; compare lanes with arrows**). With this, HA-Dynlt1 was detected with GST-Dync1i2 (dynein 1 intermediate chain 2; which has previously been shown to interact with Dynlt1) (Lo et al., 2007) but not with GST-OX1R CTD (**FIGURE 3.5B**).

Since no direct interaction was seen under the previous conditions and to solve the solubility issue, a GST fusion with only the last 30 a.a. of the 56 a.a. OX1R CTD was cloned in pGEX4T1. The 30 a.a. segment was chosen since by yeast two-
hybrid, the last 20 a.a. of OX1R CTD were deemed crucial for the interaction with Dynlt1. Indeed, this did increase the solubility of the GST-OX1R fusion (FIGURE 3.5C; compare lanes with arrows), but still no direct interaction was detected between GST-OX1R 30 a.a. CTD and HA-Dynlt1 (FIGURE 3.5D).

3.2 AIM 2: STUDY THE EFFECT OF DYNEIN LIGHT CHAINS ON OREXIN 1 RECEPTOR INTERNALIZATION

Agonist stimulation of OX1R leads to rapid internalization and to activation of the ERK pathway (Kukkonen et al., 2002). Previously, in our lab (done by Dr. David Duguay), we have shown that Dynt11 modulates the activation of the ERK pathway following stimulation by orexin. This suggests the implication of Dynlt1 in the internalization of OX1R, as ERK proteins are downstream of the OX1R signaling cascade. In view of this and in an attempt to identify the functional consequence of the OX1R-Dynlt1 interaction, we studied OX1R intracellular trafficking.

3.2.1 Dynlt1 has no effect on the population of OX1R at the membrane

In order to study the effect of Dynlt1 on orexin receptor cell surface expression, we used a surface Enzyme Linked ImmunoSorbent Assay (ELISA) to assess the amount of orexin receptor at the plasma membrane before and after stimulation with OX-A peptide. To realize this, HEK293 cells were transfected with a V5-tagged OX1R expression vector, with or without Dynlt1 expression vectors (protein expression confirmed by western blot; data not shown), or with Dynlt1 siRNAs (targeted to the 3’ untranslated region of Dynlt1 mRNA). Following
stimulation with OX-A, the V5 Ab was used to specifically label the population of orexin receptors at the plasma membrane (on unpermeabilized cells).

Upon over-expression of Dynlt1 with OX1R in HEK293 cells, there was no difference in the amount of OX1R at the membrane in basal conditions or following addition of 100 nM OX-A compared to control (without Dynlt1 over-expression) (FIGURE 3.6A). The greatest loss of receptor at the membrane was immediate, within 5 minutes with agonist, although internalization continued thereafter. Additionally, down-regulation of Dynlt1 RNA did not significantly change the population of OX1R at the membrane compared to control; in basal conditions or after stimulation with OX-A (FIGURE 3.6B). Once again, OX1R continued to be internalized over the course of 30 minutes with agonist. Thus, in both cases similar amounts of receptor were targeted to the membrane (as assessed at basal conditions) and internalized (once agonist is present). Furthermore, to ensure not to overlook the effect of Dynlt1 on orexin receptor endocytosis, due to saturation of the system, the same Dynlt1 over-expression studies were done with different concentrations of receptor and agonist. When decreasing the concentration of OX1R transfected and/or OX-A used, no significant effect of Dynlt1 on OX1R internalization was seen (data not shown).

### 3.2.2 The dynein light chains have no effect on the amount of OX1R at the membrane

Since both dynein light chains interact with OX1R and they also interact with each other (Lo et al., 2007), they may be involved in the similar regulation of the
receptor. To verify if Dynlt3 could be compensating for Dynlt1 when the RNA expression of the latter is reduced, the same ELISAs were performed on HEK293 cells co-transfected with pSG5-V5-OX1R and siRNAs against both Dynlt1 and Dynlt3.

The simultaneous down-regulation of both dynein light chains at the RNA level did not significantly affect basal levels of OX1R at the membrane nor OX1R endocytosis at any of the OX-A stimulation times shown (FIGURE 3.7).

3.3 AIM 3: INVESTIGATE THE EFFECT OF DYNLT1 ON OREXIN 1 RECEPTOR INTRACELLULAR LOCALIZATION

In view that one of dynein’s major roles is to move cargo towards the minus end of microtubules (towards the nucleus in most cells) (Desai & Mitchison, 1997), Dynlt1 may be the dynein subunit mediating the regulation of where orexin receptors go once internalized from the membrane.

