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Regulation of the orexin 1 receptor by β-arrestins

A thesis presented for degree of
Doctor of Philosophy

by

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Abbreviations

A	adenine
α	alpha subunit of G protein
aa	amino acid
ADP	adenosine-5'-diphosphate
Ala (A)	alanine
Arg (R)	arginine
Asn (N)	asparagine
Asp (D)	aspartic acid
ATP	adenosine-5'-triphosphate
β	beta subunit of G protein
β-arr2	beta-arrestin 2
BCA	bicinchoninic acid
βγ	beta-gamma dimer of G protein
8-bromo-cGMP	8-bromoguanosine 3`-5`-cyclic monophosphate
BSA	bovine serum albumin
C	cytosine
cDNA	complementary DNA
CHO	chinese hamster ovary
Ci	Curie
C-terminus	carboxy-terminus
Cys (C)	cysteine
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside triphosphate
DTT	dithriothreitol
E. Coli	*Escherichia Coli*
EDTA	ethylenediamine tetra-acetic acid
EGTA	ethylene glycol tetra-acetic acid
ERK	extracellular signal-regulated kinase
FBS	foetal bovine serum
γ	gamma subunit of G protein
g	gram

G	guanine
GDP	guanosin 5-diphosphate
G protein	guanine nucleotide binding protein
G_i	inhibitory G protein
G_s	stimulatory G protein
GF109203X	bisindolylmaleimide I hydrochloride
GFP	green fluorescent protein
Gln (Q)	glutamine
Glu (E)	glutamic acid
Gly (G)	glycine
GPCR	G protein-coupled receptor
GRK	GPCR kinase
GTP	guanosin 5-trisphosphate
h	hour
H89	N-[2-((*p*-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide, 2HCl
HA	haemagglutinin
HCl	hydrochloric acid
HEK	human embryonic kidney
HEPES	(N-[2-hydroxeythl] piperazine-N'-[2-ethanesulphonic acid])
His (H)	histidine
HRP	horseradish peroxidase
Ile (I)	isoleucine
IPTG	isopropyl β-D-thiogalactopyranoside
i2 loop	intracellular loop 2
JNK	c-jun N-terminal kinase
kb	kilo base
kDa	kilo Dalton
L (l)	litre
LB	L-broth (Luria-Bertani medium)
Leu (L)	leucine
Lys (K)	lysine
m	milli
μ	micro

M	molar
MAPKKK	MAPK kinase kinase
MAPKK	MAPK kinase
MAPK	mitogen-activated protein kinase
Met (M)	methionine
min	minute(s)
mol	mole
MOPS	4-morpholinepropanesulfonic acid
mRNA	messenger ribonucleic acid
NBCS	new born calf serum
n	nano
N-terminus	amino-terminus
ox1R	orexin 1 receptor
p	pico
^{32}P	phosphorus-32
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
PMA	phorbol 12-myristate
Pro (P)	proline
PVDF	polivinylidenflouride
R	receptor
RFP	red fluorescent protein
RGS	regulators of G protein signalling
RIPA	radioimmunoprecipitation
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
S.E.	standard error
sec	second
Ser (S)	serine
T	thymine

TBS	tris-buffered saline
TCA	trichloroacetic acid
Thr (T)	threonine
TM	transmembrane
Tris	tris(hydroxymethyl)aminomethane
Trp (W)	tryptophan
Tyr (Y)	tyrosine
Val (V)	valine
VSV-G	vesicular stomatitis virus glycoprotein
YFP	yellow fluorescent protein

Summary

The orexin 1 receptor was identified as an orphan G protein-coupled receptor (GPCR) in 1998 (Sakurai *et al.,* 1998). There is great interest in the orexin receptor system since it is involved in the control of feeding and energy metabolism (Sakurai *et al.,* 1998), the modulation of neuroendocrine function (van den Pol *et al.,* 1998; Smart, 1999) and the regulation of the sleep –wake cycle (Smart, 1999). However not much is known about the regulation of the orexin 1 receptor following stimulation. β-arrestins bind agonist-activated, phosphorylated GPCRs and mediate their desensitisation and internalisation. They may also function as GPCR signal transducers. The aim of this thesis was to investigate internalisation and signalling of the orexin 1 receptor and the involvement of β-arrestins in these processes.

In HEK293T cells expressing wild type orexin 1 receptor, orexin A stimulation triggered β-arrestin 2 binding to the receptor and co-internalisation of receptor-β-arrestin complexes *via* clathrin-coated vesicles into acidic endosomes, in a dynamin-dependent manner. Moreover, studies of receptor internalisation in wild type, β-arrestin-, Src family kinase-or $G_{q/11}$-deficient mouse embryo fibroblasts revealed sequestration of the orexin 1 receptor to be β-arrestin-dependent, but G protein-and Src-independent.

Mutational analysis of the orexin 1 receptor demonstrated that high affinity binding between the receptor and β-arrestin 2 was conferred by a single cluster of Ser/Thr residues at the extreme C-terminus. Although this mutant form of the receptor was no longer able to co-internalise with β-arrestin 2, the pathway and time course of receptor internalisation was unaltered.

In CHO cells, orexin A challenge induced rapid receptor phosphorylation which was partly mediated by protein kinase A (PKA) and protein kinase C (PKC). Surprisingly the levels of phosphorylation were similar for the cluster C1 mutant indicating the principal phosphorylation site to be distinct from the cluster of Ser/Thr residues essential for agonist-induced recruitment of β-arrestins.

To investigate the signalling pathways elicited by addition of orexin A, mutant forms of the orexin 1 receptor unable to stimulate G protein signalling were generated. Activation of the orexin 1 receptor caused an increase in ERK1/2 activity by a process depending on an intact endocytic pathway since inhibition of endocytosis by concanavalin A or dominant negative dynamin resulted in attenuated ERK1/2 phosphorylation. However hyperosmolar levels of sucrose had no effect on ERK1/2 activation. In addition, orexin A challenge of

Src family kinase knock out MEF cells expressing the orexin 1 receptor resulted in ERK1/2 stimulation. There was significant difference in the time course of ERK1/2 phosphorylation upon stimulation of the wild type receptor and the cluster C1 mutant. On the other hand, no increase in ERK1/2 phosphorylation could be observed for the mutants unable to activate G proteins. Agonist challenge of the wild type receptor also caused stimulation of the JNK MAPK pathway. In contrast to the ERK1/2 MAPK pathway stimulation of all mutants tested resulted in increased JNK activity. p38 another member of the MAPK family was not activated after agonist challenge of the orexin 1 receptor excluding an involvement of this MAPK in orexin 1 receptor signalling.

Taken together these results show that a single cluster of hydroxy amino acids within the C-terminus of the orexin 1 receptor determines the affinity of the interaction with β-arrestin 2. They also indicate a key role of β-arrestin scaffolding in fine tuning the kinetics of orexin 1 receptor-mediated, G protein-dependent ERK1/2 activation.

Chapter 1

Introduction

1.1 Orexins

1.1.1 Orexin neuropeptides

Sakurai *et al.* (1998) identified two novel hypothalamic neuropeptides. Since these peptides stimulated food consumption when administered centrally, and their production was influenced by the nutritional state of the animal, they were named orexin A and B, after the Greek word orexis, which means appetite. At the same time as Sakurai *et al.*, (1998) De Lecea and colleagues (1998) used the subtraction cloning method to identify hypothalamic peptides. During this process they found two neuropeptides produced from a common precursor. Since these peptides are predominantly localised in the hypothalamus and their sequence is homologue to secretin, they called the peptides hypocretin-1 and -2. Hypocretin-1 and –2 turned out to be identical to orexin A and B, respectively.

Orexin A and B are hypothalamic neuropeptides encoded by a single 130 (rat) and a 131 (human) amino acid mRNA transcript transcribed into prepro-orexin, which is proteolytically cleaved to give orexin A and orexin B (Sakurai *et al.* 1998). Orexin A consists of 33 residues with an N-terminal pyroglutamyl residue and C-terminal amidation. It also contains four Cys residues forming two disulphide bonds. The primary structure of orexin A is identical between human, rat, and mouse (Sakurai *et al.*, 1998). Orexin B, also carrying an amidated C-terminus, has 28 residues and shares 46 % sequence homology with orexin A. Mouse and rat orexin B peptides are identical whereas human orexin B has two amino acid substitutions compared with the rodent sequences (Sakurai *et al.*, 1998).

1.1.2 Orexin receptors

The orexins activate two closely related G protein-coupled receptors (GPCRs), orexin 1 receptor and orexin 2 receptor (Figure 1.1). The orexin 1 receptor is the orphan GPCR used to identify and then purify the orexins. The orexin 1 and 2 receptors share 64 % homology to each other and are structurally most similar to other neuropeptide receptors like the Y_2 neuropeptide Y receptor, followed by the thyrotropin-releasing hormone receptor, the cholecystokinin type-A receptor and the NK_2 neurokinin receptor. Both the orexin 1 receptor as well as the orexin 2 receptor seem to be highly conserved between species since 94 % of the amino acids in case of the orexin 1 receptor and 95 % in case of the orexin 2 receptor are identical between the human and rat homologue.

Competitive radioligand binding assays revealed that orexin A binds to the orexin 1 and the orexin 2 receptor with high affinity whereas orexin B has a 10-100 fold lower affinity and potency for the orexin 1 receptor compared to the orexin 2 receptor. Therefore the orexin 1 receptor is selective for orexin A, while the orexin 2 receptor is a non-selective receptor for orexin A and orexin B. Binding of these ligands is associated with an increase in intracellular calcium concentrations. However, the mechanism involved is still not clear, since one group reported mobilisation from intracellular stores (Sakurai *et al.*, 1998) and another reported PKC-mediated calcium influx (van den Pol *et al.*, 1998). Furthermore Smart *et al.* (1999) showed that activation of the orexin 1 receptor with orexin A or B resulted in a biphasic calcium response. This response consisted of a phospholipase C mediated calcium mobilisation from intracellular stores, and a secondary influx of extracellular calcium. On the other hand Lund and colleagues (2000) described the orexin 1 receptor to activate a novel calcium influx pathway from extracellular calcium stores and to directly stimulate phospholipase C. They further claimed that these two responses converged at the level of phospholipase C where the first response enhances the potency of the second one.

Experiments carried out in receptor-transfected cell lines and isolated receptor-expressing hypothalamic neurons indicated that the orexin 1 receptor is exclusively coupled to the G_q subclass of heterotrimeric G proteins. In contrast the orexin 2 receptor seems to be able to couple to $G_{i/o}$ and G_q (Sakurai *et al.*, 1998; van den Pol *et al.*, 1998; Zhu *et al.*, 2003).

1.1.3 Neuroanatomy of the orexin system

The mRNA for the precursor peptide is synthesised in neurons in the lateral and posterior hypothalamic areas and the perifornical nucleus of the adult rat brain (Sakurai *et al.*, 1998; de Lecea *et al.*, 1998; Broberger *et al.*, 1998). The lateral hypothalamus is a region of the brain, which has been implicated in feeding, energy homeostasis, arousal and motivated behaviour (Bernardis and Bellinger, 1993, Bernardis and Bellinger 1996). This is supported by the fact that animals with lesions in the lateral hypothalamus exhibit hypophagia, an increased metabolic rate and decreased arousal that frequently leads to death by starvation.

Interestingly, orexin neurons also express mRNAs for the orexigenic opioid dynorphin, the appetite stimulating neuropeptide galanin and the leptin receptor, the latter being a hormone produced mainly by adipocytes (Risold *et al.*, 1999; Hakansson *et al.*, 1999).

Neurons expressing melanin–concentrating hormone, also an orexigenic peptide, like orexin neurons are found in the lateral hypothalamus. However, orexin and melanin–concentrating hormone neurons are distinct and independent neuronal populations within the lateral hypothalamic area (Broberger *et al.,* 1998; Elias *et al.*, 1998).

Orexin-containing neurons project from the point of origin to numerous brain regions. The limbic system, monoaminergic and cholinergic nuclei of the brainstem and hypothalamic sites such as the locus coerulus, the arcuate nucleus, the paraventricular nucleus and the dorsal raphe receive particularly strong innervations (Peyron *et al.,* 1998; Date *et al.*, 1999; Nambu *et al.,* 1999). Orexin peptides are unique among hypothalamic neuropeptides as they, by directly acting on axon terminals of neuroendocrine cells in the arcuate nucleus, can increase the release of the major inhibitory transmitter, γ-aminobutyric acid (GABA), as well as the major excitatory transmitter, glutamate, which are together regulating almost all synaptic activity in the hypothalamus (van den Pol *et al.,* 1998).

In situ hybridisation data confirm that the orexin receptors are expressed in a pattern consistent with orexin projections, but that they are differently distributed (Trivedi *et al.,* 1998). The orexin 1 receptor mRNA is highly expressed in the prefrontal cortex, hippocampus, paraventricular thalamus, ventromedial thalamus, arcuate nucleus, dorsal raphe nucleus and locus coerulus. Orexin 2 receptor mRNA on the other hand is mainly expressed in nucleus accumbens, subthalamic and paraventricular thalamic nuclei and anterior pretectal nucleus. Apart from the central nervous system, orexin receptor mRNA expression has also been reported in the adrenal gland, enteric nervous system and pancreas (Malendowicz *et al.,* 1999; Kirchgessner and Liu, 1999).

