Orexin Signalling in Brown Adipose Tissue Precursor Cells

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Tiivistelmä — Referat — Abstract

Orexins are neuropeptides for which signalling effects have been noted in multiple functions of the central nervous system, and also potentially in the periphery of the body. Orexin receptors couple to a number of different proteins eliciting cellular responses such as activation/inhibition of ion channels, kinase activation, and second messenger generation, and downstream effects such as neuronal excitation, synaptic plasticity, and cell death. The majority of knowledge on orexin signalling has been obtained from recombinant expression systems, thus studies of signalling responses in specific cell or tissue types are desired. The goal of this master's thesis project was to investigate orexin signalling in two mouse brown adipocyte precursor cell lines (C3H10T1/2 and HIB1b) with native OX_1 receptor expression. p38 mitogen-activated protein kinase (MAPK) activation was assessed by western blot analysis, while phospholipase D (PLD) activity, arachidonic acid (AA) and 2-arachidonoylglycerol (2-AG) release, and adenylyl cyclase activity were assessed by radioactive prelabelling, extraction and separation of the molecular species, and quantification of radioactivity. p38 was activated by orexin in C3H10T1/2 cells, but not HIB1b cells; PLD, AA, and 2-AG showed no response to orexin; and adenylyl cyclase appears to be both stimulated and inhibited by orexin, at different concentrations. The results indicate that orexin signalling in these cell lines significantly differs from recombinantly expressed orexin receptors. This raises interesting questions regarding the variability of responses to orexin in different tissue types.

Avainsanat — Nyckelord — Keywords

orexin, OX_1 receptor, GPCR, brown adipose tissue, lipids, second messengers

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Abbreviations

2-AG = 2-arachidonoylglycerol

AA = arachidonic acid

ATP = adenosine triphosphate

ADP = adenosine diphosphate

BAT = brown adipose tissue

BMP = bone morphogenic protein

BMPR1A = bone morphogenic protein receptor 1A

BSA = bovine serum albumin

cAMP = cyclic adenosine monophosphate

CHO = Chinese hamster ovary

CHX = cyclohexamide

$cPLA_2$ = cytosolic phospholipase A_2

CTx = Cholera toxin

DAG = diacylglycerol

DAGL = diacylglycerol lipase

EDTA = ethylenediaminetetraacetic acid

ERK = extracellular signal-regulated kinase

FBS = fetal bovine serum

FCS = fetal calf serum

GDP = guanosine diphosphate

GEF = guanine nucleotide exchange factor

GFP = green fluorescent protein

GPCR = G-protein-coupled receptor

GTP = guanosine triphosphate

GTPase = guanosine triphosphatase

IBMX = 3-isobutyl-1-methyl-xanthine

IP_3 = inositol-1,4,5-trisphosphate

IPs = inositolphosphates

MAGL = monoacylglycerol lipase

MAPK = mitogen activated protein kinase

Myf5 = myogenic regulatory factor 5

PA = phosphatidic acid

PBS = phosphate-buffered saline

PCA = perchloric acid

PI3K = phosphoinositide-3-kinase

PIs = phosphatidylinositols

PIP_2 = phosphatidyl-inositol-4,5-bisphosphate

PIP5K = phosphatidylinositol-4-phosphate 5-kinases

PKA = protein kinase A

PKC = protein kinase C

PLA_2 = phospholipase A_2

PLC = phospholipase C

PLD = phospholipase D

PPO = prepro-orexin

PtdBut = phosphatidylbutanol

PTx = Pertussis toxin

REM (sleep) = rapid eye movement (sleep)

RGS = regulator of G-protein signalling

SAPK = stress-activated protein kinase

SDS-PAGE = sodium dodecyl sulfate – polyacrylamide gel electrophoresis

TBS = Tris-buffered saline

TLC = thin layer chromatography

TPA = 12-*O*-tetradecanoyl-phorbol-13-acetate

TRP (channels) = transient receptor potential (channels)

UCP1 = uncoupling protein 1

WAT = white adipose tissue

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1 Introduction

Orexins (also known as hypocretins) were first discovered as neuropeptides in 1998 by two independent research groups (de Lecea et al., 1998; Sakurai et al., 1998). They are produced by specific neurons in the lateral region of the hypothalamus, from where orexin-immunoreactive fibres project to many different regions of the brain and various neuronal signalling systems (Peyron et al., 1998). Orexin signalling is involved in many different functions of the central nervous system, including regulation of sleep and wakefulness, appetite and metabolism, reward and addiction, stress response, and analgesia. In addition it may have roles in the periphery of the body (reviewed in Kukkonen, 2013). Orexin receptors are G-protein coupled receptors (GPCRs) and signalling through them has the possibility to initiate a wide range of cellular responses. The receptors couple to a number of different proteins which regulate phospholipases and kinases, among other things, generating short term effects such as neuronal excitation, second messenger generation, and kinase signalling cascades, and long term effects such as synaptic plasticity and cell death or survival (reviewed Kukkonen, 2013). The cell or tissue type can therefore be of great importance in determining the response. Thus while many of the initial studies on orexin signalling have been done in recombinant expression systems, it is now crucial to study these signalling pathways in specific natural systems.

1.1 Orexin Peptides

There are two native orexin peptides, orexin-A and orexin-B, both of which are derived from the cleavage of the precursor molecule prepro-orexin (PPO) (de Lecea et al., 1998; Sakurai et al., 1998). One copy of each peptide is encoded in the PPO gene (de Lecea et al., 1998; Sakurai et al., 1998). Both peptides are amidated at the C-terminus and orexin-A also has an N-terminal glutamine cyclized to pyroglutamate and two disulfide bridges in its structure (Sakurai et al., 1998). The sequences of the orexin peptides are highly conserved in mammals. Lower vertebrates also produce orexins, however the sequences are less conserved (reviewed in Kukkonen, 2013). Invertebrates have not yet been found to produce orexins (Scammell & Winrow, 2011). Currently, no other neuropeptides are known to bind the orexin receptors (reviewed in Kukkonen, 2013).

In 1999, Kastin & Akerstrom showed that orexin-A is more stable and lipophilic than orexin-B, and that orexin-A is capable of crossing the blood brain barrier via simple diffusion. Subsequent studies however have found contradicting results, with little or no penetrance of orexin-A to the brain (Bingham et al., 2001; Fujiki et al., 2003).

1.2 Physiological Significance of Orexins

1.2.1 Regulation of Sleep and Wakefulness

Orexins are necessary for the regulation of normal sleep-wake cycles and the maintenance of wakefulness. In both rodents and primates, orexin-producing neurons are active during wakeful periods and high levels of orexin-A are present. During sleep periods the neurons are non-active and extracellular levels of orexin-A drop by approximately half (Taheri et al., 2000; Zeitzer et al., 2003). Forced wakefulness during time periods normally associated with sleep causes the orexin concentrations to remain high, suggesting a direct effect of orexin rather than being mediated through circadian signals (Zietzer et al., 2003).

Narcolepsy is a disorder in which patients are unable to maintain proper sleep-wake cycles. Those suffering from this disorder can experience increased daytime urges to sleep, sleep attacks, cataplexy, sleep paralysis, hypnagogic/hypnopompic hallucinations, and frequent transitions between sleep and wakefulness (Tsujno & Sakurai, 2009). Thannical et al. (2000) showed that human narcolepsy is caused by orexin deficiency. In different studies using rat, mouse, and canine models, elimination of the orexinergic neurons or orexin/orexin receptor knockout results in narcolepsy (Chemelli et al., 1999; Lin et al., 1999; Gerashchenko et al., 2001; Kalogiannis et al., 2011). It is the current belief that human narcolepsy is caused by death of the orexinergic neurons, resulting in decreased orexin levels, but the mechanism of cell death has yet to be proven (reviewed in Kukkonen, 2013). Thus orexin agonists could potentially become a target for therapeutic drugs aimed at treating narcolepsy. However, small molecule agonists for orexin receptors remain unknown and orexin-A itself lacks the desirable properties to

make it a good drug, with high concentrations required to get an effect, potentially limited blood-brain penetrance, and suboptimal metabolism rates (Kukkonen, 2013).

Conversely, orexin antagonists could prove successful as treatment for insomnia. In 2012 Merck completed phase III clinical trials for treatment of insomnia with an orexin receptor antagonist, suvorexant, and the New Drug Application has been accepted for review by the U.S. Food and Drug Administration. If approved, suvorexant would be the first orexin receptor antagonist medication in clinical use (http://www.mercknewsroom.com/press-release/prescription-medicine-news/merck-announces-fda-acceptance-new-drug-application-suvorex). Current treatments for insomnia include benzodiazepine receptor agonists (most common), sedating antidepressants or antipsychotics, and antihistamines. The problem is that all of these carry large risks for undesired side effects such as carryover of sleepiness the following morning, mental fog, confusion, amnesia, imbalance and falls, sleepwalking, hypotension, weight gain, and arrhythmias, as well as a risk of dependence and addiction (Scammell & Winrow, 2011). One major problem occurring with the use of benzodiazepine/benzodiazepine-like receptor agonists is decreased sleep quality and disturbed sleep architecture, for example a reduction in the time period spent in rapid eye movement (REM) sleep (Tsoi, 1991). REM sleep deprivation is associated with enhanced negative emotional reactivity, such as frustration, fear, and anxiety, and also with a decreased capability for concentration, decision making, and emotional processing and coping abilities (Rosales-Lagarde et al., 2012). Targeting the orexin system may produce fewer side effects since orexin more selectively promotes wakefulness, supported by the currently available data from the phase III suvorexant trials. There is also the idea that addiction and dependence risk should be lower because orexin antagonists also reduce drug seeking behavior, and overdose consequences would be less severe since orexin antagonists shouldn’t have a large effect on respiration or blood pressure (Scammell & Winrow, 2011). One study by Vermeeren et al. (2012) demonstrated that suvorexant does not impair next-day driving ability in healthy people under age 65, which again supports this theory of less severe side effects.

1.2.2 Regulation of Appetite and Metabolism

As mentioned earlier, orexinergic neurons are confined to the lateral hypothalamic area of the brain. This region has historically proved important for feeding and energy homeostasis by coordinating behavioral, metabolic, and neuroendocrine responses (Bernardis & Bellinger, 1993; Willie et al., 2001). In response to reduction in food availability and intake, both animals and humans adapt by assuming a state of increased alertness and disruption of normal sleep patterns, which in nature would allow for better chances of finding food (Borbely, 1977; Danguir & Nicolaidis, 1979; Dewasmes et al., 1989; Karklin et al., 1994). Thus appetite and sleep are inversely related, such that in times of hunger the animal needs to be in a state of arousal to seek out food, and when food is plenty then sleep may be indulged. This reasoning provides a link to the sleep/wake cycle regulatory functions of orexins. Another link is the association of obesity and narcolepsy, noted already in the 1930's (Cave, 1931; Daniels, 1934), although challenged in some recent studies (reviewed in Kukkonen, 2013).

The orexins were originally named as such due to the discovery that injection of orexin into rats leads to increased feeding behavior (Sakurai et al., 1998). Multiple studies have shown that after 1–2 days of fasting orexinergic neurons are activated and PPO mRNA levels increase (Sakurai et al., 1998; Yamanaka et al., 2003; Johnstone et al., 2006). The paradox however is that while lack of orexin decreases feeding, it also increases susceptibility to weight gain (Funato, 2009). In addition, ectopic expression of orexin yields a protective effect against weight gain on a high-calorie diet (Funato, 2009). Therefore the current belief is that the role of orexin lies more in metabolism regulation, rather than direct feeding behavior (reviewed in Kukkonen, 2013). Injection of orexin into the central nervous system increases metabolic rate (reviewed in Kukkonen et al., 2002), and Sellayah et al. (2011) demonstrated that orexin-null mice are unable to raise their metabolic rate in response to increased caloric intake, resulting in rapid weight gain in mice fed a high fat diet. It should be noted however that these mice were from homozygous PPO-KO mothers and thus this effect could partially be explained by the impaired orexin-driven brown adipose tissue (BAT) development (discussed later) rather than the actual loss of orexin signalling; orexin-null mice from heterozygous PPO-KO mothers still have functional BAT thermogenesis (Zhang et al., 2010). The loss of orexin did not have a large effect on mice fed a low fat diet. For both diets the

mice lacking orexin displayed reduced food intake, confirming that the weight gain is not due to overconsumption. It was also demonstrated that mice of both genotypes had similar levels of physical activity on both diets (Sellayah et al, 2011).

1.2.3 Orexins and Brown Adipose Tissue

The ability to raise metabolic rate and energy expenditure in response to caloric intake is called diet-induced thermogenesis and acts as a mechanism to maintain energy balance. BAT plays an essential role in this. These cells express a unique protein, uncoupling protein 1 (UCP1), which allows for the uncoupling of metabolism from oxidative respiration and directs energy to heat production instead of adenosine triphosphate (ATP) production (reviewed in Cannon & Nedergaard, 2004). In rodents BAT is present throughout the life cycle, whereas in humans it is mainly present at birth and early childhood as a defense mechanism to maintain body temperature (Langin, 2010). It was originally thought that BAT disappeared from humans within the first years of life, but recent evidence has shown its continued presence and activity in adults as well (reviewed in Nedergaard et al., 2007).

Unlike other organs, adipose tissue has the ability to expand or retract to vastly different states, based on the organism's energy balance and disease situation. This expansion is due to both an increase in size and number of the adipocytes (Algire et al., 2013). Both white adipose tissue (WAT) and BAT are mesodermal in origin and the progression to a mature adipocyte involves two phases, determination and differentiation. At the determination stage, pluripotent mesenchymal stem cells become committed to either the adipogenic or myogenic lineage, based on the expression of myogenic regulatory factor 5 (Myf5), to become preadipocytes (also called adipocyte progenitor cells) (Figure 1). The preadipocytes can then divide many times before undergoing differentiation into mature adipocyte cells (Algire et al., 2013). It is also possible to induce BAT formation from WAT lineage cells, possibly either by transdifferentiation of mature WAT cells or differentiation from the preadipocytes. While these cells are similar morphologically and functionally to regular BAT they are of different lineage, as seen by the expression of distinct markers (Algire et al., 2013). These have also been called "brite" or "beige" adipocytes.