3.3.1 Dynlt1 is involved in OX1R intracellular trafficking

To define at which level Dynlt1 has an effect on orexin receptor intracellular localization, we created a C-terminal GFP fusion of OX1R. HEK293 cells stably expressing OX1R-GFP were generated to allow visualization of OX1R by fluorescent microscopy. As a first step, early endosomes were labelled using the marker EEA1 with a fluorescent ALEXA 568 secondary Ab, as this is one of the first compartments encountered after endocytosis (FIGURE 3.8; middle
column). Images containing several cells were quantified for the degree of co-localization between orexin receptors and endosomes, using Manders co-localization coefficient for the green channel and assessing whether this changed when adding or removing Dynlt1.

At basal conditions (t=0), OX1R was localized to the periphery (plasma membrane) as expected for a GPCR and consistent with previous work (Milasta et al., 2005) (FIGURE 3.8; upper left corner). Furthermore, minimal co-localization (overlap of OX1R and endosomes) was observed (FIGURE 3.8; merge, top row). Upon addition of agonist for up to 30 min, OX1R was internalized; revealed by punctuate staining within the cytoplasm (FIGURE 3.8; left column) and co-localized with endosomes (FIGURE 3.8; merge, right column). The maximum co-localization between OX1R and endosomes occurred after 15 min OX-A (FIGURE 3.8A; right column, FIGURE 3.8C; white bars). When Dynlt1 was over-expressed (FIGURE 3.8B) and co-localization quantified, the amount of OX1R co-localized with endosomes after 15 min OX-A was decreased (similar to levels at 5 and 30 min OX-A) (FIGURE 3.8C; black bars). These data suggest that Dynlt1 leads to a faster exit of OX1R from this compartment and thus, is involved in its intracellular trafficking.
Amino acid sequences of putative Dynlt1-binding domains of mouse orexin 1 receptor (OX1R) (accession # NP_945197), mouse orexin 2 receptor (OX2R) (accession # NP_945200) (A,B; upper) and known Dynlt1-binding domains of parathyroid hormone receptor (PTHR) (Sugai et al., 2003), Rhodopsin (Tai et al., 1999) and Dynein Intermediate Chain (DIC) (Mok et al., 2001) (A,B; lower) are aligned. Amino acids corresponding to the proximal consensus motif; R/K-R/K-X-X-R/K (A) or discernable distal motif; V-S-K/H-T/S-X-V/T-T/S-N/Q-V (B) are underlined in boldface letters. Spaces in OX2R and DIC are introduced for alignment purposes. Amino acid residues of the proximal (found in the 3rd intracellular loop) and distal motif (found in the of C-terminal domain) are present in both orexin receptors despite weak amino acid identity in these regions.

**FIGURE 3.1 AMINO ACID ALIGNMENT OF SELECTED DYNLT1-BINDING DOMAINS**
FIGURE 3.2 SCHEMATIC REPRESENTATIONS OF OREXIN RECEPTOR C-TERMINAL DOMAIN MUTANTS

Schematic diagram of wild type and deletion mutants of distinct parts of the carboxy-terminal domain of OX2R (A) and OX1R (B; upper). Threonine residues in the putative Dynlt1-binding domain of OX1R CTD were mutated to alanine (B; lower). OX1R, orexin 1 receptor; OX2R, orexin 2 receptor; CTD, C-Terminal Domain.
FIGURE 3.3
INTERACTION OF
OREXIN 2
RECEPTOR
C-TERMINAL
DOMAIN
MUTANTS
WITH DYNLT1

Yeast two-hybrid assays using Y187 or AH109 yeast strains to assess \( \beta \)-galactosidase activity or histidine synthesis, respectively. Constructs were co-transformed in yeast as indicated in the figures to assess where Dynlt1 binds. Upper panel, \( \beta \)-galactosidase assays: cells lysed using freeze/thaw cycles were incubated with ONPG and stopped with 1M sodium carbonate when a yellow color appeared. Absorbance was read at 420 nM. Lower panel, histidine synthesis: all co-transformations succeeded and grew on plates lacking leucine (L) and tryptophan (W), SD-LW. Protein-protein interactions were detected on plates lacking L, W, and histidine (H), SD-LWH. 3-AT (3-aminotriazole) was added to assess the strength of positive interactions. Only the strongest interactions persisted at the highest 3-AT concentrations. The strongest interaction as per both assays was that of Dynlt1 with OX2R lacking the extra a.a. found in the CTD (as compared to OX1R CTD). Results represent mean \( \pm \) SEM of 3 experiments, each performed in triplicate. AD, GAL4 activation domain; DBD, GAL4 DNA Binding Domain; OX2R CTD, orexin 2 receptor C-terminal domain. **: \( p<0.01 \) when compared to wild type OX2R CTD.
FIGURE 3.4 INTERACTION OF OREXIN 1 RECEPTOR C-TERMINAL DOMAIN MUTANTS WITH THE DYNEIN LIGHT CHAINS