1.1.4. Involvement of orexin in the regulation of feeding

The localisation of orexin neurons in the hypothalamus, a key site for regulating appetite and satiety, indicates an involvment of these two neuropetides in the regulation of feeding. This hypothesis is supported by the fact that orexin A stimulated food consumption in a dose dependent manner within 1 hour, when given intracerebroventricularly in the early light phase. Orexin B also increased food intake, but the effect of orexin B did not last as long as that of orexin A. Both peptides stimulated food intake significantly less than the orexigenic peptide neuropeptide Y (Sakurai *et al.*, 1998). As neuropepeptide Y, orexin A also stimulates food intake via activation of opioid receptors (Clegg *et al.*, 2002). The physiologic relevance of feeding effects of orexins is further supported by the finding that central administration of a neutralising anti-orexin antibody significantly and dose-dependently suppressed spontaneous feeding in fasted rats (Yamada *et al.*, 2000). Cai and co-workers (1999) found that prepro-orexin mRNA was upregulated under conditions of prolonged fasting (48 hours) and acute hypoglycemia, but only if food was withheld leading them to the conclusion that orexin neurons belong to the glucose-sensitive subpopulation of neurons in the lateral hypothalamus that are stimulated by falls in circulating glucose and inhibited by signals related to feeding.

The orexigenic effect of orexin A seems to be well established. However the role of orexin B in feeding remains controversial. In contrast to Sakurai *et al.* (1998), Haynes and co-workers (1999) could not detect any effect of orexin B on feeding whereas Edwards and colleagues (1999) saw an effect of orexin B on feeding only on some occasions. The enhanced potency of orexin A compared to orexin B suggests that the effect on feeding is mediated by the orexin 1 receptor. Using a selective orexin 1 receptor antagonist, SB-334867-A (Smart *et al.*, 2001), Haynes *et al.* (2000) were able to block the orexigenic effect of orexin A in male and female rats, thus corroborating the idea that the orexin 1 receptor mediates the orexigenic response to orexin A and stimulation of the orexin 1 receptor is necessary for normal feeding. However, since orexin A binds equally well to both receptors, an involvement of orexin 2 receptors in feeding cannot be fully excluded.

1.1.5 Orexins regulate the sleep-waking cycle

Orexin neurons send projections to monoaminergic and cholinergic centres controlling sleep/wakefullness in the hypothalamus and brainstem (Chemelli *et al.*, 1999; Peyron *et al.*, 1998). A role for orexins in this process was supported by the findings that orexin neuropeptide knock out mice have a phenotype that is remarkably similar to the human sleep disorder narcolepsy (Chemelli *et al.*, 1999). Similar findings were reported for transgenic mice in which orexin-containing neurons are ablated (Hara *et al.*, 2001). In addition, Lin and colleagues (1999) found that canine narcolepsy is caused by a mutation in the orexin 2 receptor gene. Whereas orexin 2 receptor knock out mice are similarly affected with behavioural attacks of non-rapid eye movement (REM) sleep ("sleep attacks") as orexin knock out mice, they are less severely affected with cataplexy (sudden bilateral loss of postural muscle tone triggered by emotions) like attacks (Chemelli *et al.*, 1999; Willie *et al.*, 2003). Orexin 1 receptor knock out mice on the other hand show normal behaviour and exhibit only increased fragmentation of sleep-wakefullness cycles

(Kisanuki *et al.,* 2000). Orexin 1 receptor and orexin 2 receptor double knock out mice have the same phenotype as the orexin knock out mice (Kisanuki *et al.,* 2000). This suggests that loss of signalling through both receptors is necessary for the severe narcolepsy obeserved in the orexin knock out mice.

Sleep studies in rats showed that orexin neuron activity is positively correlated with wakefullness and negatively with the amount of non-REM and REM sleep (Estabrooke *et al.,* 2001) and intracerebroventricular administration of orexin A in rats (Hagan *et al.,* 1999) and central administration of orexin A in wild type and orexin-neuron-ablated mice (Mieda *et al.,* 2004) dose-dependently increases wakefullness and suppresses non-REM and REM sleep providing further evidence that orexins are involved in the regulation of sleep-wakefullness.

Orexins therefore provide a crucial link between energy balance and arousal.

1.1.6 Additional functions of orexin

The widespread distribution of orexin fibres suggests that orexins are likely to participate in additional physiological functions apart from feeding and arousal. Threshold doses for feeding of orexin A and orexin B when injected into the lateral cerebroventricle of conscious, unrestrained rats significantly raised main arterial blood pressure and heart rate. The effects were smaller than those observed with a lower dose of angiotensin II, but displayed a similar temporal sequence to that of angiotensin II (Samson *et al.,* 1999).

Another important function of orexins is their involvement in morphine dependence and withdrawal. Georgescu and colleagues (2003) observed that a subset of orexin neurons are activated by chronic morphine (25 %) and morphine withdrawal (33%) and only morphine withdrawal, but not chronic morphine, induced orexin gene expression. Interestingly, all the orexin cells responding to either chronic morphine or morphine withdrawal also express the μ-opioid receptor, suggesting a direct mode of action. In the same study orexin knock out mice displayed attenuated morphine withdrawal suggesting that orexin neurons contribute to physical morphine dependence and the expression of withdrawal.

1.2 G-protein-coupled receptors (GPCRs)

Physiological phenomena are controlled precisely by different kinds of receptor-dependent signalling. The vast majority of these receptors belong to the superfamily of G protein-coupled receptors (GPCRs). They form one of the largest protein families with estimates that at least 700 members belong to this family in the human genome (Malnic *et al.*, 2004; Vassilatis *et al.*, 2003). About 3 % (about 750) of the genes present in a mammalian genome encode GPCRs. They act as recognition sites for a wide array of external stimuli like neurotransmitters, hormones, lipids, photons, odorants, taste ligands, nucleotides, and calcium ions (Bockaert and Pin, 1999). Hence they have been widely studied and agents that act on GPCRs, either as agonists or antagonists, are widely used in drug therapy (Wilson *et al.*, 1998).

The principal function of GPCRs is to transduce information provided by extracellular stimuli across the plasma membrane into the interior of the cell. They achieve this by interacting with heterotrimeric G proteins and the subsequent regulation of a diverse variety of effector systems.

1.2.1 Classification of GPCRs

GPCRs can be classified into four main groups based on sequence similarity. Family 1 (rhodopsin-like family) is the largest and contains the odorant receptors of which 339 have been identified in humans so far (Malnic *et al.*, 2004). Family 2 (glucagon/VIP/calcitonin family) comprises 50 GPCRs. Family 3 (metabotropic glutamate/chemosensor family) receptors number 17 and family 4 (frizzled/smoothened family) contains 11 members (Gether, 2000; Vassilatis *et al.*, 2003). The families are themselves classified into subclasses that are defined by sequence similarity, ligand binding properties and functional domains (Horn *et al.*, 1998; Bockaert and Pin, 1999).

Family 1, to which the orexin 1 receptor belongs, is the largest and best characterised out of the four families. Sequence alignment of receptors in this class shows approximately 20 conserved amino acids that are predominantly located within the transmembrane segments. These include two Cys residues in extracellular loop 2 and the top of transmembrane region 3 that form a disulphide bridge necessary for maintaining correct receptor conformation, the Asp-Arg-Tyr (DRY) motif in the proximal region of intracellular loop 2, an Asn/Asp-Pro-X-X Tyr (N/DPXXY) motif in transmembrane domain 7 and a Cys residue in the C terminal domain. Palmitoylation of the latter results in generation of a fourth intracellular loop. Amongst these, the Arg residue that forms part of the conserved Asp-Arg-Tyr (DRY) motif is the only residue that is totally conserved within members of this family.

Family 1 is further grouped into three subclasses: a, b and c. To class 1a belong the receptors for small ligands such as photons and biogenic amines. It includes rhodopsin, β-adrenergic and serotonin receptors and the ligand-binding site is located within the transmembrane spanning domains. Class 1b comprises receptors that bind peptides such as chemokines to the N terminal region, the extracellular loops and the upper part of the transmembrane domains. Finally class 1c includes receptors for glycoprotein hormones such as luteinising hormone, follicle stimulating hormone and thyroid stimulating hormone. The major characteristic is the large extracellular N terminus involved in ligand binding. Apart from the N-teminus the ligand must also bind to at least one of extracellular loops 1 or 3.

Family 2 GPCRs are the second largest group of GPCRs. They have a similar morphology to the family 1c receptors but exhibit no sequence homology except for the conservation of the disulphide bridge between Cys at the top of transmembrane region 3 and the middle of extracellular loop 2. High molecular weight peptides such as glucagon, secretin, VIP-PACAP and calcitonin bind to this family of receptors, as does the black widow spider toxin α-latrotoxin (Krasnoperov *et al.*, 1997; Davletov *et al.*, 1998). The receptors have long N-terminal regions (>100 amino acids) that contain six conserved Cys residues which seem to be involved in the formation of disulphide bridges thus forming a globular domain that is suggested to be involved in ligand binding. They also have two conserved Cys residues in extracellular loops 1 and 2 and approximately 15 other residues that are conserved in all members of this class.

The third family of GPCRs contains the metabotropic glutamate receptors and the Ca^{2+} sensitive receptors as well as recently identified putative taste receptors. (Pin and Bockaert, 1995). This family also contains the $GABA_B$ receptors (Kaupmann *et al.*, 1997) and a group of putative pheromone receptors coupled to the G_o protein, termed VRs and G_o-VN (Bargmann, 1997). Receptors in this family all possess extremely long N terminal regions (500-600 amino acids) that are involved in ligand binding and several conserved Cys residues in the transmembrane spanning and extracellular regions. Like family 1 and 2 receptors they have a conserved disulphide bridge between extracellular loops 2 and 3.

The fourth family contains the"frizzled" and "smoothened" receptors that are involved in embryonic development.

1.2.2 Structural features of GPCRs

Despite the different nature of their ligands and their diverse biological functions, GPCRs share many characteristics. They comprise seven domains of 20-25 hydrophobic residues in the form of α-helices, which span the plasma membrane. They possess an extracellular N-terminus, three extracellular loops (eloop), three intracellular loops (iloop), and an intracellular C-terminus. The solution of the crystal structure of rhodopsin in 2000 revealed a highly organised heptahelical transmembrane bundle. The crystal structure gave a more detailed picture of GPCR organisation and therefore provided an improved model for the study of GPCR structure-function relationship (Palczewski *et al.,* 2000).

The N terminus (7-595 residues) varies considerably in size between the GPCRs. The N-terminus of the orexin 1 receptor possesses 46 amino acids. This region was suggested to play a role in trafficking of the receptor to the plasma membrane and it contains in most receptors a consensus Asn-X-Ser/Thr sequence for potential N-linked glycosylation (Petaja-Repo *et al.*, 2000; George *et al.*, 1986; Hughes *et al.*, 1997). This motif is absent in the orexin 1 receptor indicating that this receptor may not be subject to N-linked glycosylation. However, the orexin 1 receptor has three Ser and Thr residues that may be O-glycosylated. In some GPCRs but not in the orexin 1 receptor, the N-terminal domain also contains Cys residues implicated in protein folding (Green *et al.*, 1990). As indicated in the classification of GPCRs, the N-terminus is also involved in ligand binding in all families except the class 1a.

The next common structural feature are the seven transmembrane spanning domains each consisting of 20-25 predominantly hydrophobic amino acids that form an α-helix. The seven helices are thought to be arranged as a tight ring shaped core (Baldwin, 1993; Ji *et al.*, 1998) with the hydrophobic amino acid residues facing the lipid bilayer and the more hydrophilic residues the core. This barrel shape is achieved by the domains being orientated roughly perpendicular to the plane of the membrane in an anti-clockwise orientation (Baldwin *et al.*, 1997). A low-resolution density map of frog rhodopsin also indicates that the seven transmembrane helices are packed much more densely on the intracellular side of the membrane than on the extracellular one. As a result the area enclosed by transmembrane regions 1-7 is about 25 % smaller on the cytoplasmic than on the extracellular side of the transmembrane receptor core (Unger *et al.,* 1997). Some of the most highly conserved residues amongst GPCRs are several Pro present in transmembrane regions 4, 5, 6 and 7. In the orexin 1 receptor they are Pro178, Pro272, Pro313 and Pro355. These residues are suggested to introduce kinks into the α-helices, which are thought to be important in the formation of the ligand binding pocket and also in allowing flexibility of the ligand binding pocket of the receptor.

Studies on GPCRs such as rhodopsin revealed that the switch from the inactive to active conformation and the unmasking of the G protein binding site is associated with a change in the relative orientation of transmembrane regions 3 and 6, with a rotation of transmembrane region 6 and a separation from transmembrane region 3 (Farrens *et al.*, 1996; Bourne *et al.*, 1997; Javitch *et al.*, 1997). This conformational change alters the orientation of intracellular loops 2 and 3 affecting what constitutes one of the key sites involved in G protein recognition and activation (Spengler *et al.*, 1993; Pin and Bockaert, 1995; Wess, 1997).