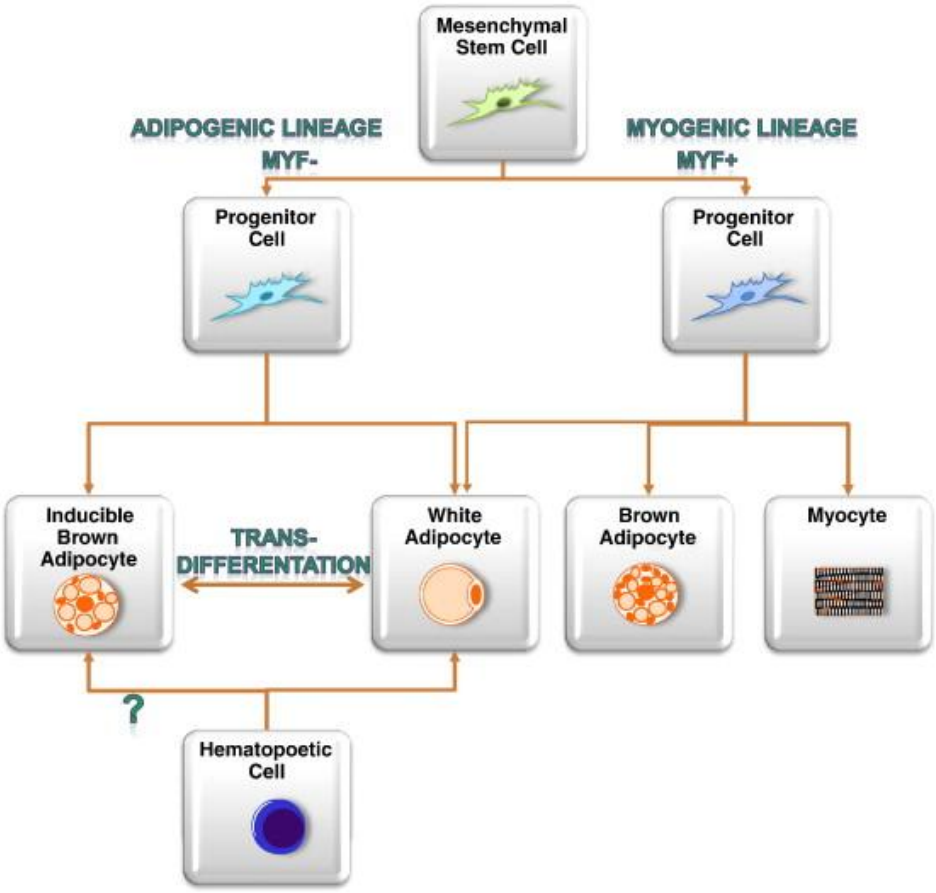

Figure 1. Origin of adipocytes. Adipocytes (white, inducible brown and brown) are mainly derived from mesenchymal stem cells of mesodermal origin. White adipocytes are derived from progenitors of adipogenic lineage or hematopoietic progenitors. Inducible brown adipocytes differentiate directly from adipocyte progenitors or may be produced through transdifferentiation from white adipocytes. Whether or not these cells can be derived from hematopoietic progenitors is not known. Finally, brown adipocytes originate from progenitors of myogenic lineage. Myf, myogenic regulatory factor. Reprinted from Biochemica et Biophysica Acta 1831, Algire, C., Medrikova, D. & Herzig, S., White and brown adipose stem cells: from signaling to clinical implications, p898, 2013, with permission from Elsevier.

Sellayah et al. (2011) suggested impaired BAT function as the mechanism for obesity associated with orexin deficiency. Without orexin present the preadipocyte cells fail to differentiate into mature brown adipocytes in mice. Thus orexin is necessary for functional BAT thermogenesis (although contradicted by results obtained by Zhang et al. (2010)), triglyceride storage, and proper BAT development. This is specific however

to BAT, as WAT development remains normal even in the absence of orexin (Sellayah et al., 2011). As BAT development in mice occurs during embryogenesis, the newborns' phenotype may be solely dependent upon the maternal genotype, i.e. the presence or absence of placental orexins (Zhang et al., 2010; Sellayah et al., 2011; Kukkonen, 2013). This is further supported by the observation that placental injection of orexin is capable of rescuing the phenotype to normal BAT (Sellayah et al., 2011).

Traditional signalling for brown adipogenesis involves the binding of bone morphogenic proteins (BMPs) to bone morphogenic protein receptor 1A (BMPR1A) to activate a signalling cascade through Smad 1/5, and a parallel activation of p38 mitogen-activated protein kinase (MAPK), both of which are necessary for differentiation into mature brown adipocytes (Hata et al., 2003; Cao et al., 2004; Tseng et al., 2008). Orexin acts through OX_1 receptors (discussed later) to induce differentiation of precursor cells to brown adipocytes in a similar manner, via activation of both p38 MAPK and BMPR1A signalling (Sellayah et al., 2011). The suggested mechanism is that orexin activates phospholipase C (PLC), as has been previously shown (Ammoun et al., 2006; Johansson et al., 2007), which sets off a signalling cascade eventually leading to the phosphorylation of p38 MAPK to its active state to trigger adipogenesis (Sellayah et al., 2011). In parallel, orexin stimulates increased expression of both BMP-7 and BMPR1A mRNA and decreased expression of mRNA for adipogenic inhibitory factors, and signalling proceeds through Smad 1/5 phosphorylation. Both of these pathways are necessary for differentiation of the precursor cells to mature adipocytes (Sellayah et al., 2011).

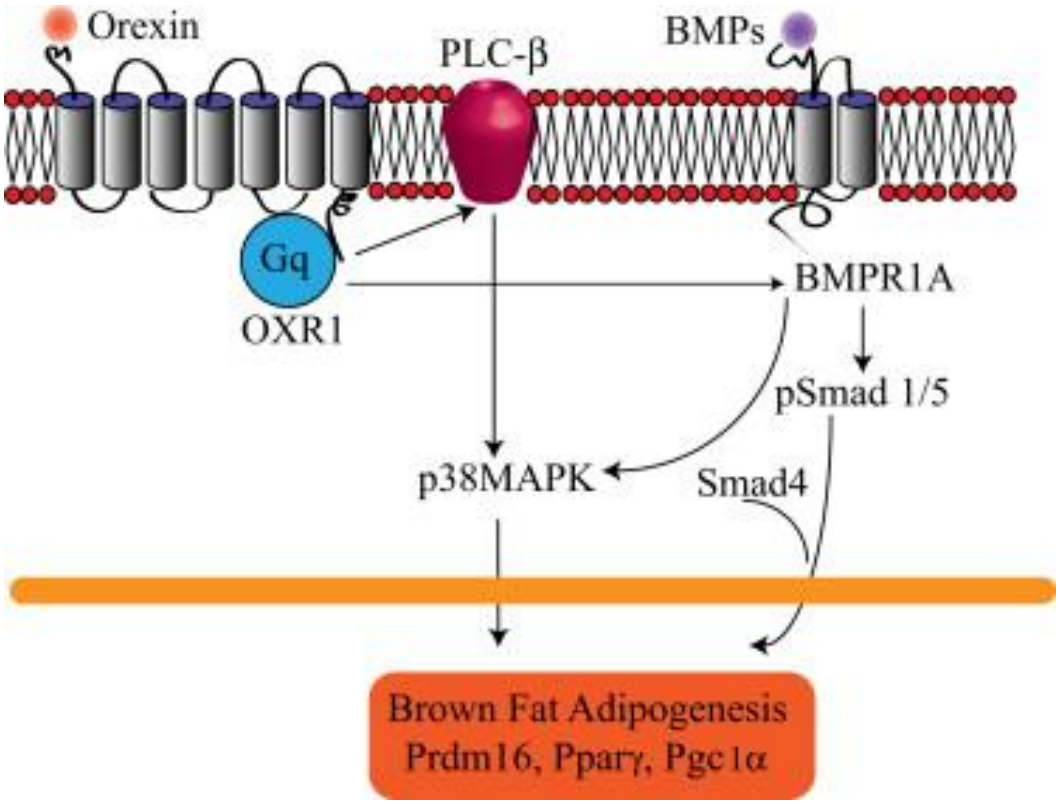

Figure 2. Model of orexin-induced differentiation. Orexin binding to orexin receptor 1 (OXR1, OX_1) induces phospholipase C (PLC) activation, which then stimulates p38 MAPK (mitogen activated protein kinase) to activate brown-fat adipogenesis. The second arm of OX signalling is relayed via Smad 1/5 phosphorylation in a bone morphogenic protein receptor 1A (BMPR1A)-dependent manner. Both the signalling arms contribute to OX-dependent differentiation such that inhibition of either pathway impairs brown-fat differentiation. Reprinted from Cell Metabolism 14, Sellayah, D., Bharaj, P. & Sikder, D., Orexin is required for brown adipose tissue development, differentiation, and function, p487, 2011, with permission from Elsevier.

1.3 G-Protein-Coupled Receptors

GPCRs are possibly the most numerous and diverse class of receptors, and can be found in a wide variety of eukaryotic organisms from yeasts to invertebrates to plants and mammals. With more than 800 identified human GPCRs, they make up approximately 2% of the human genome and represent the largest known gene family in humans (Frederiksson et al., 2003). GPCRs are involved in a huge range of biological functions and can be found in the systems for vision, smell, taste, neurotransmission, hormonal signalling, chemotaxis, cell growth, embryogenesis, and differentiation (Palczewski & Orban, 2013). The stimuli they respond to includes both physical and chemical entities

such as photons, ions and other small soluble molecules, lipids, nucleotides, peptides, and proteins (Frederiksson et al., 2003; Palczewski & Orban, 2013). GPCRs make good drug targets and it has been estimated that 30% of the current prescription drugs available act on GPCRs (Hopkins & Groom, 2002).

GPCRs share a common structure of seven transmembrane α-helices, an N-terminal extracellular domain, and a C-terminal cytoplasmic domain. The name comes from their association with the cytoplasmic heterotrimeric G-proteins, which transmit the signal from the cell membrane to the intracellular environment to elicit the response. The minimum GPCR signalling system consists of the receptor, a G-protein, an effector protein, and a regulator of G-protein signalling (RGS) protein (Ross & Wilkie, 2000).

Different classification systems exist for GPCRs, the major one being the A–F system (Kolakowski, 1994), which is based on sequence similarity. Another commonly used way to classify GPCRs is the GRAFS system (Fredriksson et al., 2003), which is based on phylogenetic similarities, yielding the glutamate, rhodopsin, adhesion, frizzled/taste2, and secretin families.

1.3.1 Orexin Receptors

Orexins belong to the class A or rhodopsin-like family of GPCRs. They comprise their own subgroup with only about 30% homology to other GPCRs (Kukkonen et al., 2002). There are two orexin receptors, OX_1 and OX_2. OX_1 binds orexin-A preferentially at up to 100 times over orexin-B, where as OX_2 binds both orexin-A and orexin-B equally (Sakurai et al., 1998). Figure 3 shows the peptide sequences of the two orexin receptors and demonstrates the classic GPCR structure exhibited by both.

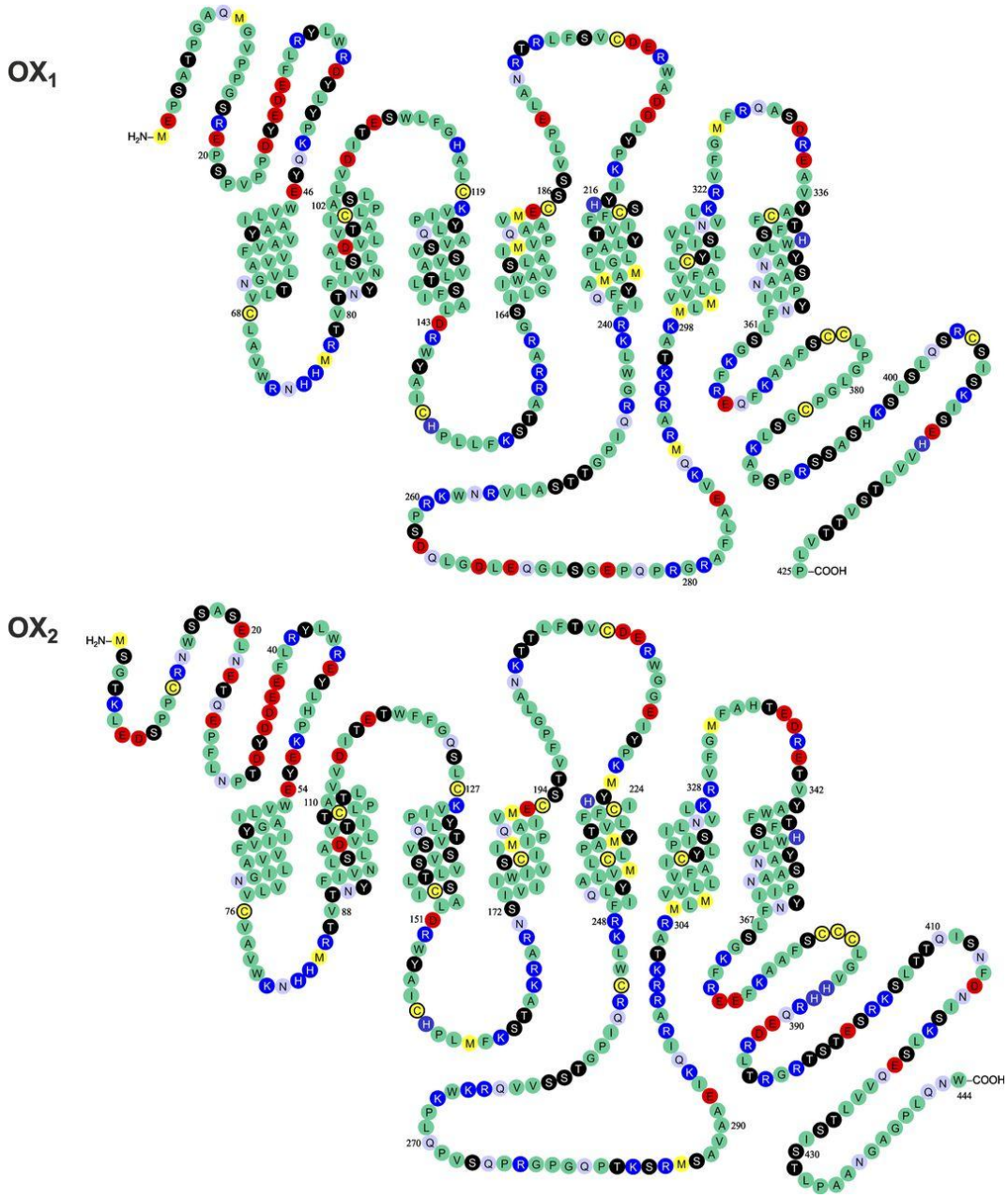

Figure 3. Peptide sequences for human orexin receptors OX_1 and OX_2. The sequences presented are only one of many known sequence variants/polymorphisms. Reprinted from American Journal of Physiology Cell Physiology 304, Kukkonen, J.P., Physiology of the orexinergic/hypocretinergic system: a revisit in 2012, pC5, 2013, with permission from The American Physiological Society.

The orexin receptors have not been crystallized, therefore predictions for structure and important regions for binding affinity are based on computer models, receptor mutagenesis, and domain exchange mutagenesis between OX_1 and OX_2 (reviewed in Kukkonen, 2013). Two isoforms (α and β) of OX_2 are expressed in mice as a result of alternative splicing in the C-terminal region. It's possible the splicing regulation is

specific to brain nuclei or tissue type (Chen & Randeva, 2004; Chen et al., 2006). Multiple sequence variants can be found in humans, some of which segregate with disorders but causative mechanisms have yet to be described (reviewed in Kukkonen, 2013).

1.4 Cellular Signalling of Orexins

The cellular responses to orexins are quite varied depending on cell type and even within a single cell. Orexin signalling proceeds through G-proteins as well as other mediators. Activation/inhibition of ion channels, kinase activation, and second messenger generation are among the major responses to orexins (Figure 4) (reviewed in Kukkonen, 2013).