Yeast two-hybrid assays using Y187 or AH109 yeast strains to assess β-galactosidase activity or histidine synthesis, respectively. OX1R CTD T409,412A point mutant (mut) and deletion mutants (Δ) were used in these assays to delineate Dynlt1 binding. For assay details see FIGURE 3.3. The bulk of the Dynlt1 interaction lies in the last 10 a.a. of the carboxy-terminus of OX1R, as their removal, or mutation of 2 threonines in this region of the CTD, blunted the interaction. The last 20 a.a. also serve for binding as their removal abolished the interaction (left). For Dynlt3, most of the interaction is in the last 10 a.a. of OX1R CTD, as further a.a. removal results in residual activity (right). Results represent the mean ± SEM of 3 experiments, each performed in triplicate. AD, GAL4 activation domain; DBD, GAL4 DNA Binding Domain; OX1R CTD, orexin 1 receptor C-terminal domain. **: p<0.01 when compared to wild type OX1R CTD.
FIGURE 3.5 IN VITRO BINDING ACTIVITY OF OREXIN 1 RECEPTOR C-TERMINAL DOMAIN WITH DYNLT1

GST pulldown assay of Dynlt1 with carboxy-terminal region of OX1R. GST-OX1R CTD fusion protein and HA-Dynlt1 were first mixed together and then with glutathione-sepharose beads. Proteins bound to beads were separated by SDS-PAGE and analyzed by Coomassie blue stain (A) to assess amounts of GST fusions, or by immunoblotting (B) with anti-HA antibody. A: Coomassie stain indicates that GST-OX1R CTD fusion has low solubility (right, indicated by arrow) compared to the other GST fusion (left), although both proteins were produced in similar amounts. B: immunoblot shows HA-Dynlt1 bound to GST-Dync1i2 but not GST-OX1R CTD. OX1R CTD, orexin 1 receptor C-terminal domain; Dync1i2, cytoplasmic dynein 1 intermediate chain 2.
FIGURE 3.5 CONTINUED. IN VITRO BINDING ACTIVITY OF LAST 30 RESIDUES OF OREXIN 1 RECEPTOR C-TERMINAL DOMAIN WITH DYNLT1

GST pulldown assay of Dynlt1 with the last 30 a.a. of the carboxy-terminal region of OX1R. GST-OX1R 30 a.a. CTD fusion protein and HA-Dynlt1 were first mixed together and then with glutathione-sepharose beads. Proteins bound to beads were separated by SDS-PAGE and analyzed by Coomassie blue stain (C) to assess amounts of GST fusions or by immunoblotting (D) with anti-HA antibody. C: Coomassie stain indicates that GST-OX1R 30 a.a. CTD fusion has better solubility (right, indicated by arrow) compared to full length CTD of OX1R (see panel A). D: immunoblot shows HA-Dynlt1 bound to GST-Dync1i2 but not GST-OX1R 30 a.a. CTD. OX1R 30a.a. CTD, amino acids 387 to 416 of orexin 1 receptor C-terminal domain; Dync1i2, cytoplasmic dynein 1 intermediate chain 2.
FIGURE 3.6
EFFECT OF DYNLT1 ON OREXIN 1 RECEPTOR AT THE PLASMA MEMBRANE

Cell surface detection of OX1R was measured by ELISA. HEK293 cells were transfected with control pSG5-V5 or pSG5-V5-OX1R vectors in combination with: (A) control pCS2-Myc or pCS2-Myc-Dynlt1, (B) 10 nM of either control siRNA or Dynlt1 siRNA (80% down-regulation). Cells stimulated with 100 nM OX-A were fixed in formaldehyde, incubated with anti-V5 Ab, followed by a horseradish peroxidase-coupled Ab and finally OPD (1 mg/mL) substrate. Corrected OD values refer to background-subtracted data at 490 nM (background refers to HEK293 cells transfected with empty pSG5-V5). At basal conditions, there is maximum receptor at the membrane (0 min; without OX-A) and it is internalized (represented by loss at the membrane) after OX-A addition. Results represent the mean ± SEM of 3 experiments, each performed in triplicate. No statistical significance for the effect of over-expressing (A) or down-regulating (B) Dynlt1, as assessed by two-way ANOVA, when compared with control.
FIGURE 3.7 EFFECT OF DOWNREGULATING DYNLT1/3 ON OREXIN 1 RECEPTOR AT THE PLASMA MEMBRANE