At the boundary between transmembrane region 3 and the second intracellular loop is an Asp-Arg-Tyr (DRY) motif, which is highly conserved in all family 1 GPCRs. In the orexin 1 receptor this motif is located between amino acids 143 and 146. However the orexin 1 receptor contains an additional Trp residue at position 145, which is highly unusual. This region together with the membrane proximal region of the second intracellular loop is thought to be involved in receptor-G-protein coupling.

The intracellular loops are 10-40 amino acids long. The only exception is the third intracellular loop, which can possess more than 150 residues. In the orexin 1 receptor the third intracellular loop comprises 59 amino acids. Since these regions are intracellularly located they are important for G protein coupling with the second (Wess, 1998), the third intracellular loop (Cotecchia *et al.*, 1992), and the C-terminal tail (O'Dowd *et al.*, 1988) being particularly important in this regard. Some of these regions have also been implicated in determining the selectivity of receptor-G protein coupling. This is supported by biochemical studies with hybrid receptors constructed between the vasopressin V_2 receptor, which couples to G_s and the vasopressin V_{1a} receptor, which couples to $G_{q/11}$. Replacement of the second intracellular loop of the V_2 receptor with the corresponding sequence in the V_{1a} receptor resulted in a mutant receptor that effectively coupled to $G_{q/11}$ proteins (Liu and Wess, 1996). Also, about 12 residues which are rich in positively charged amino acids at the carboxyl terminal portion of the third intracellular loop are implicated in the induction of the high affinity conformation of the receptors, since mutations in this region (just below transmembrane region 6) can lead to constitutive activity of the GPCR (Cotecchia *et al.*, 1992). The third intracellular loop also contains numerous Ser and Thr residues which are potential sites of receptor regulation via phosphorylation by kinases such as G-protein-coupled receptor kinases (GRKs), protein kinase A (PKA) and protein kinase C isoforms (PKC) (Dohlman *et al.*, 1991; Kobilka, 1992) implicating this region to be involved in receptor desensitisation and initiation of internalisation (see section 1.4 and 1.5). The orexin 1 receptor contains six such hydroxyl residues in the third intracellular loop.

On the extracellular loops, the single most important conserved amino acid is a Cys residue in extracellular loop 2, which is linked to a second Cys residue at the top of transmembrane region 3 by a disulphide bond. These two residues are important for maintaining the tertiary structure necessary for ligand binding (Green *et al.*, 1990). In the orexin 1 receptor these are Cys119 in transmembrane region 3 and Cys202 in extracellular loop 2.

The C-terminal domain (12-359 amino acids), which is also part of the intracellular receptor surface contains Ser and Thr residues that can be phosphorylated by GRKs and second messenger kinases and like the third intracellular loop are involved in receptor desensitisation (Bouvier *et al.*, 1988; Seibold *et al.*, 1998; Freedman and Lefkowitz, 1996) The C-terminus of the orexin 1 receptor contains 65 amino acids of which 17 are Ser and Thr residues. Like most GPCRs the orexin 1 receptor also contains Cys residues (Cys 375 and Cys376) at the N-terminal region of the cytoplasmic tail, which serve as a site for palmitoylation (O'Dowd *et al.*, 1989; Ovchinnikov *et al.*, 1998). Palmitate, a 16-carbon fatty acid chain, can be linked to Cys residues through a labile, reversible thioester linkage, regulation of which can be determined by the activation state of the receptor (Wedegaertner *et al.,* 1995). Insertion of the palmitate into the plasma membrane introduces an additional intracellular loop that might affect G protein interaction with the receptor (Ganter *et al.*, 1992; Milligan *et al.*, 1995). Since this loop has a helical conformation it is also referred to as helix 8. The process of palmitoylation seems to be dynamically regulated by receptor occupancy (James and Olsen, 1989) and it appears that these Cys residues play a role in regulation of the receptor-G-protein interaction, receptor turnover, expression and subcellular localisation (Kennedy and Limbird, 1993; Eason *et al.*, 1994).

Additionally the C-terminal tail might possess sites for interaction with a variety of other proteins, which can mediate GPCR signalling, such as PDZ domain-containing proteins

(Kornau *et al.*, 1997), Homer/Vesl proteins (Brakeman *et al.*, 1997), and calcyon (Lezcano *et al.*, 2000).

1.3 G-proteins

G-proteins bind to GPCRs and effectors and therefore act as mediators of receptor-stimulated effector activation. G-proteins that bind to GPCRs are heterotrimeric consisting of an α-subunit (38-52 kDa), which contains the GTP-binding site and intrinsic GTPase activity, a β- (35-36 kDa) and a γ-subunit (6-10 kDa) (Gilman, 1987). In the GDP-bound state, the α-subunit associates with the βγ–subunit and forms an inactive heterotrimer that is bound to the receptor. Receptor activation leads to conformational changes within the α-subunit and bound GDP is released and exchanged for GTP as the concentration of GTP in the cells is much higher than GDP. Once GTP is bound, the α-subunit assumes its active conformation and dissociates from the receptor as well as from the βγ–subunit. This lasts until the GTP is hydrolysed to GDP by the intrinsic GTPase activity of the α-subunit (Figure 1.2) (Gilman, 1987; Clapham and Neer, 1993; Neer, 1994). Once GTP is hydrolysed to GDP, the α-subunit and βγ–complex reassociate, become inactive, and return to the receptor.

1.3.1 G protein α–subunit

So far more than 20 different G protein α–subunits have been described corresponding to 16 gene products divided into four families based upon sequence similarity: G_s, $G_{i/o}$, $G_{q/11}$, and $G_{12/13}$. To the G_s family belong $G_s\alpha$ and $G_{olf}\alpha$, which mediate adenylyl cyclase stimulation and closing of Ca^{2+} channels. The $G_i\alpha$ family includes $G_i\alpha 1$–3 which are generally involved in the inhibition of adenylyl cyclase and opening of K^+ channels (Jones and Reed, 1987), G_t (αt and αt2) which stimulate cGMP phosphodiesterase (Lochrie *et al.*, 1985; Tanabe *et al.*, 1985), G_o (αoA and αoB) which are involved in Ca^{2+} ion channel closure and phosphoinositide turnover (Hsu *et al.*, 1990) and the G_{gust} and G_z proteins. G_{gust} is expressed in the taste buds and is thought to couple to cGMP phosphodiesterase. G_z is expressed in neurons and it inhibits adenylyl cyclase (Taussig and Gilman, 1995). The G_q family includes $G_q\alpha$, $G_{11}\alpha$, $G_{14}\alpha$, $G_{15}\alpha$ and $G_{16}\alpha$ (Strathman and Simon, 1990; Simon *et* *al.*, 1991; Wilkie *et al.*, 1991) and are predominantly coupled to the stimulation of phosphoinositide turnover. The last G protein family, the $G_{12/13}$ family, is ubiquitously expressed and has been shown to be involved in both the regulation of Na^+/H^+ ion exchange in cells (Hooley *et al.*, 1996) and the maintenance of the cell cytoskeleton through the activation of the small GTPase Rho (Klages *et al.*, 1999).

G_α-subunits consist of two domains. The first domain is involved in binding and hydrolysing GTP to GDP. The second domain buries the bound GTP or GDP in the protein core. This domain consists of 5 α helices surrounding a 6 stranded β-sheet which bind the phosphate and the guanine moiety of GTP. There is also a binding consensus site for Mg^{2+}, essential for catalysis, present in the core (Sprang, 1997).

All G protein α-subunits are covalently modified with either palmitate and/or myristate at or near the N-terminus implicating the N-terminus in membrane anchorage (Casey, 1994; Casey, 1995; Milligan *et al.*, 1995). N-myristoylation occurs in members of the G_i family. It is a co-translational modification of the glycine residue at the extreme N-terminus after the removal of the initiating methionine residue (Gordon *et al.*, 1991). Palmitoylation occurs on all G protein α-subunits apart from α_t. Palmitate is attached through a labile, reversible thioester bond to a Cys residue near the N-terminus (Parenti *et al.*, 1993). Both palmitoylation and myristoylation are thought to be involved in membrane association of the α-subunit, with palmitoylation providing a stronger interaction with the lipid bilayer due to its greater hydrophobicity. It has been reported that palmitoylation-deficient mutants of $G_{s\alpha}$ (which are also not myristoylated) exhibit a markedly decreased capacity to associate with the membrane (Wedegaertner *et al.*, 1993). The other important role of the N-terminus is thought to be to interact with the $\beta\gamma$–complex. This is supported by the finding that $\beta\gamma$ binding is lost upon mutation or removal of the first 20-21 residues at the N-terminus of the α-subunit (Denker *et al.*, 1992; Navon and Fung, 1987). The C-terminus of the α-subunit seems to be the region important for receptor and effector interaction. Proof for this hypothesis is provided by the observation that antibodies, directed against the extreme C-terminus of the α subunits, inhibit receptor-mediated activation of G proteins (Simonds *et al.*, 1989a; Simonds *et al.*, 1989b). In addition, a Pro to Arg mutation, at the sixth amino acid from the C-terminus of $G_s\alpha$, has been shown to abolish adenylyl cyclase activation upon receptor stimulation (Sullivan *et al.*, 1987).

1.3.2 G protein βγ–complex

The β- and γ-subunits form a dimer that only dissociates when it is denatured and is, therefore, a functional monomer. At present 6 β and 12 γ-subunits have been identified. With exceptions, most $G_{\beta\gamma}$ pairs are functional. The γ_1 protein (and all the other γ proteins) can combine with β_1 but is unable to pair with β_2. The region on the γ-subunit which determines this specificity for β_1 over β_2 is located in a 14 amino acid sequence on the γ-subunit (Spring and Neer, 1994).

All G protein γ-subunits are isoprenylated via a stable thioether bond to a Cys residue of a "CAAX" motif at the C-terminus. All γ-subunits are covalently modified by the addition of the 20-carbon isoprenoid geranylgeranyl or, in the case of the retinal-specific γ_1, the 15-carbon isoprenoid farnesyl (Wedegaertner *et al.*, 1995). Following the attachment of the isoprenyl group, the C-terminal three amino acids AAX are proteolytically removed and the new C-terminus gets carboxymethylated (Higgins and Casey, 1994). Although non-prenylated γ mutants have been shown to form stable dimers with β-subunits, its βγ-dimers are not properly targeted to the plasma membrane and are therefore found in the cytosol (Spiegel *et al.*, 1991). Prenylation of the γ chains is also necessary for binding of βγ to the α-subunit, receptors and effectors (Casey *et al.*, 1994).

The interaction of the $G_{\beta\gamma}$ with the G_α unit involves binding of the G_α N-terminal helical domain to the propeller structure of the β-subunit (Lambright *et al.*, 1996). Upon receptor activation and the exchange of GDP for GTP, the G_α subunit changes its conformation. As a result the α helical content of the G_α subunit is reduced which leads to separation of the βγ-dimer from the α-subunit (Lambright *et al.*, 1996).

Several crucial roles have been assigned to the βγ dimer apart from helping to guide α-subunits to the plasma membrane. In addition to increasing the affinity of the α-subunit for GDP and thereby promoting the association of GDP-bound α-subunits with ligand-activated receptors, the βγ-subunits are shown to be positive regulators of K^+ channels (Logothetis *et al.*, 1987), phospholipase C_β isoforms (Camps *et al.*, 1992), and adenylyl cyclase types II and IV (Tang and Gilman, 1991). They might also act through *ras* to activate mitogen-activated protein (MAP) kinase pathways (Crespo *et al.*, 1994).

1.4 GPCR desensitisation

The waning of GPCR responsiveness to agonist with time is called desensitisation and represents an important physiological "feedback" mechanism that protects against acute and chronic receptor overstimulation. The process of desensitisation is a consequence of a combination of different mechanisms. These mechanisms include uncoupling of the receptor from its heterotrimeric G protein as a result of receptor phosphorylation (Bouvier *et al.*, 1988; Lohse *et al.*, 1990), the internalisation of cell surface receptors into endosomes (Oakley *et al.*, 1999; Anborgh *et al.*, 2000) and the downregulation of total receptor number due to reduced receptor mRNA and protein synthesis and the lysosomal and plasma membrane degradation of pre-existing receptors (Jockers *et al.*, 1999; Pak *et al.*, 1999). These mechanisms all occur over different time frames ranging from seconds (phosphorylation) to minutes (endocytosis) to hours (downregulation). Desensitisation can either lead to complete termination of the signal as is the case for the visual and olfactory systems or to attenuation of agonist potency and maximal responsiveness as observed for the β_2-adrenergic receptor (Pippig *et al.*, 1995; Zhang *et al.*, 1997; Sakmar, 1998).

The phenomenon of desensitisation can be subdivided into agonist-non-specific (heterologous) and agonist-specific (homologous) events.

1.4.1 Heterologous desensitisation

The kinases involved in heterologous receptor desensitisation are second-messenger-dependent kinases such as cAMP-dependent protein kinase (PKA) or protein kinase C (PKC). This form of desensitisation does not require agonist activation of the receptor. It instead depends on kinase stimulation by many different stimuli and therefore receptors that have not bound agonist, including receptors for other ligands, can be desensitised by the activation of second-messenger-dependent kinases. Phosphorylation of the receptor by these kinases may alter receptor conformation and as a result greatly impair the receptor-G protein coupling efficiency in the absence of β-arrestins.