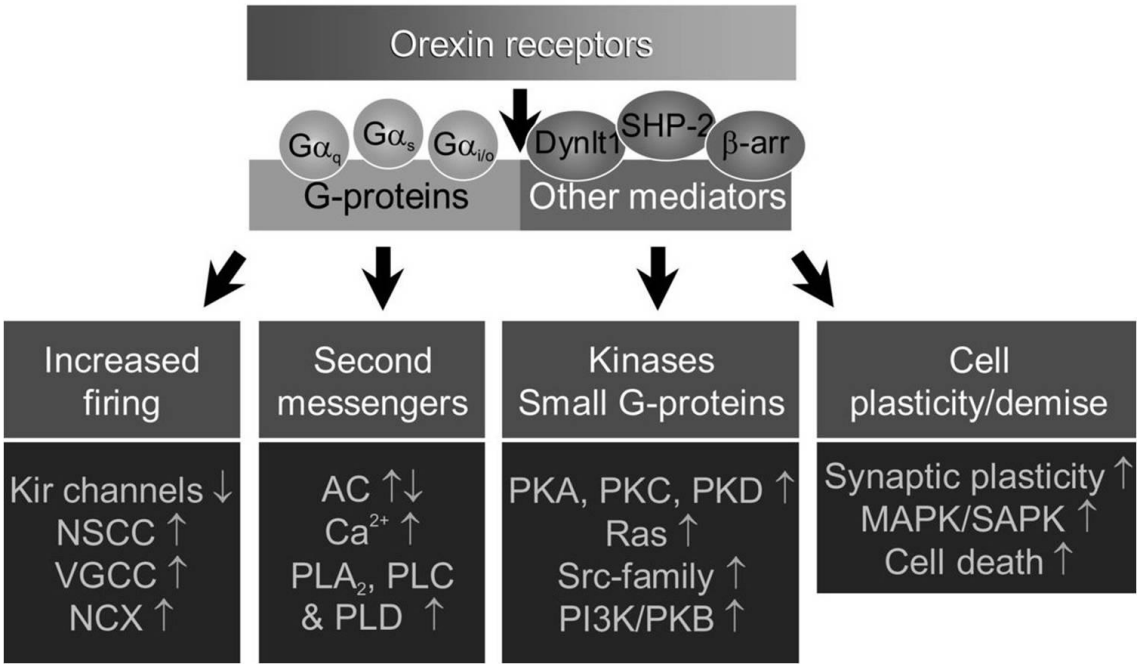

Figure 4. Cellular signalling responses to orexins. Abbreviations of importance: AC, adenylyl cyclase; PLA_2, phospholipase A_2; PLC, phospholipase C; PLD, phospholipase D; PKA, protein kinase A; PKC, protein kinase C; MAPK, mitogen activated protein kinase; SAPK, stress-activated protein kinase. Reprinted from American Journal of Physiology Cell Physiology 304, Kukkonen, J.P., Physiology of the orexinergic/hypocretinergic system: a revisit in 2012, pC7, 2013, with permission from The American Physiological Society.

1.4.1 G-Proteins

Heterotrimeric G-proteins act as the intracellular signalling partners to GPCRs. They are composed of three subunits: Gα, Gβ, and Gγ. When GDP is bound to the Gα subunit (resting state), Gα, Gβ, and Gγ are tightly bound to each other. When a GPCR binds its ligand it acts as a guanine nucleotide exchange factor (GEF) and promotes the release of GDP from Gα, leaving it free to bind GTP. GTP-bound Gα undergoes a conformational change which triggers the dissociation of Gβγ (Gilman, 1987; Wall et al., 1998). Both Gα and Gβγ can interact with effector proteins and initiate downstream signalling cascades (Figure 5). Gα has intrinsic guanosine triphosphatase (GTPase) activity, which will eventually hydrolyze GTP to GDP. Interaction with RGS proteins can speed up this process (Ross & Wilkie, 2000). At this point Gα is now back to the inactive state, reassociation with Gβγ occurs, and any interactions with effector proteins end (Gilman, 1987; Ford et al., 1998; Li et al., 1998).

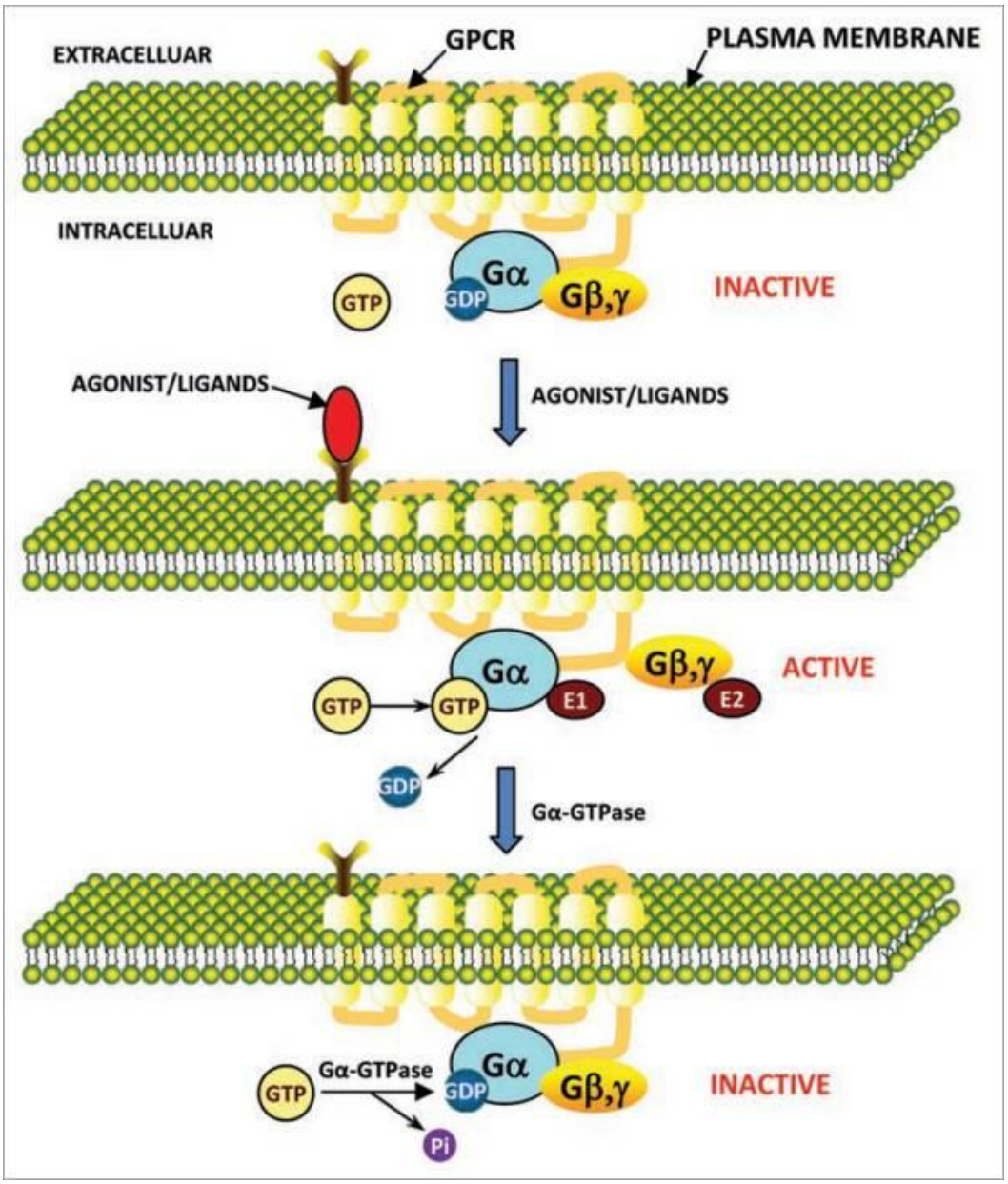

Figure 5. G-Protein-Coupled Receptor (GPCR)-activated signalling through G-proteins. Reprinted from Plant Signaling & Behavior 4, Tuteja, N., Signaling through G protein coupled receptors, p943, 2009, under open access content license.

The Gα subunits are divided into four families: $G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_q$, and $G\alpha_{12/13}$ (Simon et al., 1991). $G\alpha_s$ proteins activate adenylyl cyclase and are sensitive to cholera toxin (Milligan et al., 1989). $G\alpha_i$ proteins inhibit adenylyl cyclase and are sensitive to pertussis toxin, with the exception of $G\alpha_z$. Some members of this family are involved in taste, specifically bitter and sweet, and also vision (Ribeiro-Nato & Rodbell, 1989). $G\alpha_q$ proteins activate phospholipase Cβ (PLC) (Taylor et al., 1991). $G\alpha_q$ proteins can also activate monomeric G-proteins (Mizuno et al., 2009). $G\alpha_{12/13}$ proteins possess a

different structure than the other Gα families and can have many functions, although a major one is signalling to the Rho family of GTPases (Suzuki et al., 2009).

Direct measurement of receptor coupling to G-proteins is difficult due to a lack of specific molecular tools and the necessity of disrupting the cellular membrane, which may then disrupt signalling pathways. However, based on indirect measurements, there is evidence that both OX_1 and OX_2 can couple to the $G\alpha_s$, $G\alpha_{i/o}$, and $G\alpha_q$ families (reviewed in Kukkonen, 2013).

1.4.2 Kinase Activation

Orexin activates extracellular signal-related kinase (ERK) and p38 MAPK/stress-activated protein kinase (SAPK) cascades in recombinant cells (Ammoun & Johansson et al., 2006; Ammoun & Lindholm et al., 2006) and some native cells/cell lines, for example in BAT precursor cells (Sellayah et al., 2011). The Ras family proteins are suggested as the upstream activators of ERK, most likely regulated by protein kinase C (PKC), phosphoinositide-3-kinase (PI3K), and Src signalling (Ammoun & Johansson et al., 2006). The PKCδ isoform has also been identified as a target of orexin signalling, possibly activated through PLCβ mediated diacylglycerol (DAG) production (Holmqvist et al., 2005) although this view has also been challenged (Jäntti et al., 2012). These kinase pathways have an influence on cell growth, plasticity, cell death/survival, and in the case of the BAT precursors, they seem to have importance for differentiation and development to mature brown adipocytes (Sellayah et al., 2011; Kukkonen, 2013).

1.4.3 Influence on Ion Channels

From their first discovery orexins have been known to be neuroexcitory (deLecea et al., 1998). They employ both pre- and post- synaptic mechanisms, although the post-synaptic depolarization has been studied in more detail. Orexin causes an inhibition of K^+ channels and activation of cation influx channels, resulting in the post-synaptic depolarization of the neuron (reviewed in Kukkonen, 2013). In recombinant Chinese hamster ovary cells (CHO) orexin activates a receptor-operated Ca^{2+} influx pathway (Lund et al., 2000; Larsson et al., 2005; Turunen et al., 2010), which may be mediated

partially by transient receptor potential (TRP) channels (Larsson et al., 2005). It is still unclear as to the activation mechanism for the non-selective cation channels but the phospholipase A_2 (PLA_2) pathway may be involved, at least in the recombinant cells (Turunen et al, 2010; Turunen et al., 2012).

1.4.4 Generation of Lipid Messengers

Lipid messengers arise from membrane phospholipids upon hydrolysis by a phospholipase or phosphorylation by a lipid kinase. These signalling molecules can act in intracellular pathways and/or extracellular signalling, or they may be precursors to other signalling molecules. In addition, changes in the amount of a normal membrane lipid itself can be a signal (reviewed in Kukkonen, 2011).

PLC hydrolyzes phosphoinositides, typically phosphatidyl-inositol-4,5-bisphosphate (PIP_2), producing DAG and inositol-1,4,5-trisphosphate (IP_3). The major target of DAG is activation of protein kinase C (PKC), although other proteins can also be activated by this messenger; DAG can also be a substrate to produce other signalling molecules (Turunen et al., 2012; reviewed in Kukkonen, 2011). IP_3 is important for the opening of endoplasmic reticulum Ca^{2+} release channels and the subsequent increase of intracellular Ca^{2+} concentration (reviewed in Kukkonen, 2011). Studies in various recombinant cell systems have shown that both OX_1 and OX_2 receptors activate PLC (Lund et al., 2000; Holmqvist et al., 2002; Johansson et al., 2008; Putula & Kukkonen, 2012).

Phospholipase D (PLD) hydrolyzes phosphatidylcholine, producing choline and phosphatidic acid (PA). PA itself is an intracellular signalling molecule and among other things activates type I phosphatidylinositol-4-phosphate 5-kinases (PIP5K), necessary for PIP_2 production (van den Bout & Divecha, 2009). Alternatively, PA can be converted into DAG (Brindley & Pilquil, 2009) which has many different possible targets, as already discussed. PLD is strongly activated by orexin in recombinant CHO-hOX_1 cells (Jäntti et al., 2012). The activation occurs through PKCδ but it does not seem to be linked to PLC-dependent DAG generation (Jäntti et al., 2012). Figure 6 outlines this pathway and the method for selective monitoring of it.

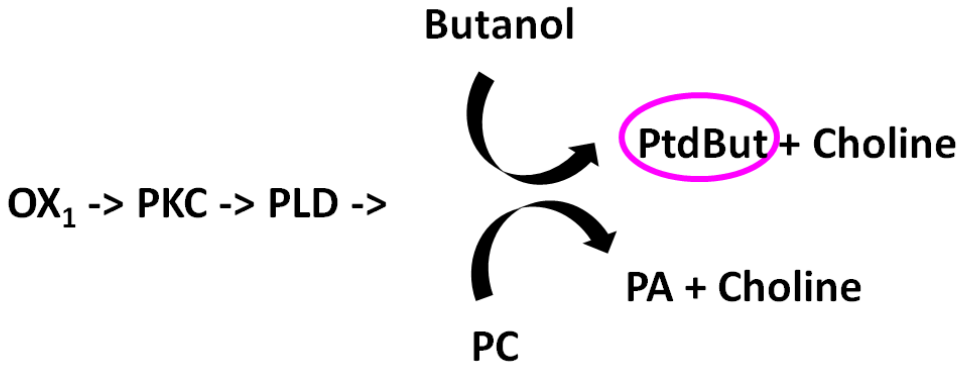

Figure 6. Schematic representation of signalling from orexin binding to activation of PLD, and selective monitoring using the butanol transphosphatidylation method (further described and justified under Materials and Methods). Circled item is the molecule measured in the assay. Other pathways have been excluded for clarity. PKC, protein kinase C; PLD, phospholipase D; PC, phosphatidylcholine; PA, phosphatidic acid; PtdBut, phosphatidylbutanol.

Arachidonic acid (AA) and 2-arachidonoyl glycerol (2-AG) are both released upon orexin treatment in recombinant cells (Turunen et al., 2012). AA can be produced by multiple pathways, such as the action of phospholipase A_2 ($cPLA_2$) on membrane glycerophospholipids or PA, or the breakdown of 2-AG by monoacylglycerol lipase (MAGL) (Kukkonen, 2011). 2-AG is produced by mainly by the action of diacylglycerol lipase (DAGL) on DAG (Kukkonen, 2011). Figure 7 provides a graphical depiction of these pathways. 2-AG is an endocannabinoid. Endocannabinoids bind to the CB_1 and CB_2 GPCRs to regulate appetite, nociception, memory, reward, and mood; at the synaptic level they perform retrograde transmission, where the endocannabinoids produced postsynaptically act on the presynaptic inhibitory CB_1 receptors (Kano et al., 2009). There is an overlap in some of the physiological functions of endocannabinoids and orexins, and also in their neuroanatomical distribution (reviewed in Kukkonen, 2013). It has also been proposed that OX_1 and CB_1 receptors form heteromeric complexes together to increase signalling (Hilairet et al., 2003; Ellis et al., 2006; Ward et al., 2011).