Cell surface detection of OX1R was measured by ELISA. HEK293 cells were transfected with control pSG5-V5 or pSG5-V5-OX1R, in combination with: either 20 nM control siRNA or a combination of Dynlt1 and Dynlt3 siRNAs at 10 nM each. For assay details see FIGURE 3.6 legend. There is maximum receptor at the membrane at basal conditions (0 min; without OX-A) and it is internalized (represented by loss at the membrane) after OX-A addition. Results represent the mean ± SEM of 3 experiments, each performed in triplicate. No statistical significance for the effect of down-regulating Dynlt1/3 at any time point when compared to control, as assessed by two-way ANOVA.
FIGURE 3.8 LOCALIZATION OF OREXIN 1 RECEPTOR FOLLOWING LIGAND-INDUCED INTERNALIZATION

PANEL A: Fluorescent microscopy analysis of internalized OX1R without Dynlt1. HEK293 cells stably expressing OX1R-GFP were transfected with control pCS2-FLAG and stimulated with 100 nM OX-A or left un-stimulated (0 min). Cells were fixed with 4% PFA and labelled with an antibody specific for the early endosome marker, EEA1. Images were taken with a Zeiss Axio Observer.Z1 microscope with ApoTome attachment at 40X. Maximum co-localization (green pixels co-localized with red pixels divided by the total # of green pixels) occurs after 15 min OX-A (compare remaining green pixels in merged images).
FIGURE 3.8 CONTINUED. LOCALIZATION OF OREXIN 1 RECEPTOR FOLLOWING LIGAND-INDUCED INTERNALIZATION

PANEL B: Fluorescent microscopy analysis of internalized OX1R with Dynlt1. HEK293 cells stably expressing OX1R-GFP were transfected with pCS2-FLAG-Dynlt1 and stimulated with 100 nM OX-A or left un-stimulated (0 min). For further details, see PANEL A. Maximum co-localization (green pixels co-localized with red pixels divided by the total # of green pixels) no longer occurs after 15 min OX-A (compare to green pixels in merged image at 15 min; PANEL A) but rather, is similar across OX-A stimulation times (compare remaining green pixels in merged images).
FIGURE 3.8 CONTINUED. LOCALIZATION OF OREXIN 1 RECEPTOR FOLLOWING LIGAND-INDUCED INTERNALIZATION

PANEL C: Manders co-localization coefficient for green channel (OX1R-GFP) analysis. Plotted is the ratio of the # of co-localizing green and red pixels divided by the total # green pixels over threshold. Images taken at the microscope were randomly cropped (45000 X 45000 pixels) using Openlab Software to include 1-3 cells (representative images shown in panels A,B). These images were then co-localized in Openlab and the co-localization coefficient for the green channel (OX1R-GFP) was measured. Results represent the mean ± SEM of triplicates from one experiment, where at least 5 fields with 1-3 cells in each were analyzed for each time point. This experiment was done twice as above and once with only 2 OX-A times (0,15 min), with similar results. The co-localization of OX1R-GFP with endosomes after 15 min OX-A is decreased upon Dynlt1 over-expression. Statistical significance for the effect of Dynlt1 as assessed by two-way ANOVA, * p<0.05 when compared to control (without Dynlt1) after 15 min OX-A.
4. DISCUSSION

In this study, we reveal the dynein light chains of the Dynlt family as protein partners of orexin receptors. To our knowledge, there are no other known interactors and regulators of orexin receptor function beyond the \( \beta \)-arrestins and CB1. Towards characterizing this interaction, we have identified that a Dynlt1 consensus motif in the extreme CTD of OX1R is indeed involved in the association with Dynlt1. Although this interaction is not important for membrane targeting of the receptor or for its internalization, we have found that Dynlt1 regulates the transition of OX1R in early endosomes. This has important implications for both protein partners involved.

4.1 THE IMPORTANCE OF THIS NOVEL INTERACTION FOR DYNEIN

Although these data suggest that OX1R is a novel target (cargo) of dynein, the other avenue has not been studied, but will be discussed here. Moreover, due to the fact that OX1R interacts with both Dynlt1/3, the possibility of them functioning together or independently will be considered here.