Second-messenger-dependent kinases are phosphotransferases that catalyse the transfer of the γ-phosphate group of ATP to Ser and Thr residues contained within specific amino acid consensus sites. They are activated in response to GPCR stimulated increases in intracellular messengers such as cAMP, Ca^{2+}, and diacylglycerol and mediate the phosphorylation of downstream targets. In addition, these kinases also phosphorylate any

GPCRs containing an appropiate PKA and /or PKC consensus phosphorylation site within their intracellular loops or C-terminal tail domains. For example, the β_2-adrenergic receptor has two PKA phosphorylation sites, one within the third intracellular loop and one within the proximal part of the C-terminus. The first of these two sites is essential for receptor coupling to G proteins and is proposed to be the preferred site for PKA phosphorylation leading to β_2-adrenergic receptor desensitisation (Bouvier *et al.*, 1988; Yuan *et al.*, 1994; Moffett *et al.*, 1996). PKC activation leads to the phosphorylation and desensitisation of many G_i- and G_q-linked GPCRs (Diviani *et al.*, 1997; Liang *et al.*, 1998; Tang *et al.*, 1998).

1.4.2 Homologous desensitisation

This is a major cellular mechanism mediating rapid desensitisation of GPCRs. It is agonist-specific and involves phosphorylation of activated receptors by G protein-coupled receptor kinases (GRKs) ensuring that only those receptors that have been stimulated will be desensitised. GRKs phosphorylate GPCRs at several Ser and Thr residues contained within the C-terminus (rhodopsin, β_2-adrenergic receptor) or third intracellular loop (m_2 muscarinic acetylcholine receptor). In contrast to second-messenger-dependent kinase phosphorylation, GRK-mediated phosphorylation seems not to be sufficient to promote desensitisation of many GPCRs on its own but its role is to facilitate the binding of cytosolic cofactor proteins named arrestins, which in turn sterically uncouple receptors from G proteins (Benovic *et al.*, 1987; Lohse *et al.*, 1990; Pippig *et al.*, 1993) and it is therefore the binding of arrestins to the receptor rather than the phosphorylation by GRKs that leads to homologous desensitisation of the receptor. Binding of β-arrestins not only uncouples receptors from heterotrimeric G proteins but also targets GPCRs for internalisation in clathrin coated vesicles (Ferguson and Caron, 1998;) (Figure 1.3). Moreover, GRK-mediated phosphorylation of GPCRs proceeds somewhat faster than second-messenger-dependent phosphorylation (Roth *et al.*, 1991).

1.4.3 G protein-coupled receptor kinases (GRKs)

There are seven known GRKs each sharing a similar functional organisation with a central catalytic domain flanked by an amino-terminal domain that is thought to be important for substrate recognition and that contains an RGS-like domain, and a variable C-terminal domain critical for plasma membrane targetting (Figure 1.4). The presence of a regulator of G protein signalling (RGS)-like domain in the N-terminal part of the kinase suggests that GRKs may not only regulate GPCR signalling at the receptor level, but also regulate the activity of the G protein as well (Carmann *et al.*, 1999; Sallese *et al.*, 2000).

The members of the GRK family can be subdivided into three groups according to sequence homology and functional similarity: 1) GRK1 (rhodopsin kinase) and GRK7, a candidate for cone opsin kinase (Weiss *et al.*, 1998), are retinal kinases involved in the regulation of photoreceptors; 2) GRK2 (β-adrenergic receptor kinase 1 or βARK1) and GRK3 (βARK2), which exhibit a more widespread tissue distribution; and 3) the GRK4 subfamily comprising GRK4, GRK5 and GRK6. GRK4 is localised primarily to the testes, whereas GRK5 and 6 are more widespread expressed.

GRK1-3 are localised to the cytosol in unstimulated cells and upon receptor activation translocate to the plasma membrane to phosphorylate their receptor targets. GRK1 and 7 each possess a C-terminal CAAX motif. Light-induced translocation of GRK1 from the cytosol to the plasma membrane is facilitated by the post-translational farnesylation of this site (Inglese *et al.*, 1992). The activity of GRK1 can also be regulated by the calcium sensor protein recoverin (Iacovelli *et al.*, 1999). GRK2 and 3 are not isoprenylated. They have an 125 amino acid βγ-subunit binding domain at the C-terminal, that bears striking sequence homology with pleckstrin homology domains (Koch *et al.*, 1993; Touhara *et al.*, 1994), and their plasma membrane translocation is in part regulated by their association with free βγ-subunits of G proteins (Pitcher *et al.*, 1992; Boekhoff *et al.*, 1994). The translocation of GRK2 and 3 to the plasma membrane is also influenced by binding of phosphatidylinositol 4,5-bisphosphate to the C-terminal pleckstrin homology domain (Pitcher *et al.*, 1995). Recently it emerged that GRK2 activity seems to be also regulated by a complex series of phosphorylation events. Phosphorylation of the C-terminus by mitogen-activated protein kinase (MAPK) decreases the efficacy of GRK2 toward the receptor (Pitcher *et al.*, 1999; Elorza *et al.*, 2000). However GRK2 activity and plasma membrane translocation are enhanced in response to Ser phosphorylation by PKC and Tyr phosphorylation by c-Src (Chuang *et al.*, 1995; Sarnago *et al.*, 1999).

GRK4 subfamily members do not bind βγ-subunits, but they share a conserved N-terminal phosphatidylinositol 4,5-bisphosphate-binding domain that appears to facilitate receptor phosphorylation (Pitcher *et al.*, 1996). In the absence of GPCR activation, GRK4, 5 and 6 exhibit substantial membrane localisation. Both GRK4 and 6 are palmitoylated at C-terminal Cys residues and this post-translational modification leads to constitutive membrane localisation (Stoffel *et al.*, 1994; Premont *et al.*, 1996; Stoffel *et al.*, 1998). Targetting of GRK5 to the membrane is thought to involve the electrostatic interaction of a highly basic 46 amino acid domain in the C-terminus with membrane phospholipids (Kunapuli *et al.*, 1994). As for GRK2, the activity of GRK5 seems be regulated by a complex series of events. The activity of GRK5 is not only influenced by autophosphorylation of Ser and Thr residues in the C-terminus but also by the interaction with membrane phospholipids (Kunapuli *et al.*, 1994). PKC also phosphorylates GRK5, but in contrast to GRK2 this phosphorylation event decreases GRK5 activity (Chuang *et al.*, 1996). In addition, calmodulin binds to the N-terminal domain of GRK5. This association not only reduces the ability of GRK5 to bind receptor and phospholipids but also stimulates autophosphorylation of Ser and Thr residues that are distinct from the ones involved in kinase activation, therefore inhibiting kinase activity (Pronin and Benovic, 1997; Pronin *et al.*, 1997; Iacovelli *et al.*, 1999).

1.4.4 Other kinases involved in phosphorylation of GPCRs

In addition to serving as substrates for PKA, PKC, and GRK phosphorylation, GPCRs have been shown to serve as substrates for phosphorylation by other kinases. The m3 muscarinic acetylcholine receptor can be phosphorylated by casein kinase 1α on the third intracellular loop upon agonist stimulation of the receptor (Tobin *et al.*, 1997) and this phosphorylation could be inhibited by either the expression of a catalytically inactive casein kinase 1α mutant or a peptide corresponding to the third intracellular loop domain of the m3 muscarinic acetylcholine receptor (Budd *et al.*, 2000). Nonetheless the functional consequence of casein kinase 1α phosphorylation remains to be fully elucidated since receptor mutants lacking the potential casein kinase 1α phosphorylation sites still undergo agonist-mediated desensitisation (Budd *et al.*, 2000). Casein kinase II was shown to phosphorylate the thyrotropin-releasing hormone receptor on its C-terminus, a process that seems to play a role in receptor internalisation but not desensitisation (Hanyaloglu *et al.*, 2001). Mutagenesis of Tyr residues in the C-terminus of the μ-opioid receptor caused a reduction in agonist-stimulated receptor downregulation (Pak *et al.*, 1999). Tyr phosphorylation has also been described for the bradykinin B_2 receptor, where it seems to be involved in receptor signalling leading to arachidonic acid release (Jong *et al.*, 1993).

1.4.5 Arrestins

Arrestins are a class of soluble proteins that play, together with GRKs, an important role in the regulation of GPCR desensitisation, internalisation and resensitisation (Lohse *et al.*, 1990; Lefkowitz, 1993; Pippig *et al.*, 1993; Ferguson *et al.*, 1996). They are cytoplasmic proteins, which, following agonist stimulation, translocate rapidly to the plasma membrane in a GRK dependent manner.

GRK mediated phosphorylation on its own is not enough to promote complete inactivation of either rhodopsin or the β_2 adrenergic receptor. This observation led to the identification of a 48 kDa arresting protein in rod outer segments, where this protein, now called visual arrestin (S antigen), was demonstrated to bind light-activated rhodopsin (Pfister *et al.*, 1985). Visual arrestin is highly restricted in its localisation. It is a major protein constituent of rod outer segments and is localised primarily to the retina with low expression in the pineal gland (Smith *et al.*, 1994). Subsequently additional members of this protein family have been cloned. Cone arrestin (C-arrestin or X-arrestin) is another retinal specific arrestin, which was found to be about 50 % homologous to visual arrestin (Murakami *et al.*, 1993; Craft *et al.*, 1994). C-arrestin is highly enriched in the retina and pineal gland, but is localised primarily within cone photoreceptors in the retina (Craft *et al.*, 1994). A visual arrestin-like protein, β-arrestin 1 (β-arrestin), was identified as a cofactor required for GRK-mediated β_2 adrenergic receptor desensitisation *in vitro*. It shares 59 % sequence homology with visual arrestin (Benovic *et al.*, 1987; Lohse *et al.*, 1990). Another nonvisual arrestin, β-arrestin 2 (arrestin 3), was cloned from bovine brain (Sterne-Marr *et al.*, 1993), human thyroid (Rapoport *et al.*, 1992), and rat brain (Attramadal *et al.*, 1992). β-arrestins are ubiquitously expressed outside the retina, but are predominantly localised to neuronal tissues and the spleen (Attramadal *et al.*, 1992). While β-arrestin 1 appears to be the major nonvisual arrestin expressed in many tissues (Sterne-Marr *et al.*, 1993), β-arrestin 2 is the predominant form in the olfactory epithilium (Dawson *et al.*, 1993). The ubiquitous expression pattern of β-arrestin 1 and 2 suggest that these proteins have a relativley broad receptor specificity in contrast to visual arrestin and cone arrestin. The arrestin proteins are evolutionarily conserved and are present in all mammals, as well as in *Drosophila melanogaster* and *Caenorhabditis elegans.*

Additional members of the arrestin family might exist. Partial cDNA clones for D- and E-arrestin have been described (Craft *et al.*, 1994). However, although the mRNAs for these proteins are expressed in a broad range of tissues, it is still debated whether full-length D- and E- arrestin actually exist (Craft *et al.*, 1994).

Alternative splice variants for visual arrestin, β-arrestin 1 and β-arrestin 2 exist. Bovine visual arrestin is expressed as a protein containing 404 amino acids as well as two polypeptide variants. In the first variant the last 35 amino acids are replaced by an Ala residue (p44). The second variant lacks residues 338-345 encoded by exon 13 (Yamaki *et al.*, 1987; Yamaki *et al.*, 1990; Smith *et al.*, 1994). The p44 form of visual arrestin which is localised to the rod outer segment is severalfold more potent in inhibiting the signal transduction of rhodopsin compared to the long form leading to the conclusion that the C-terminal domain of visual arrestin is not involved in binding to rhodopsin. At least two alternatively spliced forms of β-arrestin 1 and 2 are expressed. The variant form of β-arrestin 1 has an insertion of eight amino acids between amino acids 333 and 334, whereas the variant form of β-arrestin 2 involves the insertion of 11 amino acids between amino acids 361 and 362 (Parruti *et al.*, 1993; Sterne-Marr *et al.*, 1993). However there are no reported differences in the functional activity of the β-arrestin splice variants.

1.4.6 Involvement of arrestins in GPCR desensitisation

Arrestins bind preferentially to agonist-stimulated and GRK-phosphorylated GPCRs as opposed to second messenger kinase-phosphorylated or non-phosphorylated receptors thereby physically uncoupling the GPCRs from the G proteins (Lohse *et al.*, 1990; Lohse *et al.*, 1992). *In vitro* translated β-arrestin 1 binds to the m2 muscarinic acetylcholine receptor in a phosphorylation dependent manner, with the highest binding observed for the agonist-activated phosphorylated form of the receptor (Gurevich *et al.*, 1993). Also *in vitro*, the affinity of β-arrestin for the β_2 adrenergic receptor is increased 10-30 fold by GRK phosphorylation (Lohse *et al.*, 1992), and this selectivity is even more pronounced for visual arrestin binding to rhodopsin (Gurevich *et al.*, 1995). In the same study Gurevich *et al.* (1995) found that in contrast to visual arrestin binding to rhodopsin, which is absolutely dependent on rhodopsin being light-activated and GRK-phosphorylated, β-arrestin 1 and 2 interact substantially with phosphorylated non-activated receptors, as well as with agonist-activated non-phosphorylated receptors. This suggests that agonist-independent β-arrestin binding might be observed depending on the GPCR isoform studied (Anborgh *et al.*, 2000).