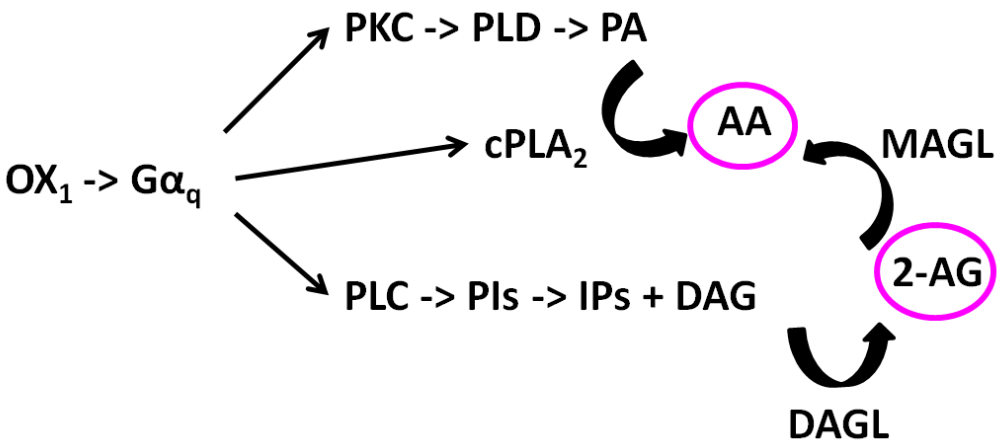

Figure 7. Schematic representation of signalling from orexin binding to release of AA and 2-AG. Circled items are the molecules measured in the assay. Other pathways have been excluded for clarity. PKC, protein kinase C; PLD, phospholipase D; PA, phosphatidic acid; $cPLA_2$, cytosolic phospholipase A_2; AA, arachidonic acid; PLC, phospholipase C; PIs, phosphatidylinositols; IPs, inositolphosphates; DAG, diacylglycerol; DAGL, diacylglycerol lipase; 2-AG, 2-arachidonoyl glycerol; MAGL, monoacylglycerol lipase.

1.4.5 Influence on Adenylyl Cyclase

Adenylyl cyclase converts ATP to cyclic adenosine monophosphate (cAMP) (Figure 8). cAMP is a ubiquitous second messenger and it is involved in regulation of a wide range of cellular processes, including glycogen metabolism, hormone synthesis, ion channels, gene transcription, and activation of other enzymes, particularly protein kinase A (PKA) (Holmqvist et al., 2005). The adenylyl cyclase family of enzymes consists of ten isoforms, nine of which are membrane-bound and the other one soluble (Patel et al., 2001). All of the membrane-bound adenylyl cyclases are regulated by G-proteins, with $G\alpha_s$ having a stimulatory effect and $G\alpha_i$ an inhibitory effect (Milligan et al., 1989; Ribeiro-Nato & Rodbell, 1989). Adenylyl cyclase regulation is also accomplished via other intracellular messengers, most importantly Ca^{2+}, PKC and $G\beta\gamma$ (reviewed in Hurley, 1999). The response of adenylyl cyclase to orexin appears to be complex, with evidence for activation from both OX_1 and OX_2 in recombinant cells (Holmqvist et al., 2005; Tang et al., 2008) and native cells (Malendowicz et al., 1999; Gorojankina et al., 2007), inhibition from OX_1 in some recombinant cells (Holmqvist et al., 2005), and no response in some other cell types (van den Pol et al., 1998; Larsson et al., 2003; Magga et al., 2006). Since each isoform of adenylyl cyclase is regulated independently one

possibility is that these differences reflect differences in either the isoform expression patterns between cell lines/tissue types or the subcellular localization of isoforms (Ostrom et al., 2012).

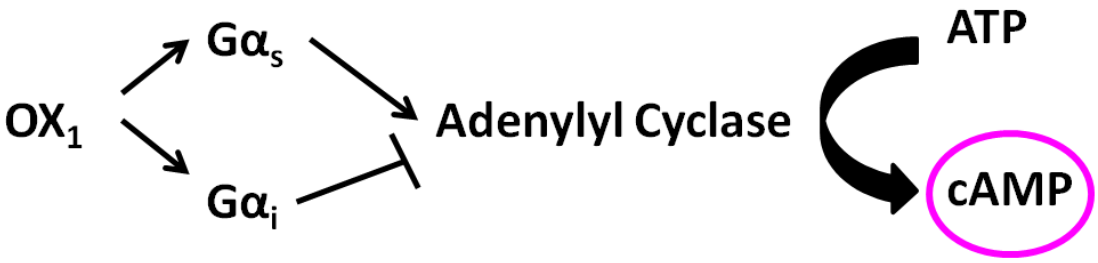

Figure 8. Schematic representation of signalling from orexin binding to activation or inhibition of adenylyl cyclase. Circled item is the molecule measured in the assay.

2 Research Objectives

The majority of knowledge on orexin signalling cascades has been obtained from recombinant expression systems, due to the fact that isolating native orexin receptor-expressing cells or cell lines has proven difficult. However, studies with natural orexin systems are necessary in order to test the significance for physiological responses in specific tissue types. With the recent discovery that orexin is capable of inducing the differentiation of precursor cells to brown adipocytes, two mouse cell lines were identified as having functional OX_1 orexin receptor expression, C3H10T1/2 mesenchymal stem cells and HIB1b preadipocytes (Sellayah et al., 2011).

The goal of this project was to investigate orexin signalling in these two cell lines. The specific responses investigated were: p38 mitogen-activated protein kinase (MAPK) activation; phospholipase D (PLD) activity; arachidonic acid (AA) and 2-arachidonoylglycerol (2-AG) release; and adenylyl cyclase activity.

3 Materials and Methods

3.1 Cell Lines

C3H10T1/2 mouse mesenchymal stem cells (Reznikoff et al., 1973) and HIB1b mouse brown preadipocyte cells (Ross et al., 1992) were from Dr. Devanjan Sikder (Diabetes and Obesity Research Center, Sanford-Burnham Medical Research Institute, Orlando, FL, USA). CHO-hOX_1 Chinese hamster ovary cells expressing human OX_1 receptors have been described earlier (Lund et al, 2000).

3.2 Cell Culture

C3H10T1/2 and HIB1b cells were cultured in Dulbecco's Modified Eagle's Medium DMEM with 4.5 g/L glucose without L-glutamine (Lonza, Verviers, Belgium), supplemented with 10% fetal calf serum (FCS), 1% penicillin + streptomycin, 10% glutamine, and 10 mM HEPES Buffering Solution (Gibco by Life Technologies, Paisley, UK). CHO-hOX_1 cells were cultured in HAM's F-12 Nutrient Mixture (Gibco), supplemented with 10% fetal bovine serum (FBS), 1% penicillin + streptomycin, and 10 mM HEPES Buffering Solution (Gibco). All cells were incubated at 37°C with 5% CO_2. To split cells, they were first washed with phosphate buffered saline (PBS), detached with PBS + 0.02% (w/v) ethylenediaminetetraacetic acid (EDTA) for 3–4 minutes at 37°C, centrifuged (4 min, 1500 rpm), and re-suspended in cell culture medium.

3.3 p38 Western Blot Analysis

Activation of p38 MAPK was assessed by Western blotting with antibodies against the dually phosphorylated (activated) p38 (Table 1). Cells were plated on 6-well plates (CellStar by Greiner Bio-One, Frickerhausen, Germany) at 150 000 cells/well in cell culture medium and changed to serum-free medium one day prior to the experiment.

The cells were activated in serum-free medium containing the activators (Table 2) for 10 or 30 min. After the activation medium was removed the cells were placed on ice, washed once with ice-cold PBS, then collected in 100 µL lysis buffer (Appendix 1) to

Eppendorf tubes. The samples were centrifuged (2 min, 16 000 rpm) to remove the insoluble fractions and the supernatants collected.

The samples were separated by 10% sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE) (Appendix 1), then transferred to Hybond™-C Extra nitrocellulose membranes (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK). The membranes were blocked with Odyssey Infrared Imaging System Blocking Buffer (LI-COR Inc., Lincoln, NE, USA):Tris-buffered saline (TBS) (Appendix 1) (50:50) for 1 h at room temperature. The membranes were incubated with the primary anti-active p38 antibody and, as the loading control, anti-total ERK antibodies (Table 1) overnight at +4ºC, then with the secondary antibodies (Table 1) for 1 h at room temperature. Odyssey Infrared Imager and software (LI-COR Inc.) was used for detection of the antibodies. Band mean intensities were measured with Nikon Imaging Software NIS-Elements AR (Version 3.1, Nikon, Tokyo, Japan) and background level intensity subtracted.

Table 1. List of antibodies used in western blot analysis.

Antibody	**Type**	**Working dilution**	**Source**
anti-ACTIVE® p38 pAb, Rabbit, (pTGpY)	primary	1:1500	Promega, Madison, WI, USA
anti-p44/42 MAPK (ERK1/2), Mouse mAb, (L34F12)	primary	1:2500	Cell Signalling Technology, Boston, MA, USA
IRDye 680RD Goat anti-Rabbit IgG (H+L)	secondary	1:10 000	LI-COR Inc., Lincoln, NE, USA
IRDye 800CW Goat anti-Mouse IgG (H+L)	secondary	1:10 000	LI-COR Inc., Lincoln, NE, USA
Precision Plus Protein™ Western C™ Standard	size marker	N/A	BIO-RAD, Hercules, CA, USA

Table 2. List of drugs and chemicals used.

Drug/Chemical	**Use**	**Source**
Adenosine triphosphate (ATP)	P2 receptor ligand for adenylyl cyclase and AA/2-AG assays	Sigma-Aldrich, St. Louis, MO, USA
Arachidonic acid (AA)	Standard for TLC separation	Cayman Europe, Tallinn, Estonia
2-arachidonoylglycerol (2-AG)	Standard for TLC separation	Cayman Europe, Tallinn, Estonia
Cholera toxin (CTx)	$G\alpha_s$ activator and therefore adenylyl cyclase stimulator	Calbiochem, La Jolla, CA, USA
Cycloheximide (CHX)	Protein synthesis inhibitor and possible p38 MAPK stimulator via cell stress	Sigma-Aldrich, St. Louis, MO, USA
Forskolin	Adenylyl cyclase stimulator	Sigma-Aldrich, St. Louis, MO, USA
3-isobutyl-1-methyl-xanthine (IBMX)	Cyclic nucleotide phosphodiesterase inhibitor	Sigma-Aldrich, St. Louis, MO, USA
Ionomycin (from *Streptomyces conglobatus*)	Ca^{2+} ionophore for AA/2-AG assay	Calbiochem, La Jolla, CA, USA
Isoproterenol	β-adrenoceptor agonist for adenylyl cyclase assay	Sigma-Aldrich, St. Louis, MO, USA
Orexin-A (human/bovine/rat/ mouse)	Orexin receptor agonist	PolyPeptide Laboratories Group, Strasbourg, France
Pertussis toxin (PTx)	$G\alpha_i$ inhibitor for adenylyl cyclase assay	Sigma-Aldrich, St. Louis, MO, USA
Phosphatidylbutanol (PdtBut)	Standard for TLC separation	BIOMOL by Enzo Life Sciences, Plymouth Meeting, PA, USA
Thapsigargin	Stimulant of Ca^{2+} release and influx for AA/2-AG assay	Sigma RBI, Natick, MA, USA

12-*O*-tetradecanoyl-phorbol-13-acetate (TPA)	Protein kinase C stimulant for PLD assay	Sigma-Aldrich, St. Louis, MO, USA

3.4 Phospholipase D Assay

The method used was based on that described by Jäntti et al. (2012) with some modifications. The transphosphatidylation assay enables specific measurement of PLD activity by providing primary alcohols (here 1-butanol), the preferred substrate of PLDs, to be utilized instead of water for the hydrolysis reaction (reviewed Morris et al., 1997). This has the advantage over the normal PA-producing pathway, as PA can be generated in multiple ways and therefore does not solely indicate PLD activity. Production of phosphatidylbutanol (PdtBut) was thus used as the indicator of PLD activity. Cells were plated on 6-well plates (Cellstar) at 150 000 cells/well (C3H10T1/2, HIB1b) or 200 000 cells/well (CHO-hOX_1) one day prior to labelling. For OX_1 overexpression, cells were transduced with bac410-OX_1-GFP baculovirus (described below) for 5 h prior to labelling.

Cells were labelled with 0.02 μCi/mL [^{14}C]-palmitic acid (Perkin Elmer, Boston, MA, USA) in cell culture medium overnight for approximately 18 h. The labelling medium was then removed and replaced with normal cell culture medium, in which the cells were allowed to settle for 1 h. The cells were activated in cell culture medium + 0.3% 1-butanol with the activation treatments (Table 2) for 30 min at 37°C. The activation medium was then removed, the cells washed once with PBS, and replaced with 300 μL ice-cold methanol. While keeping the plates on ice, the cells were scraped from the wells and collected to 2 mL Eppendorf tubes. The wells were then washed with another 300 μL methanol and collected and combined with the first samples.

For lipid extraction, 500 μL of chloroform was added to the Eppendorf tubes and the samples mixed by vortexing, then incubated for 15 min at room temperature. Next, 400 μL of water was added and the samples mixed by vortexing. The samples were then centrifuged (5 min, 10 000 rpm), after which the upper phase was removed and the lower phase (containing the lipids) was dried under a stream of nitrogen gas. The lipids were dissolved in chloroform:methanol (19:1) and 3 μL of cold PtdBut standard added

to some of the samples. The samples were loaded onto thin layer chromatography (TLC) plates (Silicagel 60, Merck, Darmstadt, Germany) pretreated with 1% K^+-oxalate in methanol:water (2:3) and oven dried at 110°C for at least 1 h. The plates were developed using the upper phase of ethylacetate:isooctane:acetic acid:water (110:50:20:100) in an unlined chromatography tank.

3.5 AA and 2-AG Release Assay

The methods used were based on those described by Turunen et al. (2012) with some modifications. Production of 2-AG indicates DAGL activity whereas AA may be released via multiple pathways, including phospholipase A_2 ($cPLA_2$) activity and 2-AG breakdown. Cells were plated on 6-well plates (Cellstar) at 150 000 cells/well (C3H10T1/2, HIB1b) or 200 000 cells/well (CHO-hOX_1) one day prior to labelling. For OX_1 overexpression, cells were transduced with bac410-OX_1-GFP baculovirus (described below) for 5 h prior to labelling.

Cells were labelled with 0.3 µCi/mL [^{14}C]-AA (Perkin Elmer, Boston, MA, USA) in cell culture medium overnight for approximately 18 h. The labelling medium was then removed and cells washed twice with Na^+-Elliot (Appendix 1) + 10 mM glucose, 1 mM $CaCl_2$, 0.1% lipid-free bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO, USA). The cells were then activated in this same buffer with the activators (Table 2) for 7 min at 37ºC, after which the supernatant was collected to Eppendorf tubes.