4.1.1 Is Dynlt1’s role dynein-dependent or independent?

The protein targets of Dynlt1 are ever-growing and the dynein light chain subunits are surpassing the other dynein subunits (LICs and ICs) in terms of the number of partner interactions. While Dynlt1 is principally known for its role as an adaptor to link cargo to dynein for retrograde transport (Desai & Mitchison, 1997), it has
dynein-independent functions as well; notably in mediating G-protein signaling (Takesono et al., 1999) and in regulating the actin cytoskeleton (Chuang et al., 2005). Although, we have not specifically addressed the role of Dynlt1 in this respect, our data suggest a dynein-dependent function as we have evidence that over-expression of Dynlt1 alters OX1R trafficking. Dynlt1 promotes the faster exit of OX1R from early endosomes, in accordance with dynein’s minus-end directed cargo transport (from the periphery to the nucleus in most cells) (Paschal et al., 1987). Further studies focusing on inhibiting dynein function would be interesting to confirm Dynlt1’s dynein-dependent role in OX1R regulation. This can be achieved either chemically, using erythro-9-[3-(2-hydroxynonyl)]adenine (EHNA) (inhibits dynein ATPase activity) or by knockdown of p150\textsubscript{glued} (the dynactin subunit required for many of cytoplasmic dynein’s functions) (Cheung et al., 2004).

Towards strengthening the role of Dynlt1 as being dynein-dependent in the regulation of OX1R, another experimental model could have been used. Since we have centered on dynein’s role in the transport of vesicles, specifically in a retrograde manner along microtubules, the study of Dynlt1’s role in neurons may have yielded stronger effects on OX1R regulation. It is possible that our HEK293 cell model did not allow us to see the full extent of Dynlt1’s effects, due to lack of axons and dendrites. Thus, this should be further investigated. Moreover, since we have done over-expression studies in HEK293 cells, we have largely relied on the successful expression of Dynlt1. Through Dynlt1 immunolabelling and subsequent fluorescence analysis, we have shown that some cells highly express
Dynlt1, while others have low expression (data not shown). This has made studying its effects possible but nevertheless, low Dynlt1 expression levels may have masked some of Dynlt1’s effects on OX1R localization.

4.1.2 Do Dynlt1 and Dynlt3 have independent functions?

Among the other GPCRs that have been found to interact directly with Dynlt1, the interaction was specific to this isoform, with no binding to its close homolog Dynlt3 (Sugai et al., 2003; Tai et al., 1999). In contrast, we have shown that OX1R can bind both Dynlt family members. This is the first time that an interaction between Dynlt3 and a GPCR is shown. Consequently, could these light chains be involved in the same functional regulation of OX1R or do they have distinct functions?

The common notion of cargo transport by dynein involves the dynein light chains that bridge the cargo to the dynein motor complex, through binding to the intermediate chain of dynein (Williams et al., 2006). Moreover, it is thought that the presence of different subunit isoforms allow organization of the dynein complex and thus cargo-specificity (Lo et al., 2007). Specifically, homodimers of Dynlt1 or Dynlt3 are capable of binding to the six dynein intermediate chain isoforms, conferring selectivity. Strikingly, Dynlt1/3 heterodimers were unable to bind the intermediate chain, suggesting that only homodimers assemble into the dynein complex (Lo et al., 2007). Thus far, our data hint toward a dynein-dependent role of Dynlt1. This may explain why in our ELISA study, of cell surface OX1R levels, we did not see an effect upon additional dynein light chain
knockdown (i.e. *DynLt3*) at the RNA level (compare FIGURE 3.6B with FIGURE 3.7). However, it does not exclude the possibility that the dynein light chain protein levels remained elevated, despite RNA levels being reduced, and thus may also explain why no effect was observed. Nevertheless, it can still be considered that although both Dynlt1/3 interact with OX1R, their functional roles in the dynein complex are not redundant and more likely competitive. Further evidence for this comes from studies where over-expressed Dynlt3 displaced endogenous Dynlt1 bound to the dynein complex and consequently disrupted Dynlt1-related functions (Sugai et al., 2003; Tai et al., 2001). We did not attempt Dynlt3 over-expression studies, but it would be interesting to discern its effect on Dynlt1-regulated OX1R function. Furthermore, in support of the differential strengths of interaction we have observed between OX1R and Dynlt1/3 (yeast-two hybrid studies, FIGURE 1.6; as well as co-IPs, data not shown), each dynein light chain could have independent roles in regulating OX1R. Likewise, the possibility still exists that Dynlt1-Dynlt3 heterodimers, formed in solution, could be regulating OX1R function (not dynein complex-related).