Solution of the crystal structure of arrestin, together with mutagenesis studies, provided insight into the mechanism of arrestin binding to phosphorylated, light-activated rhodopsin (Gurevich *et al.*, 1995; Granzin *et al.*, 1998; Vishnivetskiy *et al.*, 1999; Hirsch *et al.*, 1999). Mutagenesis studies revealed that with respect to receptor binding visual arrestin can be divided into three functional and two regulatory domains (Gurevich *et al.*, 1995) (Figure 1.5). The functional domains comprise a receptor activation recognition domain (amino acids 24-180), a secondary receptor binding domain (residues 180-330), and a phosphate sensor domain (amino acids 163-182). The regulatory domains include an amino-terminal regulatory domain (residues 1-24) and a carboxyl-terminal regulatory domain (residues 330-404). The crystal structure analysis of visual arrestin supports the observations made from the mutagenesis studies. Visual arrestin is comprised of three major structural and funtional domains, an N domain (residues 8-180), a C domain (amino acids 188-362), that are each constructed from a seven stranded β sandwich, and a C-tail (Granzin *et al.*, 1998; Hirsch *et al.*, 1999). The N and C domain are connected by a hinge region and the C domain is connected to the C-terminal tail (residues 372-404) by a flexible linker. The C-terminus forms various interactions with parts of the N and C domains thus maintaining a rigid structure of arrestin. The phosphate sensor domain constitutes a polar core that in the basal state is embedded between the N and C domain. Residues from the N- and C-terminal regulatory domains are also thought to contribute to the polar core. Upon receptor binding, the phosphorylated parts of the receptor displace the C-terminus in the polar core leading to a movement of the N and C domain relative to each other resulting in arrestin activation, which allows high affinity binding of arrestin to the receptor (Freedman *et al.*, 1996; Pitcher *et al.*, 1998). This model is consistent with the observation that the p44 splice variant of visual arrestin demonstrated little selectivity for phosphorylated light-activated rhodopsin and that the mutation of polar residues within the polar core of visual arrestin results in mutants that are able to bind non-phosphorylated rhodopsin (Palczewski *et al.*, 1994; Vishnivetskiy *et al.*, 1999). Celver and colleagues (2002) introduced homologous mutations into β-arrestin 1 and 2 and found that these mutants bound to the β_2-adrenergic receptor *in vitro* independent of receptor phosphorylation suggesting that the basal conformation of all arrestins and the mechanism of activation triggering arrestin transition into its high affinity binding state are conserved throughout this family. In contrast to visual and cone arrestin, β-arrestin 1 and 2 both contain a C-terminal 15-18 amino acid clathrin-binding domain.

1.5 GPCR internalisation

An important aspect of GPCR regulation is the translocation from the cell surface to intracellular membrane compartments upon agonist-activation (Figure 1.3) Although GPCR internalisation was originally thought to be the principal mediator of receptor desensitisation due to the physical separation of the receptor from its effectors (Sibley and Lefkowitz, 1985) it could be shown that receptor desensitisation occurs more rapidly than receptor endocytosis and the majority of sequestered receptors are phosphorylated and thus already desensitised.

1.5.1 Involvement of arrestins in GPCR trafficking

After the initial identification of β-arrestins and their role in GPCR desensitisation, these proteins were also found to participate in initiating the internalisation of several GPCRs including the β_2-adrenergic receptor. Overexpression of both β-arrestin 1 and β-arrestin 2 alone with the β_2-adrenergic receptor-Y326A mutant augmented receptor sequestration even in the absence of GRKs (Ferguson *et al.*, 1996). Moreover, β-arrestins promoted internalisation of C-terminal tail truncated β_2-adrenergic receptors and mutants lacking putative GRK phosphorylation sites and they do so by acting as adaptors that link the receptors to clathrin-coated pits. (Ferguson *et al.*, 1996). The relationship between GRK-mediated phosphorylation and β-arrestin recruitment is likely different for each GPCR subtype depending upon the receptor subtype and the cell type in which it is expressed. For example, internalisation of the chemokine receptors CCR-5 and CXCR1 in HEK293 cells requires overexpression of both GRKs and β-arrestins (Aramori *et al.*, 1997; Barlic *et al.*, 1999). In contrast, for the m2 muscarinic acetylcholine receptor only phosphorylation by GRKs is important for internalisation but not binding of β-arrestin, depending on the cellular environment in which it is expressed (Tsuga *et al.*, 1994; Schlador and Nathanson, 1997; Werbonat *et al.*, 2000). There are also examples of receptors that do not interact with either GRKs or β-arrestins and do not internalise upon agonist activation (Jockers *et al.*, 1996).

Both β-arrestin 1 and 2 interact with at least two components of the endocytic machinery: clathrin itself and the β2-adaptin subunit of the AP-2 complex (Goodman *et al.*, 1996; Laporte *et al.*, 1999). Critical residues mediating the interaction between β-arrestins and clathrin have been identified on both proteins. Using site directed mutagenesis, a Glu residue (E89) and two conserved Lys residues (K96 and K98) in the clathrin heavy chain were identified as being critcal in mediating binding to β-arrestins. The domain of β-arrestin involved in binding to clathrin is localised to amino acid residues 373-377 in the C-terminus of β-arrestin 2. Mutation of the residues within this region substantially reduced clathrin cage binding without altering binding to phosphorylated receptors (Krupnick *et al.*, 1997). Visual arrestin, although structurally related to the β-arrestins, does not bind to clathrin and therefore also does not promote β_2-adrenergic receptor internalisation. Laporte *et al.*, (1999; 2000) showed, that β-arrestins also bind to the β2-adaptin subunit of the heterotetrameric AP-2 adaptor complex and that this interaction is important for β_2-adrenergic receptor internalisation. The heterotetrameric AP-2 complex comprises four subunits: two large 100 kDa subunits, called α- and β2-adaptin, one medium size subunit of 50 kDa termed μ2, and one small 17 kDa subunit named σ2 (Kirchhausen, 1999). The β-arrestin 2 domain important for binding to the β2-adaptin subunit is localised in the C-terminus downstream of the clathrin-binding domain and involves specific Arg residues (R394 and R396). These Arg residues are also present in β-arrestin 1. *In vitro* binding experiments using the β-arrestin C-terminus, AP-2 and clathrin indicate that β-arrestin binds to the β2-adaptin subunit independently of clathrin-binding (Laporte *et al.*, 2000). Furthermore, the interaction between β-arrestin and AP-2 and not between β-arrestin and clathrin appears to be important for the initial targetting of receptors to coated pits, since β_2-adrenergic receptor-β-arrestin complexes lacking the clathrin-binding motif in β-arrestin translocated to coated pits, whereas β_2-adrenergic receptor-β-arrestin complexes missing the β2-adaptin binding site did not (Laporte *et al.*, 2000). β-arrestin 1 also binds and recruits the non-receptor Tyr kinase c-Src to agonist activated β_2-adrenergic receptors (Luttrell *et al.*, 1999). The binding of c-Src to β-arrestin 1 is in part mediated by an interaction between the Src-homology domain 3 (SH3) of the kinase and Pro-rich motifs located at residues 88-91 and 121-124 within the N-terminus of β-arrestin 1. Interaction between the SH1 domain and additional residues located in the N-terminal 185 residues of β-arrestin 1 also contributes to the binding of c-Src by β-arrestin 1 (Miller *et al.*, 2000). Activation of β_2-adrenergic receptors causes rapid c-Src-mediated Tyr phosphorylation of dynamin, a GTPase implicated in the "pinching off" of clathrin coated vesicles from the plasma membrane and ablation of this phosphorylation event hinders receptor internalisation (Ahn *et al.*, 1999).

Following internalisation, GPCRs are either dephosphorylated in endosomes and recycled back to the plasma membrane as fully functional receptors, retained in early endosomes or targetted to lysosomes for degradation (Anborgh *et al.*, 2000; Li *et al.*, 2000; Seachrist *et al.*, 2002) depending on their interaction with β-arrestins (Oakley *et al.*, 2000) (Figure 1.6). GPCRs were thus divided into two classes, A and B, on the basis of their internalisation properties. Class A receptors, like the β_2-adrenergic receptor, bind preferentially β-arrestin 2. The receptor and β-arrestin co-localise in clathrin-coated pits at or near the cell surface. The complex rapidly dissociates before the GPCR travels to endosomes and is efficiently recycled back to the plasma membrane. Class B receptors, such as vasopressin V2 receptor and the angiotensin II type 1A receptor, couple to β-arrestin 1 and 2 with equal efficacy and co-internalise with β-arrestin into endosomes. In contrast to the class A receptors, class B receptors are not efficiently recycled back to the cell surface (Anborgh *et al.*, 2000; Oakley *et al.*, 2000) (Figure 1.7). The factor determining which class a given GPCR belongs to seems to be the presence of clusters of Ser and Thr residues in the C-terminus of the GPCR (Oakley *et al.*, 2001). Class A GPCRs that dissociate from β-arrestin near the plasma membrane lack such a cluster of Ser and Thr residues, whereas class B receptors that co-internalise with β-arrestin into endosomes contain such clusters within the C-terminus (Oakley *et al.*, 2001). A chimeric GPCR with the seven transmembrane domains of the β_2-adrenergic receptor and the C-terminus of the vasopressin V2 receptor shares internalisation properties with the vasopressin V2 receptor. The opposite is true when the tail of the β_2-adrenergic receptor is switched to the body of the vasopressin V2 receptor. The stability of the receptor-β-arrestin complex appears to determine whether a receptor is recycled and resensitised (Oakley *et al.*, 2001). An event necessary for resensitisation is the dephosphorylation of GRK phosphorylated receptors in early endosomes. A prerequisite for the dephosphorylation to take place seems to be the dissociation of β-arrestin from GPCRs which in turn allows binding of a phosphatase to the receptor. Therefore, class A receptors that dissociate from β-arrestin at or near the plasma membrane are rapidly dephoshorylated and recycled, whereas class B receptors that remain associated with β-arrestin are slowly dephosphorylated and recycled. In case of the dephosphorylation of the β_2-adrenergic receptor the phosphatase involved is a membrane associated member of the phosphatase 2A family. It has been termed the GPCR phosphatase and, at least *in vitro*, not only dephosphorylates the GRK (but not the PKA-) phosphorylated β_2-adrenergic receptor, but also the α_{2a}-adrenergic receptor and rhodopsin (Pitcher *et al.*, 1995). *In vitro* the phosphatase is only active at acidic pH (Krueger *et al.*, 1997). These findings demonstrate that β-arrestins are not only involved in terminating the receptor-G protein coupling but also in initiating processes that regulate re-establishment of receptor responsiveness. However some diversity exists. The dissociation of β-arrestin from the angiotensin II type 1A receptor is not sufficient to allow receptor dephosphorylation indicating that additional GPCR-specific determinants may regulate receptor dephosphorylation in endosomes (Anborgh *et al.*, 2000). Also for the prostaglandin EP4 receptor, Ser and Thr residues are not required for internalisation although the C-terminus is involved (Desai *et al.*, 2000). Furthermore, the neurokinin 1 receptor, which co-internalises with β-arrestin, is efficiently dephosphorylated, recycles back to the plasma membrane and resensitises (Grady *et al.*, 1995; McConalogue *et al.*, 1999). Thus additional mechanisms may contribute to the control of GPCR trafficking between specific endosomal compartments.

1.5.2 Alternative pathways of GPCR endocytosis

Although clathrin-mediated endocytosis seems to be the prevailing mechanism of GPCR internalisation alternative pathways exist. For example, the endothelin type B receptor internalises mainly via the caveolae pathway that is dependent on dynamin, but not on β-arrestin (Claing *et al.*, 2000; Teixeira *et al.*, 1999). Caveolae, which contain a high proportion of detergent-insoluble glycolipid-enriched (DIG) regions of the plasma membrane known as rafts (Sargiacomo *et al.*, 1993), are non-clathrin-coated plasmalemmal vesicles enriched in cholesterol, glycosphingolipids and caveolin proteins. Caveolae are thought to be involved in cell surface receptor endocytosis and signal transduction as numerous receptors like the m2 muscarinic acetylcholine receptor, the β_2-adrenergic-receptor and the bradykinin receptor, as well as G proteins and effectors, are present in caveolae (Feron *et al.*, 1997; Dupree *et al.*, 1993; de Weerd and Leeb-Lundberg, 1997; Anderson, 1998).

Molecules endocytosed via caveolae can then be transported to the cytoplasm, the endoplasmic reticulum, the opposite cell surface or caveolae-derived tubular/vesicular compartment (Anderson, 1998). Some receptors, like the secretin and the N-formyl peptide receptor, are internalised via a third pathway that does not involve β-arrestin or dynamin (Walker *et al.*, 1999; Gilbert *et al.*, 2001). However, the exact mechanism and nature of vesicles involved in this pathway is still unknown.