For lipid extraction, the samples were centrifuged (1 min, 16 000 rpm) to remove detached cells and 800 µL of the supernatant transferred to Kimax tubes (Kimble Glass Inc., Vineland, NJ, USA). Then 2 mL methanol and 1 mL chloroform was added to the tubes and mixed by vortexing, followed by 1 mL water and 1 mL chloroform and mixed again by vortexing. The tubes were centrifuged (5 min, 500 rpm) and the lower phase transferred to 2 mL Eppendorf tubes and dried under a stream of nitrogen gas. The lipids were dissolved in chloroform:methanol (19:1) and loaded on to TLC plates (Silicagel 60, Merck, Darmstadt, Germany) previously oven dried at 110ºC for at least one hour, along with the AA and 2-AG standards. The plates were developed using ethylacetate:isooctane:acetic acid (144:56:20) in a chromatography tank lined with filter paper.

3.6 Baculovirus Vector and Transduction

Generation of the baculovirus expression vector for human C-terminally green fluorescent protein (GFP) tagged OX_1 receptor (OX_1-GFP) under cytomegalovirus promoter was previously described (Näsman et al., 2006). The virus stocks were grown and maintained in Sf9 insect cells by Jaana Putula (Department of Veterinary Biosciences, University of Helsinki, Helsinki, Finland). For use with C3H10T1/2 and HIB1b cells, 500 µL virus stock/well was centrifuged (30 min, 16 000 rpm) and the virus pellet re-suspended in 1 mL/well of cell culture medium before adding to the cells. Successful transduction was confirmed via fluorescent microscopy to check the presence of GFP within the cells.

3.7 TLC Quantification

In both the PLD and AA/2-AG assays, TLC plates were allowed to dry and then exposed overnight with an imaging plate (Fujifilm, Tokyo, Japan). The imaging plate was scanned with the Fujifilm Fluor-Imager FLA-5100 (Fujifilm, Tokyo, Japan). The TLC plates were placed in an iodine vapour tank to allow visualization of the standards. After marking the desired bands, the plates were then scanned into the computer to allow for image scaling and overlay of the standards with the radioactivity images. Band mean intensities and areas were measured with Nikon Imaging Software NIS-Elements AR (Version 3.1, Nikon, Tokyo, Japan) and background level intensity subtracted.

3.8 Adenylyl Cyclase Assay

Cells were plated on 24-well plates (Cellstar), pretreated with 0.5% polyethyleneimine, at 30 000 cells/well (C3H10T1/2, HIB1b, CHO-hOX_1) two days prior to the experiment. Production of cAMP was used as the indicator of adenylyl cyclase activity. For PTx and CTx treatments the cells were incubated overnight with the toxin for approximately 18 h.

The cells were labelled with 5 µCi/mL [^{3}H]-adenine (Perkin Elmer, Boston, MA, USA) in cell culture medium for 2 h. Inhibitor and activator solutions were prepared in Na^+-

Elliot. The labelling medium was removed and 200 µL Na^+-Elliot + 0.5 mM IBMX inhibitor solution (Table 2) added and cells incubated for 10 min at 37ºC. The inhibitor solution was then replaced with 200 µL activation solution containing the activators (Table 2) and cells incubated for 20 min at 37ºC. Following the incubation the supernatant was discarded and 200 µL ice-cold 0.33 M perchloric acid (PCA) added and the cells frozen immediately.

The samples were thawed at room temperature and then centrifuged to remove the precipitate (10 min, 2500 rpm). The ATP+ADP and cAMP fractions were separated and collected using sequential Dowex/Alumina chromatography (Holmqvist et al., 2005). Cells were first centrifuged (10 min, 2500 rpm) to remove the precipitate. Then equal amounts of supernatants were added to the Dowex columns along with 0.33 M PCA to obtain a total volume of 1 mL. The ADP + ATP fractions were eluted with 2 mL water into large scintillation vials. The Dowex columns were then placed on top of the Alumina columns and the samples eluted into Alumina with 10 mL water. The cAMP fractions were then eluted with 4 mL 0.1 M imidazole into large scintillation vials. To each vial scintillation cocktail (HiSafe 3, Wallac-PerkinElmer, Turku, Finland) was added and the radioactivity measured with Wallac 1414 liquid scintillation counter (Wallac-PerkinElmer, Turku, Finland). The amount of [^{3}H]-cAMP was calculated as a percentage of the total eluted [^{3}H]-ATP + [^{3}H]-ADP.

3.9 Data Analysis

All data have been presented as the mean ± standard error; *n* refers to the number of batches of cells. Each experiment was run in duplicate (Western Blot, PLD, AA, and 2-AG) or quadruplicate (cAMP) at least three times. The data presented is a summary of the data sets generated by each batch of cells, unless specifically indicated to be from a single representative experiment. The data were normalized to basal or standard stimuli to allow comparison and averaging of different data sets (see figure legends). Student's paired or non-paired *t*-test with Bonferroni correction for multiple comparisons was used for all comparisons, utilizing Microsoft Excel.

4 Results

4.1 Label Uptake

The efficiency of the cells to take up the radio-labelled tracers was tested before their use in further experiments. In all cases all cell lines showed effective cellular uptake of the labels, with efficiencies ranging from 72.1 – 89.8%.

4.2 p38 Western Blot Analysis

Different concentrations of orexin-A were tested, along with different activation time points to determine the best conditions for kinase activation. Sucrose, cyclohexamide (CHX), and 10% FCS (functions described in Table 2) were tested as positive controls, again with different time points. Sucrose and CHX produced no response from either cell line, while 10% FCS gave decent activation in C3H10T1/2 cells but not in HIB1b cells. Weak activation of p38 MAPK was observed with both 100 nM and 1 µM orexin-A concentrations after a 10 min activation period, and stronger activation after a 30 min activation period in C3H10T1/2 cells (Figure 9). No significant activation above the basal levels was observed with any concentration of orexin-A at either time point in HIB1b cells (Figure 9). The p38 assay was only performed once, since it was essentially only a control for cell function and reproduced the results of the previous study by Sellayah et al. (2011).

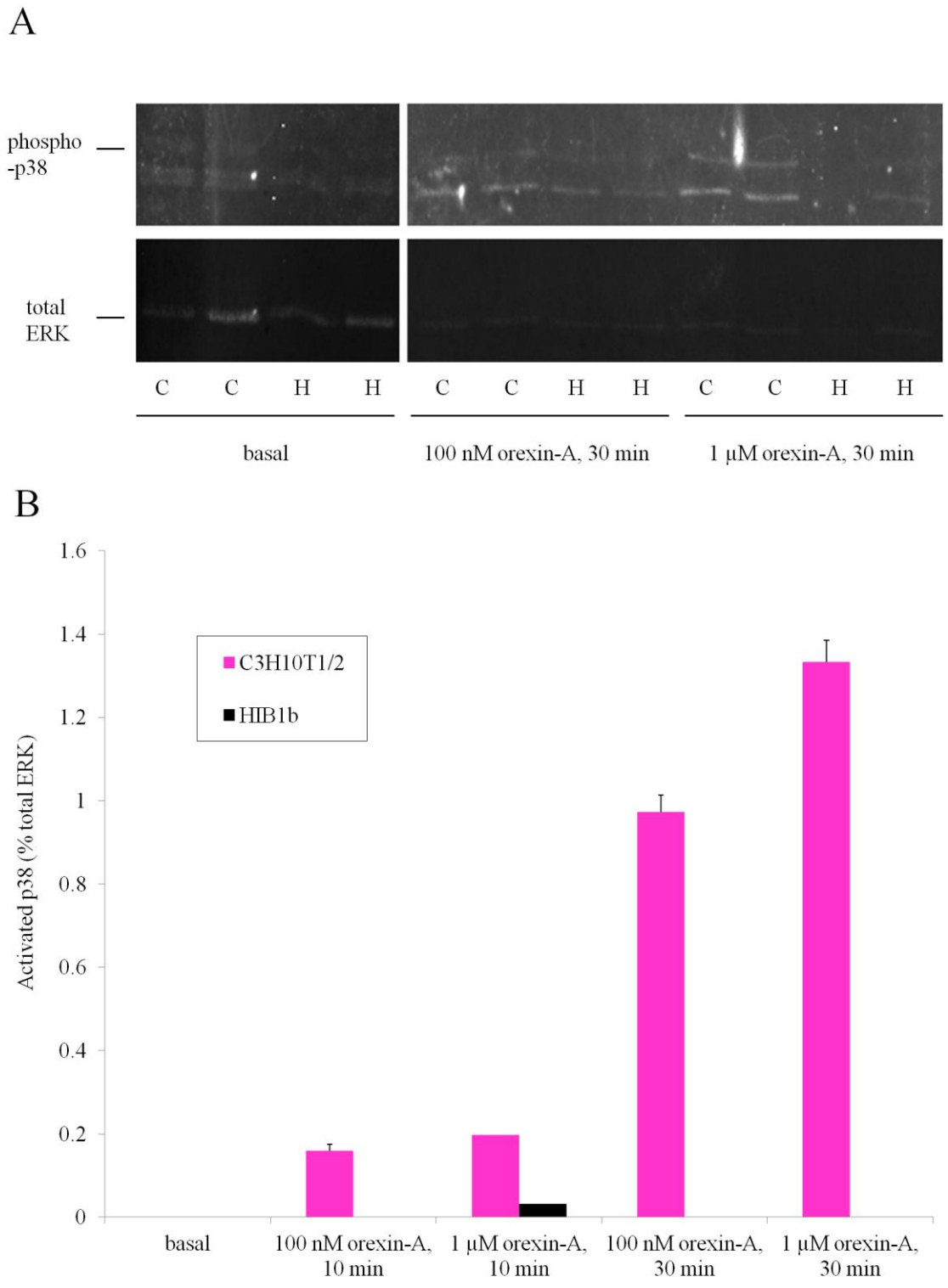

Figure 9. Western blot analysis of phosphorylated (active) p38 mitogen activated protein kinase (MAPK) and total extracellular signal-regulated kinase (ERK) (as a loading control). (A) Infrared image as captured by Odyssey Infrared Imager and software (LI-COR Inc.). C = C3H10T1/2 cells; H = HIB1b cells. (B) Activation of p38 MAPK normalized to the total ERK present in the cells. Image and data from one representative experiment; error bars represent standard deviation between two replicates.

4.3 Phospholipase D Assay

CHO-hOX_1 cells were tested in parallel with the C3H10T1/2 and HIB1b cells. The responses to the control and orexin treatments in CHO-hOX_1 cells were consistently as expected and had been previously reported (Jäntti et al., 2011) with strong activation of PLD by the both the positive control TPA and orexin-A (not shown), confirming no technical problems with the assay.

TPA stimulation in C3H10T1/2 and HIB1b cells resulted in clear activation of PLD, as can be seen from the dark PtdBut bands on the TLC plate, whereas orexin-A (1 nM – 1 µM) yielded no visible PtdBut bands (Figure 10).

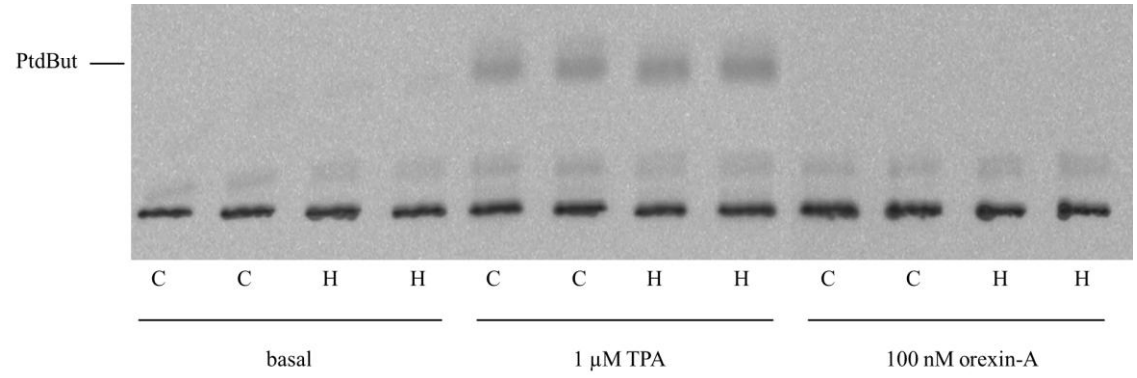

Figure 10. TLC separation of the [^{14}C]-palmitic acid containing molecules produced upon stimulation. Phosphatidylbutanol (PtdBut) is indicative of phospholipase D activity. Radioactivity image as captured by an imaging plate from TLC plate; image from one representative experiment. C = C3H10T1/2 cells; H = HIB1b cells.

After measuring the mean band intensity, the data was normalized to TPA by setting the TPA response to 100%. TPA was chosen rather than the basal controls because the orexin-stimulated responses were so low, nearly the same as basal levels, therefore any small variation may have been perceived as larger than in reality if compared to the basal. TPA gave a consistent response across all the cell lines and experiments. It is clear from Figure 11 that orexin-A produced no significant activation of PLD in either C3H10T1/2 or HIB1b cells.

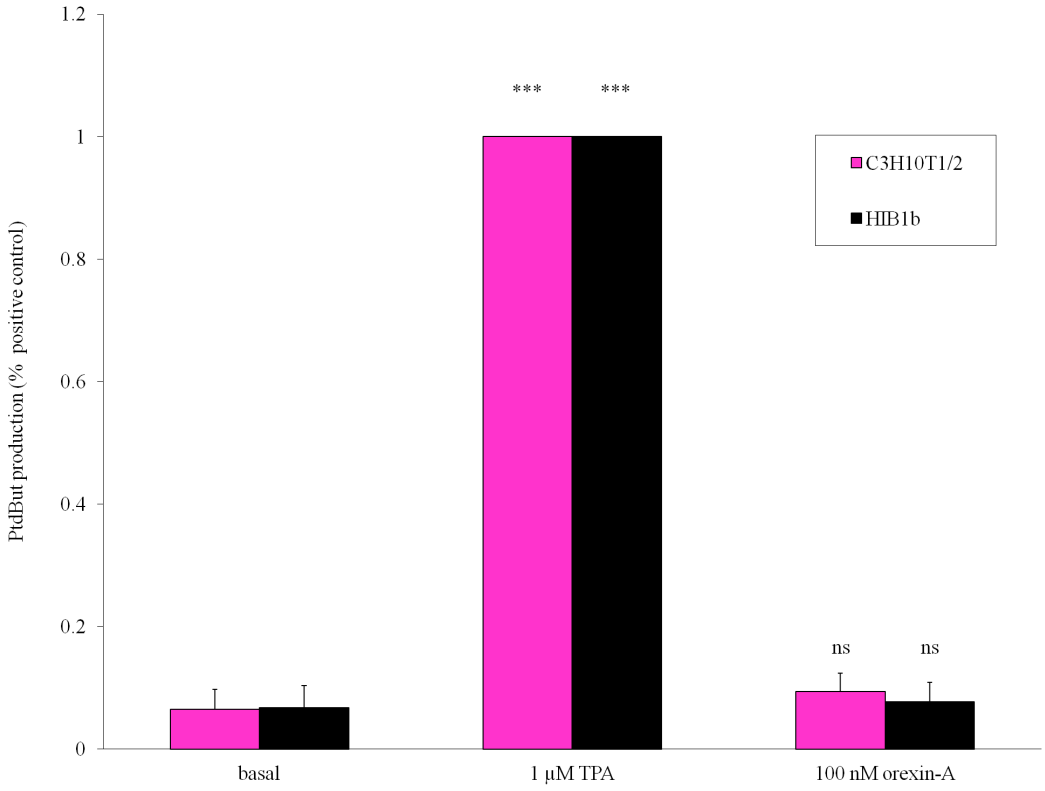

Figure 11. Effect of orexin stimulation on phospholipase D activity, as measured by phosphatidylbutanol (PtdBut) production. Data was normalized to the positive control TPA. Comparisons are to the basal controls. Error bars indicate standard error of the mean (n = 3–4); ns, not significant ($P > 0.05$); ***$P < 0.001$.