4.2 THE SIGNIFICANCE OF THIS INTERACTION FOR OREXIN RECEPTORS

We have shown that Dynlt1 is a mediator of OX1R trafficking and that it also acts as a regulator of OX1R-mediated signaling. The consequence of this for known orexin receptor function will be explored here.
4.2.1 The effect of Dynlt1 on the β-arrestin-2/orexin 1 receptor interaction

Ligand-induced internalization of GPCRs has been shown to involve β-arrestin-2, through its binding to sites on the C-terminus that have been phosphorylated by G-protein receptor kinases (Ferguson, 2001). β-arrestins are also well known for their roles in GPCR sequestration and more recently in signal transduction (Ritter & Hall, 2009). All these events are due, in large part, by the sustainability of the interaction between the GPCR and β-arrestins (Ferguson, 2001). Of interest to this project, the tight association between β-arrestin-2 and human OX1R relies on three threonines and one serine at the extreme C-terminus of the receptor (Milasta et al., 2005). Interestingly, two of these conserved threonine residues in mouse OX1R are important for Dynlt1 binding, as their mutation resulted in a blunted interaction with the receptor (yeast two-hybrid assays, FIGURE 3.4; also confirmed with co-IPs, data not shown). Thus, it is a possibility that Dynlt1 is mediating the β-arrestin-2/OX1R interaction by either acting as an adapter protein or conversely, by competing for binding on the C-terminus of the receptor.

Towards assessing the interplay between these three proteins, we have done some co-IPs in HEK293 cells that were successfully co-transfected with plasmids coding for Myc-tagged Dynlt1 or Flag-tagged β-arrestin-2 and V5-tagged OX1R treated with 100 nM OX-A or vehicle (distilled water) for 15 min (data not shown). Although the interaction between Dynlt1 and OX1R was detected by immunoprecipitation of the V5-tagged receptor using the V5 Ab, that of β-arrestin-2 and OX1R was not (data not shown). Therefore, the effect of Dynlt1 on the β-arrestin-2/OX1R interaction, to date, is inconclusive. It will be interesting to
optimize this co-IP (others have shown the β-arrestin-2/OX1R interaction (Milasta et al., 2005)) to then be able to study all three proteins in the same system; to ultimately determine if the binding of either protein to OX1R benefits or hinders its interaction with the other protein.

4.2.2 Does phosphorylation of orexin 1 receptor enhance Dynlt1 binding?

Class B GPCRs (exemplified by the angiotensin II receptor type IA) are able to form stable complexes with the β-arrestins due to specific clusters (3 out of 4 consecutive positions) of serine/threonine residues in the CTD that are phosphorylated upon agonist treatment (Oakley et al., 1999). This is a platform for β-arrestin recruitment and even more importantly, the amount of phosphorylated residues confers the sustainability of the interaction for some GPCRs (Ferguson, 2001).

Indeed, orexin receptors are Class B GPCRs (Pfegler et al., 2007) and the stability of their interaction with β-arrestin-2 may rely on the number of phosphorylated residues as well. This may be accurate for OX1R, since single a.a. substitutions required for phosphorylation (threonine or serine to alanine) in the CTD did not abolish interactions with β-arrestin-2, but double a.a. mutants did (Milasta et al., 2005). Since we have shown that Dynlt1 binds to threonine residues in the extreme carboxy end of OX1R, does the phosphorylation of the receptor strengthen its interaction with Dynlt1? It does not appear that this is true for Dynlt1 binding, as the interaction was detected to the same extent by co-IP before and after 15 min OX-A (data not shown). This is also in favor of our results.
demonstrating that Dynlt1 is not involved in the internalization of OX1R (FIGURE 3.6), but does not exclude the possibility that it regulates OX1R’s interaction with β-arrestin-2 once the receptor has been internalized.

4.2.3 Towards understanding why the interaction is not detected in vitro

A Dynlt1-binding motif has been identified in protein targets of Dynlt1, including other GPCRs (FIGURE 3.1), indicating a direct-binding site. Interestingly, even though 2 motifs have been identified (proximal and distal), only the distal motif has been found in Rhodopsin, where Dynlt1 has been implicated in the targeting of the receptor (Tai et al., 1999). This may suggest that in some cases, Dynlt1 binding to only one of the bi-partite motifs is enough for it to execute its functions (for example if different Dynlt1 molecules bind each part). Likewise, maybe Dynlt1’s binding to one motif or the other dictates the specific regulation of its target. For another target of Dynlt1, PTHR, both the proximal and distal motifs are present and at least some (or all) of these residues are important for receptor localization and internalization (Sugai et al., 2003). Thus, for some proteins, it may be that both the proximal and distal parts are needed for Dynlt1 to regulate the biological function of its target (for example if one Dynlt1 molecule is binding to both motifs).