1.5.3 Receptor down-regulation

Down-regulation of GPCRs is caused by long-term exposure of the receptor to agonist and is characterised by a persistent loss of receptors from cells or tissues. As a result cellular signal transduction is attenuated over a prolonged period of time. In contrast to the processes of receptor desensitisation and internalisation, downregulation is only slowly or incompletely reversed after agonist removal. Downregulation is the least understood mechanism involved in controlling receptor responsiveness.

In case of the β_2-adrenergic receptor, at least two pathways are involved in the downregulation process: one that is agonist-dependent and PKA-independent and a second one that is PKA-dependent (Collins *et al.*, 1992; Hadcock *et al.*, 1989). Impairment of receptor-G protein coupling results in reduced agonist-mediated downregulation of β_2-adrenergic receptors which can be partially restored by direct activation of PKA by forskolin. Moreover a β_2-adrenergic receptor mutant lacking potential PKA phosphorylation sites is more slowly downregulated than the wild type receptor. On the other hand a mutant lacking the putative GRK phosphorylation sites is downregulated to the same extent as the wild type form (Collins *et al.*, 1992; Hadcock and Malbon, 1993; Lohse, 1993). Thus long-term agonist exposure and subsequent G protein coupling may result in a distinctive phosphorylation pattern or in a particular receptor conformation that exposes lysosomal targetting sequences. Apart from the β_2-adrenergic receptor, the thrombin, thyrotropin and cholecystokinin receptors have also been shown to be sorted to lysosomes upon agonist exposure (Hein *et al.*, 1994; Petrou *et al.*, 1997; Tarasova *et al.*, 1997). Moreover there is emerging evidence that some GPCRs are degraded by non-lysosomal mechanisms. The V2 vasopressin receptor undergoes ligand-induced proteolysis in a non-endocytic pathway involving a plasma membrane-associated metalloprotease (Kojro and Fahrenholz, 1995).

The second component of downregulation is reduced receptor synthesis either by reduced gene transcription or destabilisation of the mRNA. The latter is the prevailing mechanism for the β_2-adrenergic-receptor and the m_1 muscarinic acetylcholine receptor (Hadcock *et al.*, 1989; Lee *et al.*, 1994; Tholanikunnel and Malbon 1997). This process depends on PKA suggesting either phosphorylation or induction of a factor participating in selective degradation of receptor mRNA. For the β_2-adrenergic receptor, an agonist induced receptor binding protein (β ARB) has been identified, that only binds to β_2-adrenergic-receptor mRNA but not to mRNAs encoding the α_{1b}-, the β_1-, or the β_3-adrenergic receptor, leading to dramatic downregulation of β_2-adrenergic receptor mRNA (Port *et al.*, 1992; Tholanikunnel *et al.*, 1995).

1.6 β-arrestin regulation and signalling

Cytosolic β-arrestin proteins translocate to the plasma membrane and bind to the receptor upon agonist stimulation of the latter. However, the mechanism behind this receptor-mediated response is not clear. β-arrestin translocation could either be a passive process involving diffusion-dependent interactions with agonist-stimulated phosphorylated receptors or a signal driven process. Moreover the function of β-arrestin 1 in GPCR sequestration seems to be regulated by a feedback mechanism involving phosphorylation/dephosphorylation of the β-arrestin 1 molecule (Lin *et al.*, 1997; Lin *et al.*; 1999). Cytoplasmic β-arrestin 1 is constitutively phosphorylated on Ser412 in the C-terminus. Upon recruitment to the plasma membrane by agonist-stimulation of the β_2-adrenergic receptor, β-arrestin 1 becomes rapidly dephosphorylated. This dephosphorylation is required for its function in receptor endocytosis but not for receptor binding and desensitisation. Following extracellular signal-regulated kinase (ERK) activation by receptor endocytosis, ERKs phosphorylate β-arrestin 1 at Ser412 thereby inhibiting further receptor endocytosis (Lin *et al.*, 1999). Hence, a S412D β-arrestin 1 mutant was found to function as a dominant negative mutant of β_2-adrenergic receptor endocytosis, whereas it had no effect on receptor desensitisation (Lin *et al.*, 1997). It has also been suggested that ERK-mediated phosphorylation contributes to the regulation of β-arrestin 1/β-adaptin interactions (Lin *et al.*, 1999). Interestingly Ser412 is not conserved in β-arrestin 2. In contrast, β-arrestin 2 was shown to be phosphorylated at Thr382 by casein kinase II and becomes dephosphorylated upon β_2-adrenergic receptor stimulation. However dephosphorylation of β-arrestin 2 does not seem to be involved in receptor endocytosis, but appears to regulate the formation of a large β-arrestin 2-containing protein complex (Kim *et al.*, 2002).

β-Arrestin activity is also regulated by phosphoinositides, particularly IP_6 (Gaidarov and Keen, 1999; Gaidarov *et al.*, 1999). The binding site for the phosphoinositides is located between residues 233-251 in β-arrestin 2. Mutation of basic residues within this domain reduces the phosphoinositide binding to β-arrestin 2 and produces a β-arrestin mutant defective in internalisation of the β_2-adrenergic receptor in COS 1 cells. Furthermore, the mutant did not concentrate at clathrin coated pits but was still recruited to the plasma membrane upon agonist stimulation of the β_2-adrenergic receptor. Thus, phosphoinositide binding may be involved in the routing of receptor-β-arrestin complexes to the clathrin-coated pits.

In addition to regulating endocytosis of phosphorylated receptors, β-arrestins are now being appreciated as mediators of GPCR signalling due to the discovery that they can serve as adaptor proteins for signalling proteins. As mentioned in section 1.5.1 activation of β-arrestin 1 by the phosphorylated β_2-adrenergic receptor leads to recruitment of active c-Src to the receptor. The translocation of c-Src is not only essential for endocytosis, but is also implicated in the GPCR-mediated activation of the ERK cascade (Figure 1.8). The ras dependent-activation of the ERK1/2 pathway by many GPCRs requires c-Src kinase activity, since c-Src recruitment leads to phosphorylation of Shc and formation of Shc-Grb2 complexes (Luttrell *et al.*, 1996). Hence, inhibiting the interaction between β-arrestin and c-Src with mutant forms of β-arrestin impairs β_2-adrenergic receptor stimulated ERK activation in HEK293 cells and also inhibits receptor internalisation by preventing dynamin phosphorylation (see section 1.5.1) (Luttrell *et al.*, 1999). Similar to the β_2-adrenergic receptor, activation of the neurokinin-1 receptor by substance P leads to ERK activation by a process involving β-arrestin-mediated recruitment of c-Src to the activated receptor and expression of either mutant receptor unable to bind β-arrestin or a dominant negative form of β-arrestin 1 inhibits ERK activation by the receptor (DeFea *et al.*, 2000a). In neutrophils stimulation of the chemokine receptor CXCR1 by interleukin-8 results in the formation of complexes containing β-arrestin and Hck or Fgr, two members of the Src kinase family (Barlic *et al.*, 2000). The formation of these complexes leads to Hck activation and the translocation of these complexes to granule-rich regions. In cells expressing a dominant negative β-arrestin mutant, the interleukin-8-mediated granule release is inhibited, indicating a role for β-arrestin-Hck complexes in the trafficking of the exocytotic vesicles, but not necessarily in the activation of the ERK pathway. In addition to c-Src, other components of the ERK cascade like Raf, a MAPK kinase kinase (MAPKKK), and ERK also appear to interact with β-arrestin (Figure 1.9). For example, stimulation of the proteinase-activated receptor 2 (PAR2) in KNKR cells causes the formation of multiprotein complexes comprising the internalised receptor, β-arrestin 1, Raf-1 and activated ERK1/2 and expression of a truncated form of β-arrestin that inhbibits receptor endocytosis blocked ERK activation (DeFea *et al.,* 2000b). Similar results have been obtained for the angiotensin II type 1A receptor (Luttrell *et al.*, 2001). Receptor activation results in formation of complexes consisting of c-Raf-1 as MAPKKK, MEK 1 as MAPKK and ERK2 as MAPK. In response to agonist stimulation ERK2 is localised in the same endosomes that also contain receptor-β-arrestin complexes.

In addition to serving as a scaffold in the ERK cascade, β-arrestin 2 also serves as an adaptor in the c-jun N-terminal kinase type 3 (JNK3) cascade. In cells, β-arrestin 2 interacts with both JNK3 and Ask1, a MAPKKK, to strongly enhance Ask1 stimulation of JNK3, but not of JNK1 or 2, via MKK4, a MAPK kinase (MAPKK). MKK4 does not seem to directly bind to β-arrestin 2 but rather, is recruited to the complex via Ask1 and/or JNK3 (McDonald *et al.,* 2000). Ask1 binds to the N-terminus of β-arrestin 2, whereas JNK3 binds to an RRSLHL motif in the C-terminus of β-arrestin 2 (McDonald *et al.,* 2000; Miller *et al.,* 2001). This motif is a consensus MAPK binding site and not present in β-arrestin 1. So in contrast to the ERK cascade, where both β-arrestin 1 and 2 served as adaptors, only β-arrestin 2 can form a scaffold in the JNK3 pathway. The phosphorylation of JNK3 also occurs in response to activation of the angiotensin II type 1A receptor which leads to the co-localisation of activated JNK3 and β-arrestin 2 in cytosolic vesicles bringing the activity and spatial distribution of this MAPK module under the control of a GPCR.

It is thought that the function of β-arrestins as scaffold proteins is to target both ERK and JNK away from the nucleus and into the cytosol, thus enabling the phosphorylation of non-nuclear substrates, including other kinases, which can then activate transcription (Figure 1.10). This idea is supported by recent findings concerning the PAR2, where activated ERK is retained in the cytosol and fails to promote cellular proliferation (DeFea *et al.,* 2000).

1.7 Mitogen-activated protein kinase (MAPK) signal transduction pathways

The MAPK pathways are amongst the most widespread mechanisms of eukaryotic cell regulation. Mammalian MAPK pathways relay, amplify and integrate signals from a diverse range of stimuli signalling through diverse receptor families including hormones and growth factors such as epidermal growth factor that act through receptor tyrosine kinases or cytokine receptors, vasoactive peptides like angiotensin II acting through GPCRs, and transforming growth factor-β-related polypeptides acting through Ser-Thr kinase receptors. These pathways are also activated by inflammatory cytokines of the tumour necrosis factor family and cellular stresses such as irradiation, heat shock, osmotic imbalance, DNA damage, and bacterial products such as lipopolysaccharides. Activation of MAPKs by these stimuli controls gene expression, metabolism, cytoskeletal functions and other cellular regulatory events. MAPKs therefore participate in the regulation of fundamental cellular processes such as proliferation, differentiation, survival, apoptosis and migration.

The core module of a MAPK signalling pathway consists of three protein kinases (Figure 1.10). The first kinase of the three-component activation module is a MAPK kinase kinase (MAPKKK) (Fanger *et al.,* 1997; Widmann *et al.,* 1999)). Specific MAPKKKs can be activated either by phosphorylation by a MAPK kinase kinase kinase (MAPKKKK) or by coupling to a small GTP-binding protein of the Ras or Rho family. Other possible modes of activation are oligomerisation and subcellular re-localisation. The MAPKKKs are Ser-Thr kinases that phosphorylate and activate a MAPK kinase (MAPKK), the next kinase in the module, upon activation (Siow *et al.,* 1997). MAPKKs are dual specificity kinases that recognise and phosphorylate a Thr-X-Tyr motif in the activation loop of MAPK (Gartner *et al.,* 1992). The final kinase in the module is a MAPK which phosphorylates Ser and Thr residues on their substrates. Although the vast majority of substrates are transcription factors, MAPKs can also phosphorylate other protein kinases, phospholipases, and cytoskeleton-associated proteins. This set-up not only provides amplification, but also importantly additional regulatory interfaces that allow the kinetics, duration and amplitude of the activity to be precisely tuned.

To date, 14 MAPKKKs, 7 MAPKKs and 13 MAPKs have been identified in mammalian cells. The MAPKKKs can be divided into four subfamilies. The best characterised is the Raf subfamily and comprises B-Raf, A-Raf and Raf-1. The second subfamily, the MEK kinases, contains MEKK1 to 4. Ask1 and Tpl2 form the third subfamily. The fourth group comprises Mst, Sprk, Muk, Tak1 and Mos. In case of the MAPKKs, MEK1 and MEK2 are more closely related as are MKK3 and MKK6. The MAPKs can be classified into five subfamilies based on sequence homology and different activation by agonists. These are the growth factor activated MAPKs; ERK 1 and ERK 2, which contain the signature activation sequence Thr-Glu-Tyr, p38, which contains the activation sequence Thr-Gly-

Tyr, JNK1 to 3, which contain the activation sequence Thr-Pro-Tyr, the Erk5 and Erk3/4 subfamilies. Since the p38 and the JNK subfamilies are activated by cellular stress such as exposure to DNA damaging agents, oxidative stress, pro-inflammatory cytokines and protein synthesis inhibitors, they are classified as stress-activated protein kinases (SAPK). The MAPK gives the name to the pathway that employs it for example the MAPK pathway using JNK is called the JNK MAPK pathway.