Following transduction of the cells to overexpress OX_1 receptors, HIB1b cells showed a marked increase in PLD activity upon stimulation with orexin-A (Figure 12). This demonstrates that orexin-induced PLD activation is at least possible in these cells, although it appears to not be a commonly used pathway in their native state. C3H10T1/2 cells did not show a significant difference in orexin-induced PLD activation between normal and transduced cells (Figure 12). However, C3H10T1/2 cells seemed to have more difficulty in taking up the virus, as assessed based on the GFP fluorescence using a fluorescence microscope (not shown). Various virus concentrations and incubation time periods were tried, and still these cells did not respond well to the transduction. Thus it is more probable that the lack of response is due to inefficient transduction or recombinant expression and the subsequent lack of OX_1 receptor

overexpression, rather than an incapability of OX_1 receptors to couple to PLD activation in these cells.

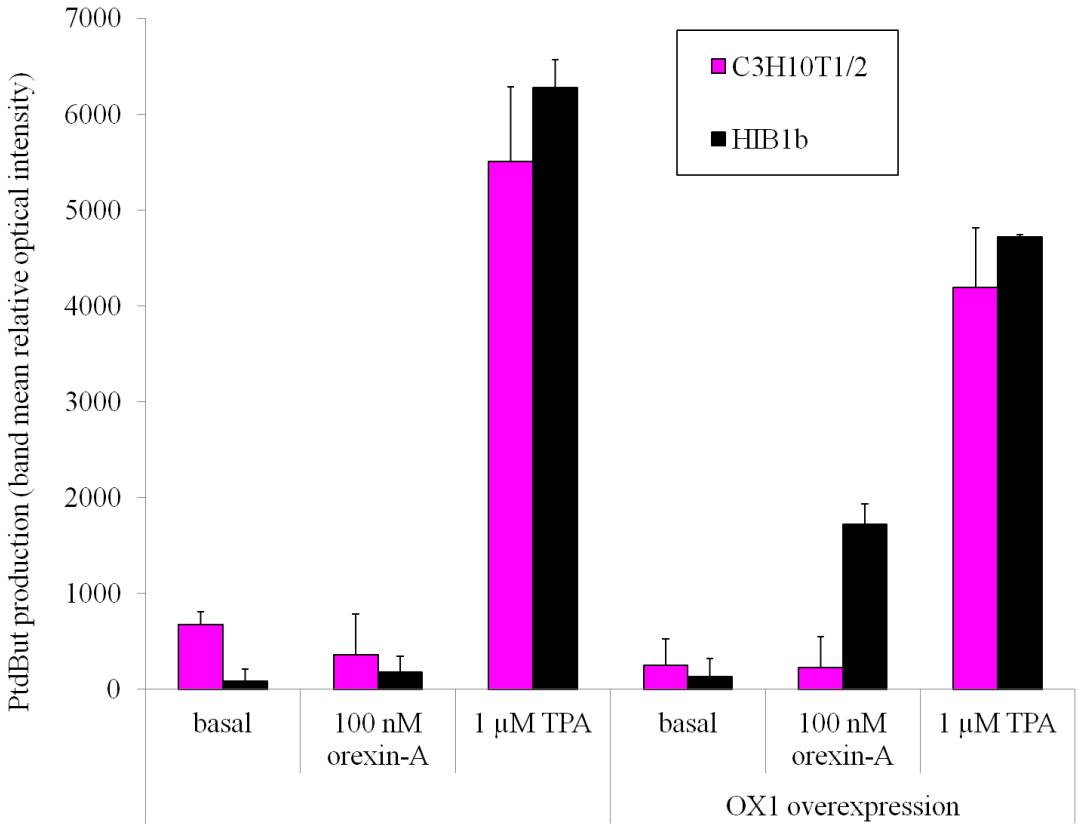

Figure 12. Effect of orexin stimulation on phospholipase D activity in cells overexpressing OX_1 receptors, as measured by phosphatidylbutanol (PtdBut) production. Data is presented as the mean relative optical intensity of the bands of interest. Representative data from a single experiment; error bars show standard deviation between two replicates.

4.4 AA and 2-AG Release Assay

CHO-hOX_1 cells were tested in parallel with the C3H10T1/2 and HIB1b cells. CHO-hOX_1 cells responded to orexin-A treatments with increased AA and 2-AG release in a concentration-dependent manner (not shown) which for the most part was consistent with the results previously published (Turunen et al., 2012). There was however some variation in the responses between experiments, raising potential concern over the efficacy of the assay.

Difficulties arose in identifying a strong positive control for this assay and no strong bands were observable on the TLC plates from any of the treatments, except for the AA band upon ionomycin treatment in C3H10T1/2 cells (Figure 13). Ionomycin was initially chosen as it stimulated release of both AA and 2-AG in the CHO-hOX_1 control cells. In C3H10T1/2 cells, ionomycin produced significant AA release, but not in HIB1b cells (Figure 14). For 2-AG release, the responses to ionomycin were not significant in either cell line (Figure 15). Other positive controls tested were thapsigargin and ATP (not shown). All drug functions are described in Table 2.

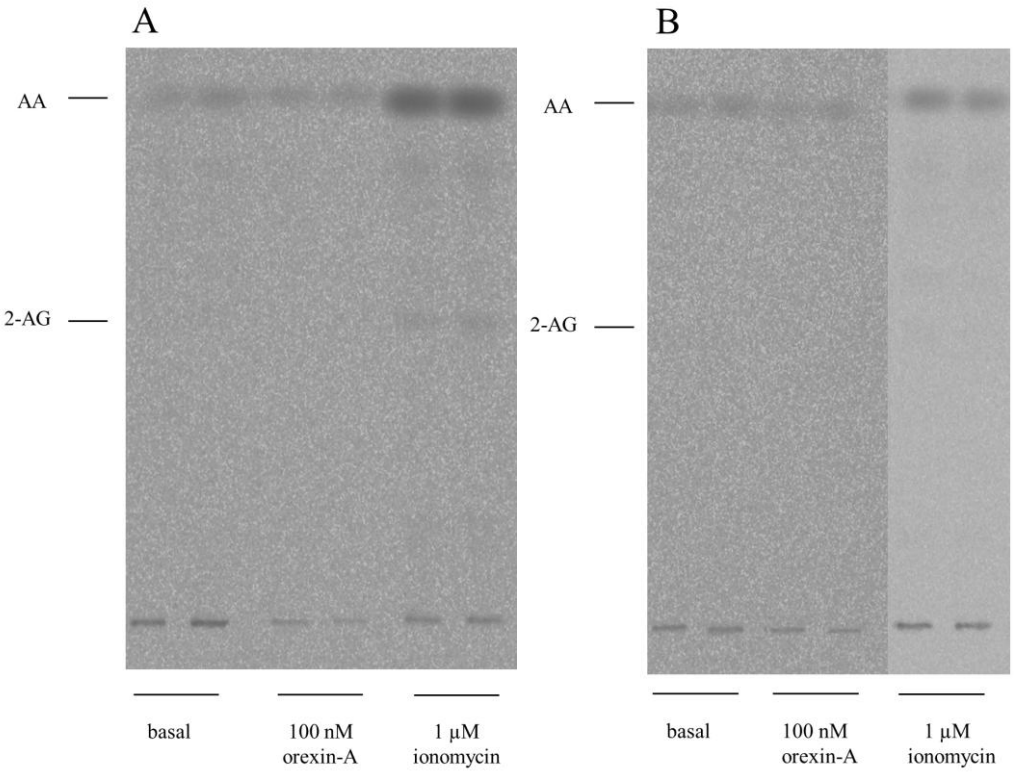

Figure 13. TLC separation of the [^{14}C]-AA lipid species arachidonic acid (AA) and 2-arachidonoyl glycerol (2-AG) released upon stimulation. Radioactivity image as captured by an imaging plate from TLC plate; images from one representative experiment. (A) C3H10T1/2 cells; (B) HIB1b cells.

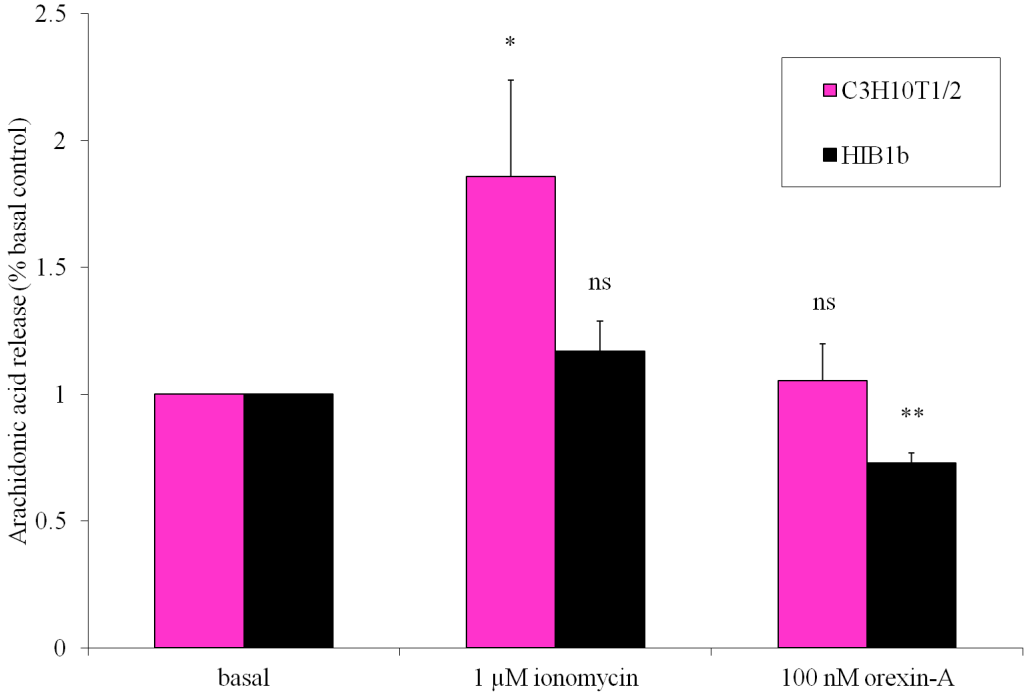

Figure 14. Effect of orexin stimulation on arachidonic acid release. Data was normalized to the basal controls. Comparisons are to the basal controls. Error bars indicate standard error of the mean (n = 3–4); ns, not significant ($P > 0.05$); *$P < 0.05$; **$P < 0.01$.

Orexin-A stimulation produced no significant change in AA release in C3H10T1/2 cells; in HIB1b cells, orexin-A significantly decreased AA release (Figure 14).

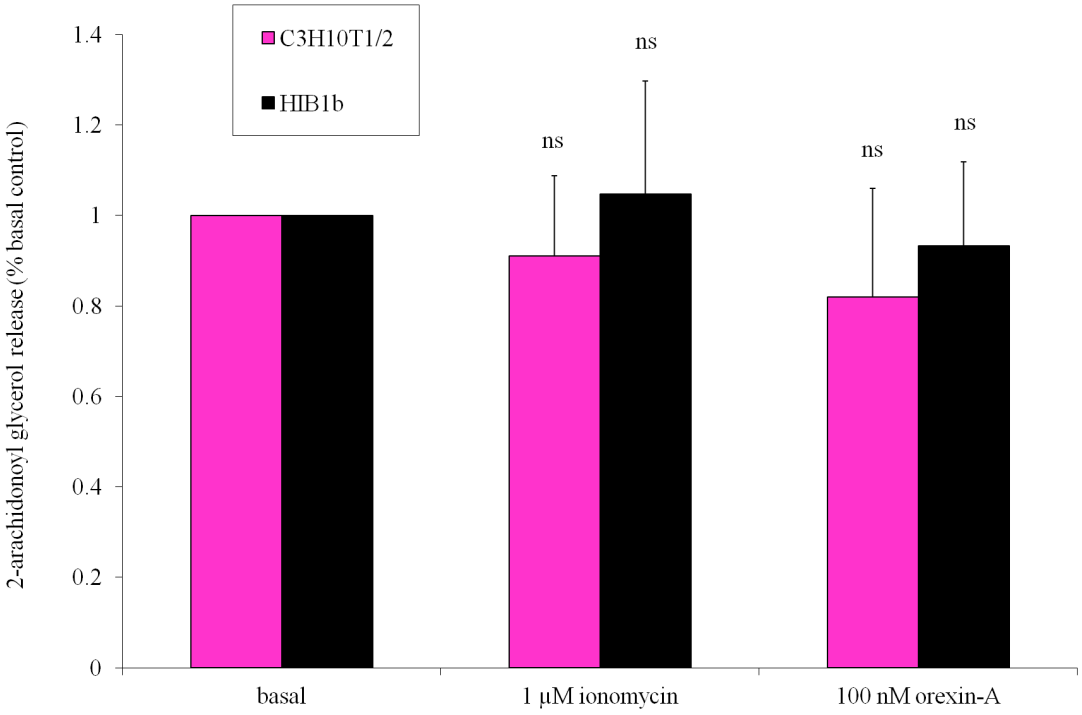

Figure 15. Effect of orexin stimulation on 2-arachidonoyl glycerol release. Data was normalized to the basal controls. Comparisons are to the basal controls. Error bars indicate standard error of the mean (n = 3–4); ns, not significant (P > 0.05).

The responses to orexin-A were not significant in either C3H10T1/2 or HIB1b cells when measuring 2-AG release (Figure 15).

When cells were transduced to overexpress OX_1 receptors, HIB1b cells displayed an increase in both AA and 2-AG release in response to orexin-A (Figures 16 & 17), demonstrating these pathways are capable of being activated by orexin in these cells. C3H10T1/2 cells did not show a change in AA or 2-AG release (Figures 16 & 17). As discussed above (4.3 Phospholipase D Assay) this may be due to low virus uptake or protein production from the virus in C3H10T1/2 cells. Therefore the same conclusion holds true, that it is probably a lack of successful transduction yielding this non-response rather than an inability of the OX_1 receptors to trigger AA and 2-AG release in these cells.