This is relevant to orexin receptors since they contain both the proximal (found in the 3rd intracellular loop) and distal (found at the extreme C-terminus) sequence motifs (FIGURE 3.1). Two possibilities exist to explain that we have not detected a direct association thus far between OX1R CTD and Dynlt1, despite having
detected an interaction by yeast two-hybrid (FIGURE 1.6, FIGURE 3.4) and co-IP (FIGURE 1.8).

First, the possibility still exists that another molecule could be mediating the interaction between OX1R and Dynlt1. This is unlikely since we have identified the Dynlt1 binding site in OX1R and, as mentioned, have seen the interaction in yeast two-hybrid assays.

Second, this may be due to missing domains in OX1R that are necessary for the interaction to occur (FIGURE 3.5B). Moreover, OX1R solubility (i.e. GST fusion packaged in inclusion bodies) issues were encountered. Although increasing the solubility of OX1R was achieved, it was so at the expense of removing part of the CTD that could be needed for the interaction to be robust (FIGURE 3.5C). This may explain why no direct interaction between the last 30 a.a. of OX1R CTD and Dynlt1 was detected (FIGURE 3.5D). Also, for some GPCRs, the third intracellular loop can be highly regulated by phosphorylation and protein partner binding (Oakley et al., 1999). Indeed, OX1R specifically has some PKA (T296) and PKC phosphorylation sites in intracellular loop III (Voisin et al., 2003). Therefore, although we have kept the important domains identified in yeast-two hybrid, the missing proximal Dynlt1-binding motif (R294-K298) may contribute to why we have not identified a direct interaction in the in vitro pulldown assay.

However, although much emphasis is cast on our yeast-two hybrid results, caution must be issued when interpreting yeast-two hybrid assays. The potential for artifact exists and may lead to the identity of false positives due to the absence of
proper protein localization, cell context in different cell types and time constraints. Thus, OX1R CTD and Dynlt1 could have associated in yeast because these proteins were targeted to the same compartment (yeast nuclei), but in physiological conditions may not even exist in the same compartment of the cell. Then, the importance (biological relevance) of the interaction is lost. For this reason, in our study, the yeast-two hybrid system was used as an initial screening for the interaction and to further identify specific domains that may be involved in the interaction before proceeding with additional assays. Hence, all data were subsequently verified in a mammalian system with the full length OX1R, using co-IPs (done by Dr. David Duguay). The interaction between OX1R and Dynlt1 was confirmed, as well as the importance of the C-terminal domain of OX1R (FIGURE 1.8). Moreover, all domains of OX1R (FIGURE 3.4) deemed crucial for interaction with Dynlt1 were confirmed by co-IPs in mammalian cells (done by Dr. David Duguay, data not shown), where both proteins were properly localized (as assessed by immunofluorescence). Therefore, it is very unlikely that the yeast-two hybrid results were artifactual and rather, served as a very accurate initial assessment.

4.2.4 The significance of Dynlt1 in the therapeutic potential of orexin 1 receptor

In view that orexin receptors are the targets for many novel pharmacotherapies, the importance of characterizing what occurs downstream to their activation becomes relevant in order to entirely exploit their therapeutic potential. There are two distinct classes of receptors with which β-arrestins can associate: Class A and
Class B. Class B receptors usually recycle to the plasma membrane slowly following internalization, due to sustained interactions with β-arrestin-2 after internalization, in opposition to Class A receptors which recycle rapidly (Drake et al., 2006). Yet, some GPCRs (e.g. somatostatin 2A receptor) exhibit characteristics of both classes of receptors (Pfleger et al., 2007). There is some evidence for OX1R that it recycles more rapidly than the typical Class B (Pfleger et al., 2007), thus displaying features of Class A receptors. This is what we would expect, more rapid recycling and thus quicker re-sensitization, if Dynlt1 competes or weakens the interaction of OX1R with β-arrestin-2. Although we have not explored the localization of OX1R beyond early endosomes, similar co-localization studies using markers for recycling endosomes as well as lysosomes would be required to further elucidate this concept. This would also be clinically significant, as slower re-sensitization of OX1R in the absence of Dynlt1 (i.e. if Dynlt1 is compromised) would mean a hyposensitive receptor (less responsive to agonist or antagonists) and would require re-evaluation of dosage of treatment for orexin-related disorders. Ultimately, Dynlt1 may be acting to regulate the intracellular location of orexin receptors beyond what we have shown here and should be addressed as this may have further implications into orexin receptor life cycle.
4.3 MODEL OF OREXIN 1 RECEPTOR REGULATION BY DYNLT1

Re-examining the life cycle of a GPCR together with our current data, we question at which level Dynlt1 is acting to regulate OX1R. In accordance with our data, there are three- not mutually exclusive- possibilities (FIGURE 4.1).