The combination of 13 MAPKs, 7 MAPKKs and 14 MAPKKKs in mammalian cells presents a very complex picture. Certain themes, however, can be detected. The MAPKK family represents the smallest group in the module. They are also highly specific for their substrate since they not only recognise the linear Thr-X-Tyr activation motif, but also the tertiary structure of a specific MAPK, thereby effectively restricting their regulation of different MAPKs allowing minimal variation of the MAPKK-MAPK part of the MAPK module. In contrast MAPKKKs are able to couple to different MAPKK-MAPK combinations. The MAPKKK family represents the highest number of members in the MAPK module. The MAPKKKs have regulatory motifs, that are absent in MAPKKs or MAPKs. These motifs comprise Pleckstrin homology (PH) domains, Pro-rich sequences for binding SH3 domains, binding sites for GTP-binding proteins, leucine-zipper dimerisation sequences, and phosphorylation sites for Tyr and Ser-Thr kinases. The large number of MAPKKKs allows for diversity of inputs from numerous stimuli to feed into specific MAPK pathways.

The MAPKKKs can thus be differentially regulated by a variety of upstream inputs for their selective regulation of the MAPKKs. Recent evidence suggests that specificity is in part achieved by the use of scaffolding or anchoring proteins to co-ordinate MAPKKK binding to specific proteins for upstream inputs as well as specific downstream MAPKK-MAPK complexes (Pawson, 1995; Pawson and Scott, 1997).

The major targets for MAPKs and SAPKs are transcription factors and to be able to phosphorylate these factors, MAPKs/SAPKs must translocate from the cytoplasm to the nucleus, which is normally associated with prolonged stimulation. Using PC12 cells it could be demonstrated that the magnitude and duration of the MAPK activation is important to determine the physiological outcome. These cells proliferate in response to epidermal growth factor and differentiate as indicated by neurite outgrowth as result of exposure to nerve growth factor. This difference in response is entirely due to the ability of nerve growth factor, but not of epidermal growth factor to cause sustained MAPK activation and nuclear translocation (Marshall, 1995).

The major point for regulating the duration and magnitude of MAPK activation occurs at the level of MAPK itself. The activity of MAPK is controlled by the balance between phosphorylation and dephosphorylation events. Since upstream kinases phosphorylate both Thr and Tyr residues, dephosphorylation of either by Tyr-specific phosphatases, Ser-Thr phosphatases or by dual specificity (Thr-Tyr) phosphatases is sufficient for inactivation.

1.7.1. The ERK MAPK pathway

The best characterised pathway in mammalian cells is the ERK pathway, which consists of Ras as G protein, Raf as MAPKKK, MEK as MAPKK and ERK as MAPK (Figure 1.11). This pathway includes a number of different MAPKKK, MAPKK and five different MAPK defined as ERK1 to 5. Out of this group ERK1 and 2, with molecular mass of 44 and 42 kDa respectively, are the most extensively studied.

After stimulation of cells through receptor tyrosine kinases, non-receptor Tyr kinases or GPCRs, the small G protein Ras initiates the cascade by recruiting Raf from the cytosol to the plasma membrane via the N-terminal regulatory domain of Raf (Koide *et al.,* 1993). Although all three Raf isoforms, Raf-1, A-Raf and B-Raf can interact with Ras, only Ras binding to B-Raf is sufficient for activation. Raf-1 and A-Raf require additional signals for activation (Marais *et al.,* 1997). Activation of Raf-1 involves Tyr phosphorylation by membrane bound Tyr kinases including c-Src (Marais *et al.,* 1995). It was also shown that Ser phosphorylation by PKC controls Raf-1 kinase activity (Kolch *et al.,* 1991). The Ser-Thr kinase Raf phosphorylates and activates MEK, which in turn phosphorylates and activates ERK. MEK is a dual Thr-Tyr kinase that phosphorylates ERK at the Thr-Glu-Tyr in the activation loop of the catalytic domain. As a consequence of activation, ERK rapidly translocates to the nucleus where it is functionally sequestered and can regulate the activity of nuclear proteins including transcription factors such as Ets-1, Elk-1 and c-Myc by phosphorylation on Ser and Thr residues within a Pro-directed motif. ERK also has substrates in the cytosol and can therefore affect gene expression indirectly by activating p90^{rsk} (ribosomal S6 kinase) family kinases, which can modify transcription factors and histones (Davie and Spencer 2001; Lewis *et al.,* 1998). Other cytoplasmic substrates are cytosolic phospholipase A_2 and the juxtamembrane region of the epidermal growth factor receptor (Lin *et al.,* 1993; Seger and Krebs, 1995). The ERK pathway therefore contributes to cellular proliferation, differentiation, cell cycle regulation, and cell survival.

1.7.2 The JNK MAPK pathway

The JNK MAPK kinase pathway was identified in 1991 and differs from the ERK pathway in two characteristics: 1) it is activated by cell stress such as ultraviolet radiation, heat shock oxidant stress and DNA damaging chemicals (topoisomerase inhibitors) and 2) it phosphorylates c-Jun at the N-terminal activating sites rather than the C-terminal inhibitory sites phosphorylated by ERK2 (Pulverer *et al.*, 1991; Derijard *et al.*, 1994; Kyriakis and Avruch, 1996). This kinase, therefore, was termed c-Jun N-terminal kinase (JNK) or stress-activated kinase (SAPK). This pathway consists of a number of different MAPKKKs like Ask1, Mst, two MAPKKs, MKK4 and 7 and three different MAPKs, JNK1 to 3 (Figure 1.12). JNK1 and 2 are ubiquitously expressed, whereas JNK 3 seems to be limited to the brain (Yang *et al.*, 1997).

Although JNK was first described as a SAPK, the response of JNK to extracellular ligands is far better characterised. JNKs have now been shown to be activated through cell surface receptors from different families including the tumour necrosis factor family, GPCRs, tyrosine kinase receptors and cytokine receptors (Fanger *et al.*, 1997). JNKs are activated by phosphorylation on Thr and Tyr of the Thr-X-Tyr activation motif by either of the dual specificity kinases, MKK4 or 7 (Sanchez *et al.*, 1994; Tournier *et al.*, 1997). These are in turn activated by MAPKKKs which include Ask1, Muk1 and Tpl-2 (Wang *et al.*, 1996; Hirai *et al.*, 1996; Salmeron *et al.*, 1996). Other signalling proteins that act as upstream activators of the JNK pathway are Rac1 and Cdc42, two members of the Rho family of small G proteins (Coso *et al.*, 1995).

To date all the described substrates for JNK are transcription factors including c-Jun, ATF-2, Elk-1, p53, DPC4, and NFAT4. As for ERK, JNKs phosphorylate their substrate at a Ser/Thr-X-Pro motif. However this sequence on its own is not sufficient to induce phoshorylation. An additional docking site is present in c-Jun. Recruitment of JNK via this docking site results in increased local concentration of the kinase and directs activity to the phosphorylation motif within the N-terminus of c-Jun.

JNK activity has been implicated in the response to cell stress, specifically apoptosis. Although JNKs do not seem to be sufficient on their own to induce apoptosis, they are necessary for apoptosis to occur in response to growth factor withdrawal, stress, and DNA damage (Ham *et al.*, 1995; Chen *et al.*, 1996; Kasibhatla *et al.*, 1998). In some instances, however, activation of JNK promotes growth or survival. BAF3, pre-B cells, undergo apoptosis in the absence of interleukin 3. Re-addition of interleukin 3 activates JNK, and inhibition of JNK activity by expression of a JNK specific phosphatase inhibits interleukin 3 –induced proliferation, while it has no effect on apoptosis induced by interleukin 3 withdrawal (Smith *et al.,* 1997). Another example where the JNK MAPK pathway is involved in survival rather than apoptosis are T89G glioblastoma cells. In these cells the JNK pathway seems to be involved in DNA repair (Potapova *et al.,* 1997).

1.7.3 The p38 MAPK pathway

The p38 MAPKs are a second family of stress-activated protein kinases in mammals. This family consists of at least four homologous proteins p38α, p38β, p38γ, and p38δ. Similar to JNK, p38 is activated by cellular stresses including ultraviolet radiation, osmotic shock, heat shock, lipopolysaccharides, protein synthesis inhibitors and certain cytokines like interleukin-1 and tumour necrosis factor α (Whitmarsh and Davis, 1996). In addition p38 can be activated through the $G_{q/11}$-coupled m1 muscarinic acetylcholine receptor, the G_i-coupled m2 muscarinic acetylcholine receptor, and the G_s-coupled β_2-adrenergic receptor Overexpression of Gβγ or $G_{11}\alpha$, but not of $G_s\alpha$ or $G_i\alpha$ can activate p38. Thus depending on the GPCR, p38 stimulation is either mediated by the βγ-subunit or the α-subunit of the heterotrimeric G protein (Yamauchi *et al.,* 1997). Similar to ERK and JNK, the p38 pathway is organised in a three-kinase architecture consisting of the MAPKKKs TAK1, ASK1, SPRK and PAK, the MAPKKs MKK3 and MKK6 and the four different MAPKs p38α, β, γ, δ (Figure 1.13).

p38 is activated by dual phosphorylation on Thr and Tyr in the Thr-Gly-Tyr activation motif by specific MAPKKs, which are in turn phosphorylated and activated by the MAPKKKs listed above. As for JNK, regulation of p38 activation can also occur through Rac1 and Cdc42 (Bagrodia *et al.,* 1995; Zhang *et al.,* 1995). Cdc42 and Rac1 cannot directly activate p38, but do so by activating upstream signalling molecules. For example in response to interleukin 1 they activate p38 through activation of a family of Ser-Thr kinases called p21-activated kinase (PAK) (Bagrodia *et al.,* 1995).

Substrates for activated p38 are the MAPK-activated protein (MAPKAP) kinases 2 and 3 which upon activation phosphorylate small heat shock proteins such as 27 kDa heat shock protein (Rouse *et al.,* 1994) and the transcription factors ATF2, Elk-1, Chop and Max (Raingeaud *et al.,* 1995; Raingeaud *et al.,* 1996; Wang and Ron, 1996; Zervos *et al.,* 1995). Max heterodimerises with c-Myc, an ERK substrate, raising the possibility that these heterodimers represent a point of integration between the ERK and the p38 cascades.

Since p38 can phosphorylate many different substrates, it seems plausible that the p38 signalling pathway affects many different biological functions. To date p38 seems to be involved in the production of cytokines in hematopoietic cells (Lee *et al.,* 1994), in cytokine-stimulated cellular proliferation (Crawley *et al.*, 1997), and in apoptosis (Whitmarsh and Davis, 1996). However, there are cells where p38 is activated without apoptosis just as the case with JNK. In WEHI-231 cells JNK/p38 activation correlates with cell survival (Sutherland *et al.,* 1996).

1.8 Project aims

The orexin receptors were shown to bind two peptides produced by the hypothalamus. The orexin peptides appear to play key roles in food intake and regulation of the sleep-wakefullness cycle. Small molecule regulators of these receptors are thus attracting great interest within the pharmaceutical industry. It is therefore surprising that only little is known about the regulation of the receptors following agonist stimulation. For that reason the aims of this study were (1) to characterise the pathway of internalisation of the orexin 1 receptor, (2) to determine the signalling pathways elicited upon agonist stimulation and (3) to identify the molecular determinants within the orexin 1 receptor important for internalisation and signalling.

Figure 1.1: Sequences of the orexin 1 and 2 receptor

Deduced amino acid sequence of the human orexin 1 receptor (ox1R) and the human orexin 2 receptor (ox2R). Putative transmembrane (TM) domains are marked, as predicted by the PredictProtein server (http://www.embl-heidelberg.de/predictprotein). Gaps introduced to obtain optimal alignment are indicated by dashes.