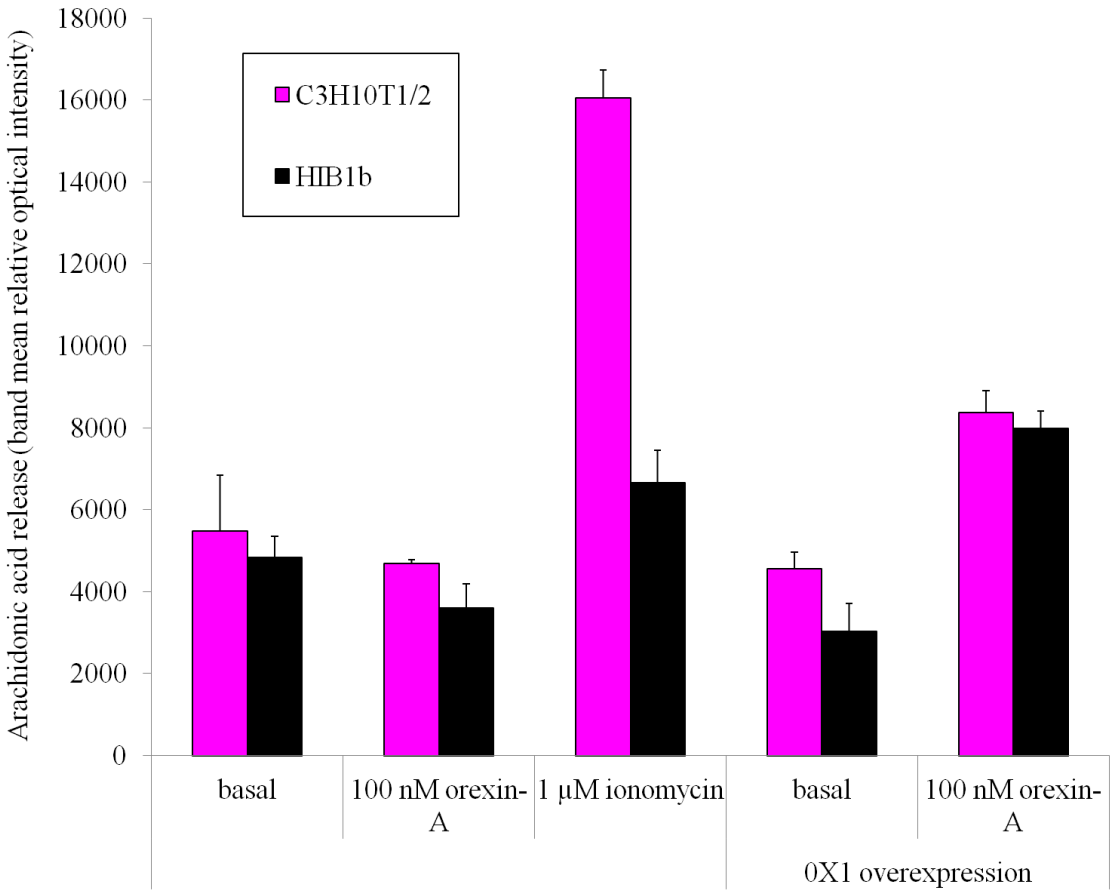

Figure 16. Effect of orexin stimulation on arachidonic acid release in cells overexpressing OX_1 receptors. Data is presented as the mean relative optical intensity of the bands of interest. Representative data from a single experiment; error bars show standard deviation between two replicates.

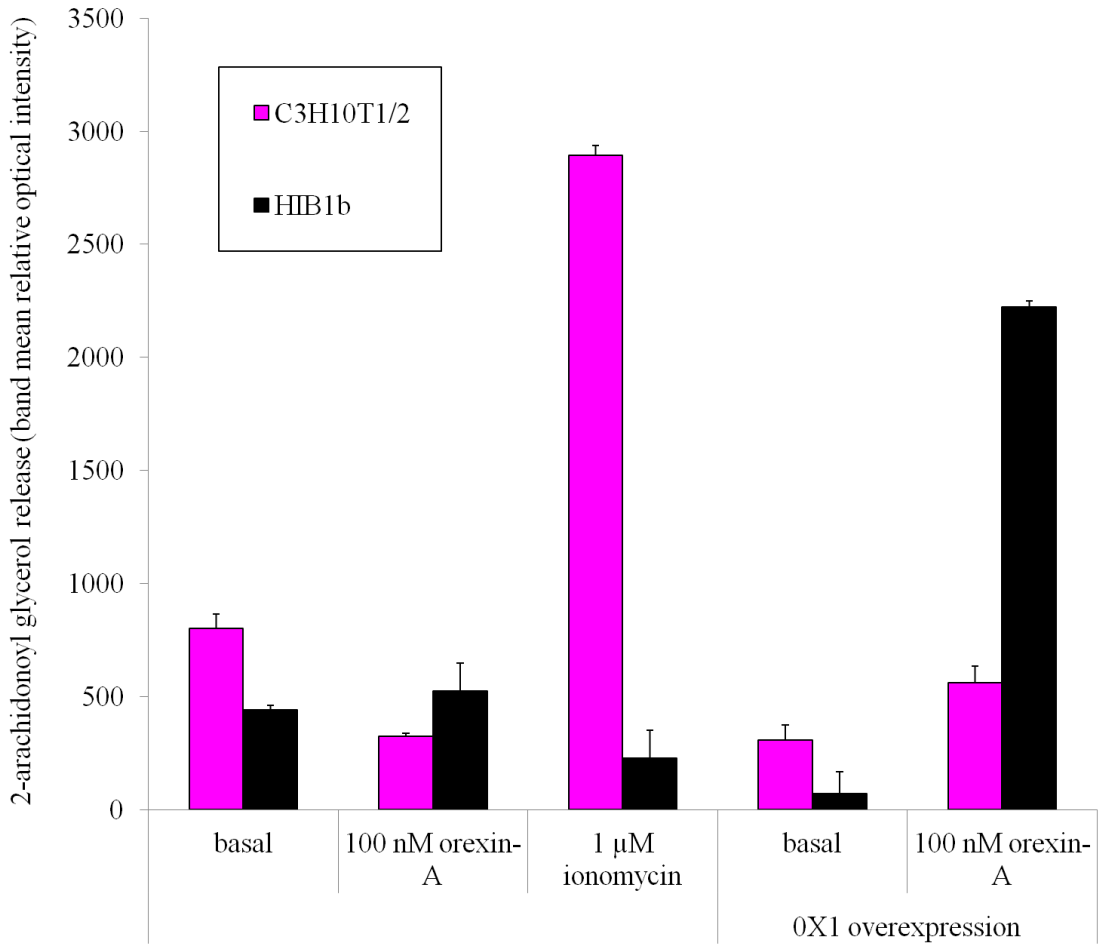

Figure 17. Effect of orexin stimulation on 2-arachidonoyl glycerol release in cells overexpressing OX_1 receptors. Data is presented as the mean relative optical intensity of the bands of interest. Representative data from a single experiment; error bars show standard deviation between two replicates.

4.5 Adenylyl Cyclase Assay

Treatment with forskolin significantly increased cAMP production in both C3H10T1/2 and HIB1b cells (Figure 18), confirming the presence of adenylyl cyclase in these cells and the feasibility of this assay. Isoproterenol, a known stimulator of the $G\alpha_s$-coupled β-adrenergic receptors, also stimulated adenylyl cyclase activation in both cell lines (Figure 19), with the response being especially strong in C3H10T1/2 cells. CHO-hOX_1 cells were tested in parallel to confirm the assay was working correctly; increased cAMP production was observed with both forskolin and orexin-A in these cells (not shown). In C3H10T1/2 and HIB1b cells, orexin-A treatments did not affect basal cAMP

production, even at concentrations as high as 1 μM, indicating that OX_1 receptors do not readily couple to $G\alpha_s$ in these cells (not shown).

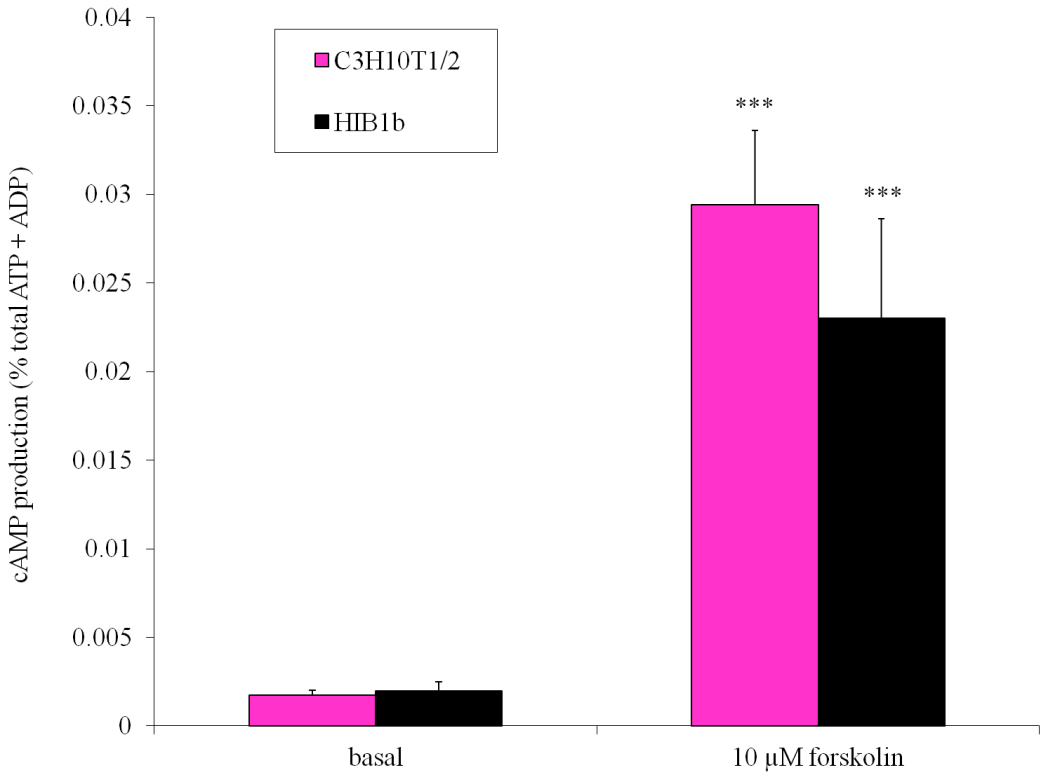

Figure 18. Activation of adenylyl cyclase by forskolin, as measured by cAMP production. Data was normalized to total ATP + ADP levels. Comparisons are to the basal controls. Error bars indicate standard error of the mean (n = 6–7); ***P < 0.001.

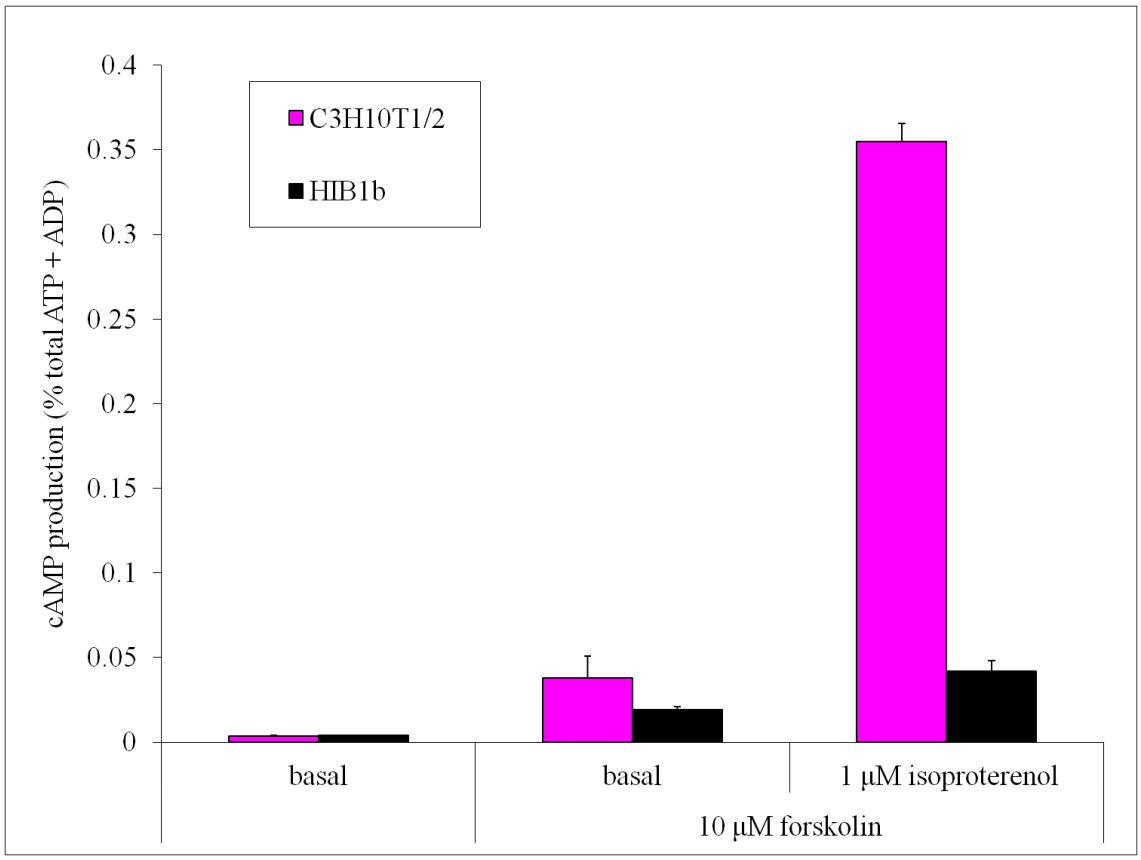

Figure 19. Effect of isoproterenol stimulation on adenylyl cyclase activity, as measured by cAMP production. Data was normalized to total ATP + ADP levels. Representative data from a single experiment; error bars show standard deviation between four replicates.

In order to further investigate this response, treatments of constant forskolin concentrations combined with various orexin-A concentrations were applied to the cells. In C3H10T1/2 cells, the result of these experiments was a biphasic curve, with increasing cAMP production at lower orexin-A concentrations and reduced cAMP production at higher orexin-A concentrations (Figure 20). Based on these results it would seem there is coupling of the OX_1 receptors to both $G\alpha_s$, producing the stimulatory effect, and to $G\alpha_i$, producing the inhibitory effect, and that this determination may be concentration-dependent. However, it must be noted that also other cascades can be involved (see 1.4.5 Influence on Adenylyl Cyclase). In HIB1b cells, no change was observed in cAMP production with any concentration of orexin-A (Figure 20).