Initially, focusing on a role for Dynlt1 in the production of a faster decline of orexin-induced ERK activation at the membrane, we speculated Dynlt1 to be involved in the de-sensitization of OX1R (FIGURE 4.1; 1). With this, we would have expected that the subsequent internalization of the receptor be affected. However, we saw no effect of Dynlt1 in the surface ELISAs (FIGURE 3.6). In addition, upon Dynlt1 treatment, the entry of OX1R in early endosomes was not accelerated (no change seen after 5 min OX-A) and a decrease, not an increase, of OX1R in endosomes (after 15 min OX-A), did not support this hypothesis (FIGURE 3.8C).

Next, we pursued the G-protein independent signaling that can occur through β-arrestin-2 (Oakley et al., 1999). GPCR-mediated signaling to ERK MAPK occurs from the plasma membrane as well as within endosomes and more importantly, the later is proportional to the amount of time the GPCR/β-arrestin complex stays in endosomes (Sorkin & von Zastrow, 2009). Also, the cluster of amino acids at the extreme C-terminal tail of OX1R, that is essential for strong association with β-arrestin-2, is also important for ERK1/2 phosphorylation (Milasta et al., 2005). This provides evidence for orexin-mediated β-arrestin bound ERK activation. Our work demonstrates a role of Dynlt1 in the transition of OX1R in endosomes and
thus supports this hypothesis (FIGURE 4.1; 2). After 15 min OX-A, there is less OX1R co-localizing with endosomes upon addition of Dynlt1, suggesting Dynlt1 is involved in the exit of OX1R from endosomes (FIGURE 3.8C). Consequently, the β-arrestin-2/OX1R interaction may become disrupted as the receptor moves internally, which would subsequently lead to a quicker ERK signal termination. Indeed, we have shown that with over-expressed Dynlt1, the orexin-induced activation of the ERK pathway is less sustained.

Finally, as mentioned, both β-arrestin-2 and Dynlt1 bind to the same residues in the extreme carboxy end of OX1R (Milasta et al., 2005, and our data). Thus, these proteins could be competing with each other, which would mean that in the presence of Dynlt1 beyond endogenous levels, OX1R’s association with β-arrestin-2 is weakened and would subsequently cause a less sustained ERK pathway activation (FIGURE 4.1; 3). We have begun exploring this hypothesis by performing independent co-IPs, where either β-arrestin-2 or Dynlt1 is present with OX1R to verify the interaction, and then co-IPs where all proteins are present to observe the effect on each protein’s interaction with OX1R. To date, the results are inconclusive, but it will be interesting to explore the significance of these interactions (see section 4.2.1, above) to more specifically elucidate how Dynlt1 achieves its regulation of OX1R.
FIGURE 4.1 MODEL OF WHERE DYNLT1 IS ACTING TO REGULATE OREXIN 1 RECEPTOR LIFE CYCLE

Schema depicting GPCR life cycle; G-protein-mediated signaling as well as arrestin mediated signaling by GPCRs once bound by agonist⁹. Depicted are 3 possibilities of where Dynlt1 is acting to regulate OX1R after OX-A treatment. 1. Dynlt1 is involved in the de-sensitization of OX1R (not likely according to our data); 2. Dynlt1 promotes the EXIT of OX1R from early endosomes (which our immunocytochemistry results support); 3. Dynlt1 competes with β-arrestin-2 for OX1R binding and thus interferes with β-arrestin-dependent signaling (our ERK signaling results corroborate this). See text for more details.

5. CONCLUSION

In the present study, we centre on assessing the role of Dynlt1 as an intracellular regulator of orexin receptor function, notably by studying its effects on orexin-induced internalization and subsequent intracellular localization.

Thus far, we have shown that Dynlt1, a subunit of the dynein motor complex, interacts with OX1R and that each protein’s carboxy-terminal is crucial for this interaction. Dynlt1 acts to regulate OX1R trafficking and signalization, reducing its time spent in endosomes and promoting signal termination.

Indeed, an improved picture emerges for the regulation of OX1R. This research has uncovered a protein involved in orexin receptor function and thus its modulation may be necessary when assessing biological functions involving orexins.
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