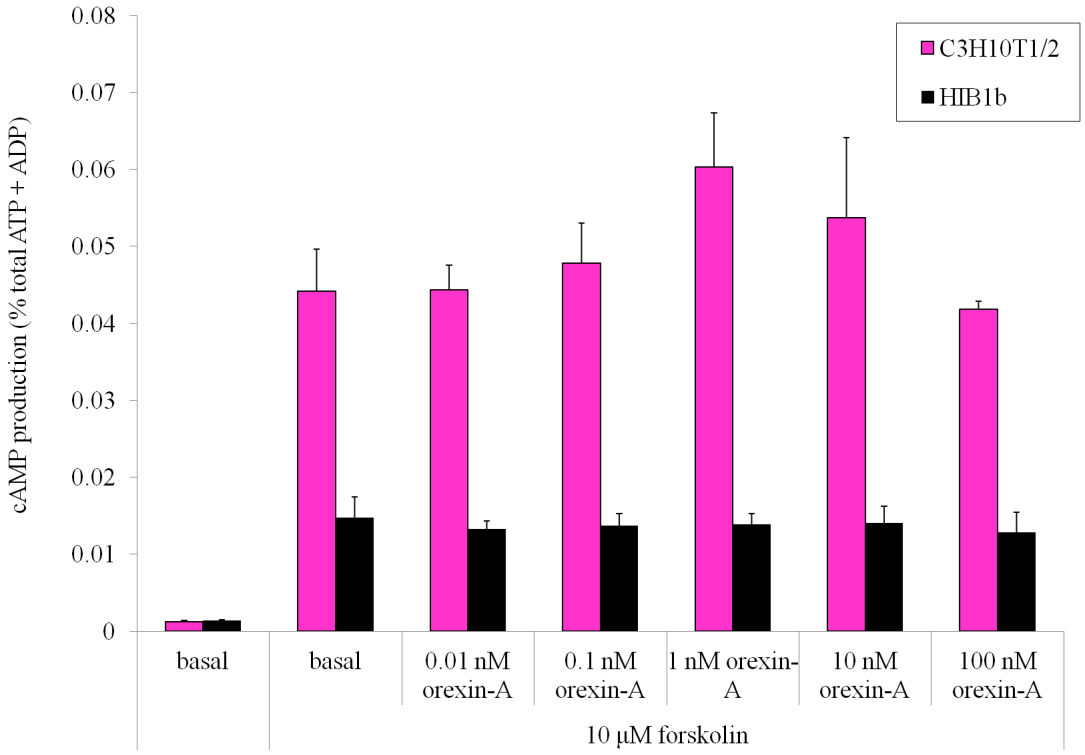

Figure 20. Effect of orexin stimulation on adenylyl cyclase activity, as measured by cAMP production. Data was normalized to total ATP + ADP levels. Representative data from a single experiment; error bars show standard deviation between four replicates.

CTx and PTx were used in an attempt to sort out the stimulatory and inhibitory effects (data not shown). The results were somewhat contradictory however, and due to time constraints further investigations were not carried out.

5 Discussion

5.1 Signalling Responses to Orexin Differ From Those Observed in Recombinant Cells

The successful activation of p38 MAPK in C3H10T1/2 cells confirmed the presence of functional orexin receptors and replicated the previous results obtained by Sellayah et al. (2011). As already discussed in the introduction (1.2.3 Orexins and Brown Adipose Tissue) the activation of p38 MAPK is necessary for these cells to differentiate into mature brown adipocytes. Orexin-induced activation of p38 MAPK has also been previously demonstrated in recombinant CHO-hOX1 cells, where it is required for induction of cell death (Ammoun & Lindholm et al., 2006).

The lack of p38 MAPK activation in HIB1b cells is concerning. However, activation had not been demonstrated previously in the HIB1b cell line and thus is potentially not a critical pathway in these cells. The alternative explanation is a lack of functional OX_1 receptor expression at the cell membrane. If this explanation is accepted, it would of course impact the rest of the studies carried out and could serve as a possible explanation for the severe lack of any response observed in HIB1b cells in any of the assays. However, orexin responses in this cell line are seen in the studies by Sellayah et al. (2011).

Failure of orexin to stimulate PLD activation in C3H10T1/2 and HIB1b cells was surprising, since there was such a strong response in the recombinant CHO-hOX_1 cells in previously published results (Jäntti et al., 2011) and our control CHO-hOX_1 cells. It was noted that orexin also failed to activate PLC in C3H10T1/2 and HIB1b cells (Putula, unpublished results); however this should not have a major impact on PLD as it was already demonstrated that orexin acts independently of PLC through PKCδ to activate PLD (Jäntti et al., 2011). Granted, these results were obtained with the recombinant cells and thus again the signalling could be operating differently in the BAT precursor cells. AA has also been linked to PKC activation (Shirai et al., 1998; Cho & Stahelin, 2006), which could be another explanation for the lack of PLD activation, based on the lack of orexin-induced AA release observed in the C3H10T1/2

and HIB1b cells. However, AA was deemed not required for PLD activation in the recombinant cells (Jäntti et al., 2011). The data from the OX_1 overexpression transduction of these cells indicates that HIB1b cells are at least capable of orexin-induced PLD activation, even if it may not be a normally significant pathway. The physiological significance of PLD activation in relation to orexins remains unknown, and therefore it is difficult to speculate as to why these BAT precursor cells would not have the same response as the recombinant cells.

The results gathered from the AA/2-AG release assay indicate no increases in release of either molecule in response to orexin in C3H10T1/2 or HIB1b cells, a result which is again in opposition to the previously available data from recombinant CHO-hOX_1 cells; HIB1b cells even showed a significant decrease in AA release upon orexin treatment. In the recombinant cells orexin stimulated an increase in release of both AA and 2-AG, resulting from the activation of the $cPLA_2$ and DAGL signalling cascades (Turunen et al., 2012). Thus once again it appears the targets of orexin signalling and cellular responses differ in the BAT precursor cells when compared to the recombinant cells. In this case however it is not so clear if the difference is entirely based on different signalling in these cells or if some part can be attributed to technical obstacles operating at the level of the assay itself. The high basal levels made sorting out true responses difficult, as did a lack of any consistent strong positive control across the cell lines. There was also some variation of the responses observed in the control CHO-hOX_1 cells. If we assume however that the differences observed are truly different cellular responses, perhaps it makes sense that AA and 2-AG release are not so important in BAT precursor cells. Brown adipose tissue acts as a defense mechanism against weight gain, where as AA content in adipose tissue has been associated with obesity in humans (Savva et al., 2004). In terms of differentiation, Peterson et al. (2003) demonstrated that AA inhibits the differentiation of 3T3-L1 preadipocytes to adipocytes. This may also be true then for the C3H10T1/2 and HIB1b precursor cells, and we should not expect to see AA increases then if orexin is triggering differentiation to brown adipocytes. Endocannabinoids such as 2-AG have been strongly linked to adipose tissue, adipocyte growth and differentiation, and overall energy balance (Pagano et al., 2008; Uberto, 2008). Increased 2-AG and overactivation of the endocannabinoid system are associated with obesity, and treatment of brown adipocytes with endocannabinoid receptor agonists decreases the expression of UCP1 (Jbilo et al., 2005; Perwitz et al., 2006), a

protein whose increased expression is one of the hallmarks of BAT; conversely endocannabinoid receptor antagonists reverse diet-induced weight gain by increasing energy expenditure and BAT function (Jbilo et al., 2005), and possibly induce transdifferentiation of WAT to BAT (Uberto, 2008). Extrapolating these observations to the C3H10T1/2 and HIB1b cells, again perhaps it makes sense that orexin does not stimulate an increase in 2-AG release. When looking at the upstream activity, AA and 2-AG release could be partially influenced by PLD and PLC activity. Since neither of these enzymes was activated by orexin in the BAT precursor cells, it would follow then that the downstream release of AA and 2-AG would also be impaired.

The adenylyl cyclase assay yielded some novel positive results. While orexin itself did not produce noticeable changes in cAMP production, when combined with forskolin (a strong activator of adenylyl cyclase) it became apparent that orexin may trigger both stimulatory and inhibitory effects on adenylyl cyclase. This was suggested due to the biphasic nature of the curve observed, which was reminiscent (although opposite) to the curve observed in CHO-hOX_1 cells upon orexin stimulation (Holmqvist et al., 2005). It has already been noted that the response of adenylyl cyclase to orexin is rather complex, as discussed in the introduction (1.4.5 Influence on Adenylyl Cyclase). The capability of GPCRs to couple to multiple G-proteins is well documented and provides a challenge when trying to determine specific receptor responses (reviewed in Kukkonen, 2004). GPCRs can assume multiple active forms, each of which interact differently with the associated G-proteins; a phenomenon termed stimulus trafficking describes how the ligands themselves can promote specific active conformations (reviewed in Kukkonen, 2004). The multiple isoforms of adenylyl cyclase in existence, each of which respond and are regulated independently, are another source of variation complicating the determination of specific receptor-triggered effects (Patel et al., 2001).

CTx and PTx can be used to separate some inhibitory and stimulatory effects on adenylyl cyclase. CTx catalyzes ADP-ribosylation of $G\alpha_s$ thereby eliminating its capability to hydrolyze GTP $\rightarrow$ GDP + Pi. This locks $G\alpha_s$ in the GTP-bound active form, causing continual activation of adenylyl cyclase (Sanchez & Holmgren, 2011). Therefore, the treatments on cells previously treated with CTx should show only inhibitory effects, assuming that adenylyl cyclase is saturated with $G\alpha_s$. PTx catalyzes ADP-ribosylation of $G\alpha_i$ which prevents it from interacting with GPCRs at the

membrane and thus prevents its ability to inhibit adenylyl cyclase (Burns, 1988). Therefore, the treatments on cells previously treated with PTx should show only stimulatory effects. Unfortunately, the studies with CTx and PTx did not bring a clearer understanding of these inhibitory and stimulatory components of the orexin response. Due to time constraints further investigation was not possible within the scope of this thesis, thus this area will be a target for future studies.

As for the physiological relevance of adenylyl cyclase activity, the expression of UCP1 in BAT and subsequent onset of thermogenesis is dependent upon increased cellular cAMP levels (Lafontan & Berlan, 1993). cAMP is a well known activator of PKA as well as other kinases; in the case of BAT increased cAMP can activate p38 MAPK, which plays a major role in regulating transcription of the UCP1 gene (Cao et al., 2004) and initiating differentiation to mature brown adipocytes (Cao et al., 2001; Cao et al., 2004; Sellayah et al., 2011).

5.2 Limitations and Sources of Error

General sources of error for all of the experiments can arise from the cell culture phase. Accurate cell counting and splitting of cells are necessary to ensure comparable concentrations. Cell growth and confluency had to be carefully monitored, as it was noted that both C3H10T1/2 and HIB1b cells have a tendency for spontaneous differentiation if they become too confluent. Differentiation would of course change the cells' properties and subsequently affect signalling and responses to the treatments. During the sample preparation and treatment incubations the cells had to be handled gently as to not induce activation by any physical force.

One problem encountered with the western blot analysis was bleed-through of signal between the two infrared channels. This became an issue because of the very close proximity of the bands from the total ERK control and the phospho-p38 MAPK bands, due to their similar size. It would have been nice to have a different baseline control that was further away from the phospho-p38 MAPK, however total ERK gave a consistent and clear signal and was convenient for normalizing the data. Thus as long as care was

taken when adjusting the parameters for scanning and measuring the band intensity, it was acceptable to continue using total ERK as the baseline control.

Potential limitations of the PLD and AA/2-AG assays lie in the extraction efficiency of the lipids from the lysed cells/supernatant. Sources of error can also be found in the assessment of the bands and targeting the area for measurement of band intensity. Additionally, it is not possible to assign a specific source to the AA/2-AG produced, as these molecules can be produced via multiple pathways (see 1.4.4 Generation of Lipid Messengers). Therefore it also holds that a lack of AA/2-AG release could be a result of a number of different upstream enzymes not being activated.

A source of variation specific to the adenylyl cyclase assay would be in the composition of the columns. Any dirt or contamination on the columns will affect the run-through or binding of the molecules and therefore the separation and final concentrations available for measuring. In addition, direct assumptions of coupling to $G\alpha_s$ and $G\alpha_i$ made from cAMP concentration measurements carry an innate risk as cAMP levels may also be regulated in other ways, however use of the inhibitor solution should prevent this.

5.3 Future Directions

It is clear from the results presented here that orexin signalling does not behave the same in all cell/tissue types, and between native and recombinant receptor expression. This suggests a need for further studies in cell lines of different origin with native orexin receptor expression. The identification of such cells will lead to more possibilities to study the effects of orexin at the cellular level, and can hopefully lead to a better understanding of the physiological significance of the various cellular responses observed. This will also help to unravel a better overall picture of the role for orexins, and potentially open up new therapeutic opportunities targeted at the various aspects of orexin signalling.

In terms of BAT, the next logical experiments would be to continue with the adenylyl cyclase assays to confirm the dynamics of the seemingly stimulatory and inhibitory

effects of orexin on that pathway, and secondly, to assess the role of cAMP in the BAT differentiation.

6 Conclusions

This study originated with the goal of examining orexin signalling in two mouse BAT precursor cell lines with native OX_1 receptor expression, to gain insight into the cellular responses induced by orexin and compare to the responses previously shown in recombinant cells. It was expected that the cellular responses to orexin in C3H10T1/2 and HIB1b cells would be the same or similar to those observed previously in CHO cells recombinantly expressing the OX_1 receptors. This was not the case however. Taken altogether, the results of this thesis indicate that responses to orexin signalling in the BAT precursor cells are not the same as when orexin receptors are recombinantly expressed, nor are they necessarily the same even between the two BAT precursor cell lines. This raises interesting questions regarding the variability of responses to orexin in different tissue types, and demonstrates the importance of finding more cell lines which natively express orexin receptors. Studying the cellular responses to orexin and determining the physiological significance of these responses in specific tissue types or cell lines will lead to a better overall understanding of orexin signalling and potentially reveal new therapeutic applications for orexin-related disorders. Additionally, it may be possible through such studies to determine the factors leading to tissue-specific signalling of GPCRs.

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9 Appendix 1. Solutions

9.1 Lysis Buffer for Western Blot Analysis, pH 7.5

TKB buffer*	desired volume
phosSTOP	1:10
protease inhibitor	1:100
PMSF	1:100

*TKB buffer = 50 mM HEPES, 150 mM NaCl (pH 7.5) + 10% (v/v) glycerol, 1% (v/v) Triton X-100, 1.5 mM $MgCl_2$, 1 mM EDTA, 10 mM Na^+-pyrophosphate, 1 mM Na^+-orthovanadate, 10 mM NaF, 250 µM *p*-nitrophenol phosphate

phosSTOP phosphatase inhibitor cocktail (Roche, Mannheim, Germany)
protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA)
PMSF, phenylmethylsulfonylfluoride

9.2 Gel Recipe used in SDS-PAGE (1 gel)

Lower Gel, 10%:

40% acrylamide	1.25 mL
H_2O	2.5 mL
1.5 M Tris-HCl buffer, pH 8.8	1.25 mL
10% SDS	50 µL
10% APS	50 µL
TEMED	2 µL

APS, ammonium persulfate
TEMED, N,N,N′,N′-tetramethylethane-1,2-diamine

Upper Gel, 5%:

40% acrylamide	250 µL
1M Tris-HCl buffer, pH 6.8	256 µL

H_2O	1.5 mL
10% SDS	21 μL
10% APS	21 μL
TEMED	2 μL

9.3 Tris-Buffered Saline (TBS) Recipe (10X), pH 7.6

Tris base	20 mM
NaCl	137 mM

Adjust the pH with a strong HCl solution.
Add H_2O to 1000 mL final volume.

9.4 Na^+-Elliot Buffer Recipe (10X), pH 7.4

NaCl	1370 mM
KCl	50 mM
$MgCl_2 \cdot 6H_2O$	12 mM
$NaHCO_3$	42 mM
KH_2PO_4	4.4 mM
HEPES	200 mM

Add H_2O to 1000 mL final volume.
Add 1 mM $CaCl_2$ and 10 mM glucose from 1 M stock solutions when used.