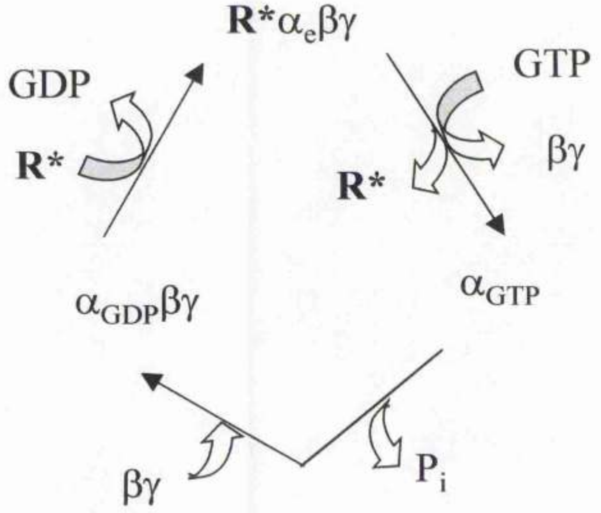
Figure 1.1

```
                                                                 -------
ox1R        MEPSATPGAQMGVPPGSREPSPVPPDYEDE-FLRYLWRDYLYPKQYEWVLIAA   52
ox2R MSGTKLEDSPPCRNWSSASELNETQEPFLNPTDYDDEEFLRYLWREYLHPKEYEWVLIAG   60

       ------TM1-----------          ----------TM2---------
ox1R YVAVFVVALVGNTLVCLAVWRNHHMRTVTNYFIVNLSLADVLVTAICLPASLLVDITESW  112
ox2R YIIVFVVALIGNVLVCVAVWKNHHMRTVTNYFIVNLSLADVLVTITCLPATLVVDITETW  120

            ---------TM3-------------                     -------
ox1R LFGHALCKVIPYLQAVSVSVAVLTLSFIALDRWYAICHPLLFKSTARRARGSILGIWAVS  172
ox2R FFGQSLCKVIPYLQTVSVSVSVLTLSCIALDRWYAICHPLMFKSTAKRARNSIVIIWIVS  180

       ---TM4-----------                          ---------TM5---
ox1R LAIMVPQAAVMECSSVLPELANRTRLFSVCDERWADDLYPKIYHSCFFIVTYLAPLGLMA  232
ox2R CIIMIPQAIVMECSTVFPGLANKTTLFTVCDERWGGEIYPKMYHICFFLVTYMAPLCLMV  240

       -------
ox1R MAYFQIFRKLWGRQIPGTTSALVRNWKRPSDQLGDLEQGLSGEPQPRGRAFLAEVKQMRA  292
ox2R LAYLQIFRKLWCRQIPGTSSVVQRKWKPL--QPVSQPRGPGQPTKSRMSAVAAEIKQIRA  298

           ----------TM6-------------               --------TM7----
ox1R RRKTAKMLMVVLLVFALCYLPISVLNVLKRVFGMFRQASDREAVYACFTFSHWLVYANSA  352
ox2R RRKTARMLMVVLLVFAICYLPISILNVLKRVFGMFAHTEDRETVYAWFTFSHWLVYANSA  358

       ---------
ox1R ANPIIYNFLSGKFREQFKAAFSCCLPGLGPCGSLKAPSP-RSSASHKSLSLQ--SRCSIS  409
ox2R ANPIIYNFLSGKFREEFKAAFSCCCLGVHHRQEDRLTRGRTSTESRKSLTTQISNFDNIS  418


ox1R KISEHVVLTSVTTVLP                                               425
ox2R KLSEQVVLTSIST-LPAANGAGPLQNW                                   444
```

Figure 1.2: The G protein cycle

Activated receptors (R*) associate with the trimer (α-GDPβγ) causing dissociation of GDP and binding of GTP to the complex of R* and the trimer in its "empty" state ($\alpha_e\beta\gamma$). This induces a conformational change which leads to the dissociation of α-GTP from the complex, releasing βγ. After GTP hydrolysis, α-GDP reassociates with βγ.

Figure 1.2

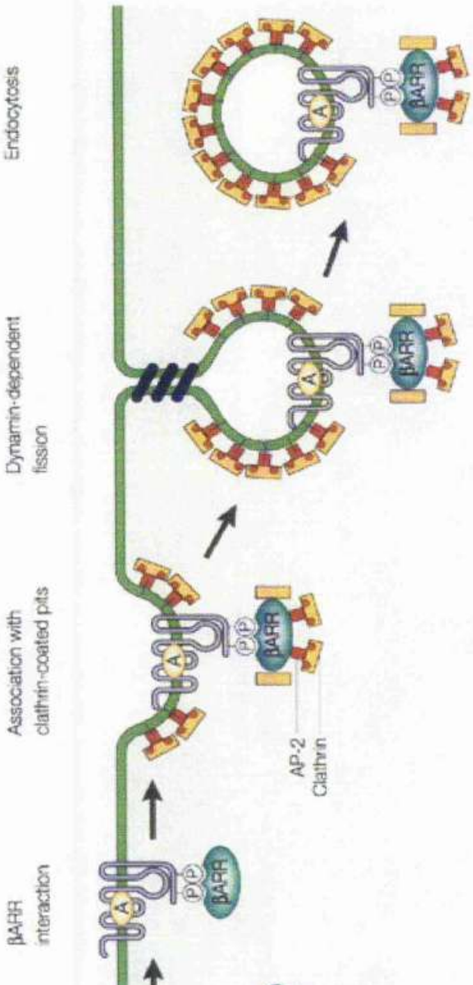


R*α_eβγ
GDP
R*
GTP
βγ
R*
α_GDPβγ
α_GTP
βγ
P_i

Figure 1.3: Desensitisation and internalisation of GPCRs

After binding of agonist (A) to the GPCR, GRKs phosphorylate residues within the third intracellular loop and the C-terminus of the receptor which leads to the recruitment of β-arrestins (βARR), which uncouples the receptor from the G protein. GPCRs are then targeted for clathrin-mediated endocytosis via recruitment of clathrin and the AP-2 complex by the β-arrestins (source: Pierce and Lefkowitz, 2001).

Figure 1.3



Ligand binding
A
Receptor phosphorylation
GRK
P P
βARR
βARR interaction
Association with clathrin-coated pits
AP-2
Clathrin
Dynamin-dependent fission
Endocytosis

Figure 1.4: Diagrammatic representation of the structure of GRKs 1-7

The amino terminal domain of the GPCR-binding domain of each GRK contains a conserved RGS domain. The GRKs are targetted to the plasma membrane via the carboxyl terminal domain. GRKs 1 and 7 are farnesylated at "CAAX" motifs in their carboxyl termini, whereas GRKs 4 and 6 are palmitoylated at cysteine residues. GRKs 2 and 3 contain βγ-subunit binding that exhibits sequence homology to a Pleckstrin homology domain and GRK5 contains a stretch of 46 basic amino acids that mediate interactions with the phospholipids in the plasma membrane.

Figure 1.4

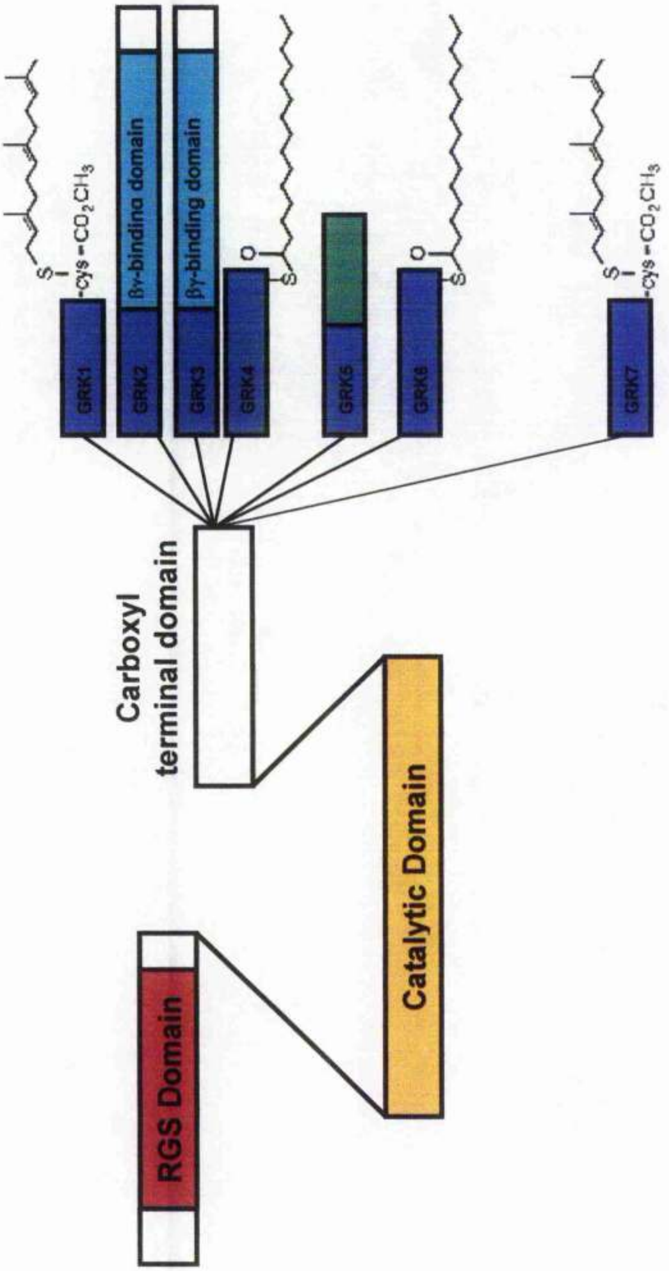
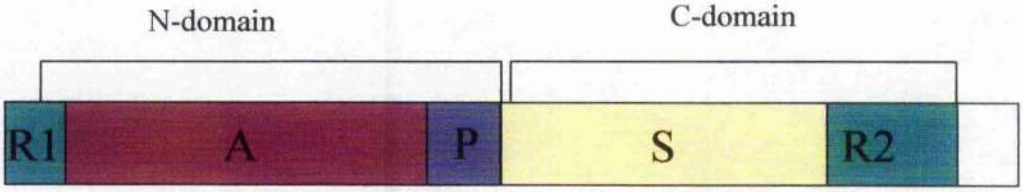

RGS Domain
Catalytic Domain
Carboxyl terminal domain
GRK1
GRK2
GRK3
GRK4
GRK5
GRK6
GRK7
βγ-binding domain
βγ-binding domain
S
-cys-CO_2CH_3
O
S

Figure 1.5: Molecular architecture of arrestins

The arrestin proteins comprise an amino regulatory domain (residues 1-24), a receptor activation domain (residues 24-180), a phosphate sensor domain (163-182), a secondary receptor-binding domain (residues 180-330), and a carboxyl terminal domain (residues 330-404). The white box highlights the clathrin- and β-adaptin-binding domains that are only conserved among β-arrestins 1 and 2.

Figure 1.5


N-domain
C-domain
R1
A
P
S
R2

R1, amino-terminal regulatory domain; A, activation-recognition-domain;
P, phosphorylation-recognition domain; S,secondary receptor-binding domain;
R2, C-terminal regulatory domain

Figure 1.6: Receptor fate after internalisation

After internalisation, the clathrin-coated vesicles shed the clathrin-coat very quickly and become early endosomes. Receptors can internalise with or without β-arrestin. In the first case, the receptors are dephosphorylated in an acidified perinuclear compartment and then recycled back to the plasma membrane where they can once again interact with their ligands. This process is called resensitisation. Receptors that co-internalise with β-arrestin are either retained in large endosomes and/or targetted for degradation by lysosomes (adopted from McDonald and Lefkowitz, 2001).

Figure 1.6

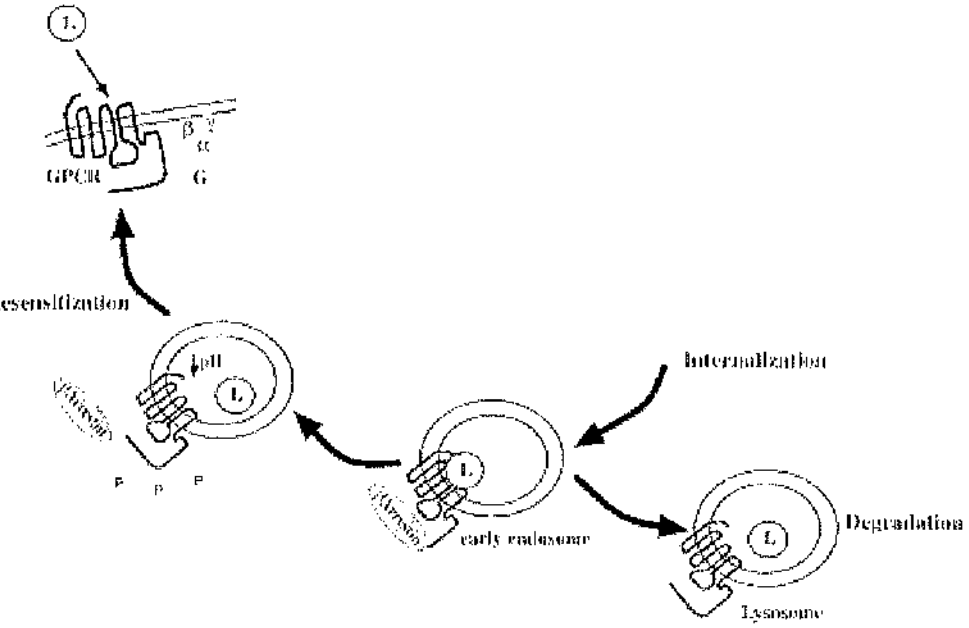

L
GPCR
β
γ
α
G
Resensitization
pH
L
P
P
P
Internalization
L
early endosome
Degradation
L
Lysosome

Figure1.7: Class A and class B GPCRs

Class A members preferentially internalise through a β-arrestin 2-mediated pathway, whereas class B receptors bind equally well to β-arrestin 1 and 2. In the case of class A receptors, the receptor interacts with β-arrestin only transiently and β-arrestin does not co-localise with the GPCR in endosomes and the receptor is resensitised and recycles rapidly. For Class B receptors, the β-arrestin-GPCR interaction is more stable, and the receptor and β-arrestin co-localise in endosomes. In this case the receptor recycles slowly or is downregulated (adopted from Pierce and Lefkowitz, 2001).

Figure 1.7

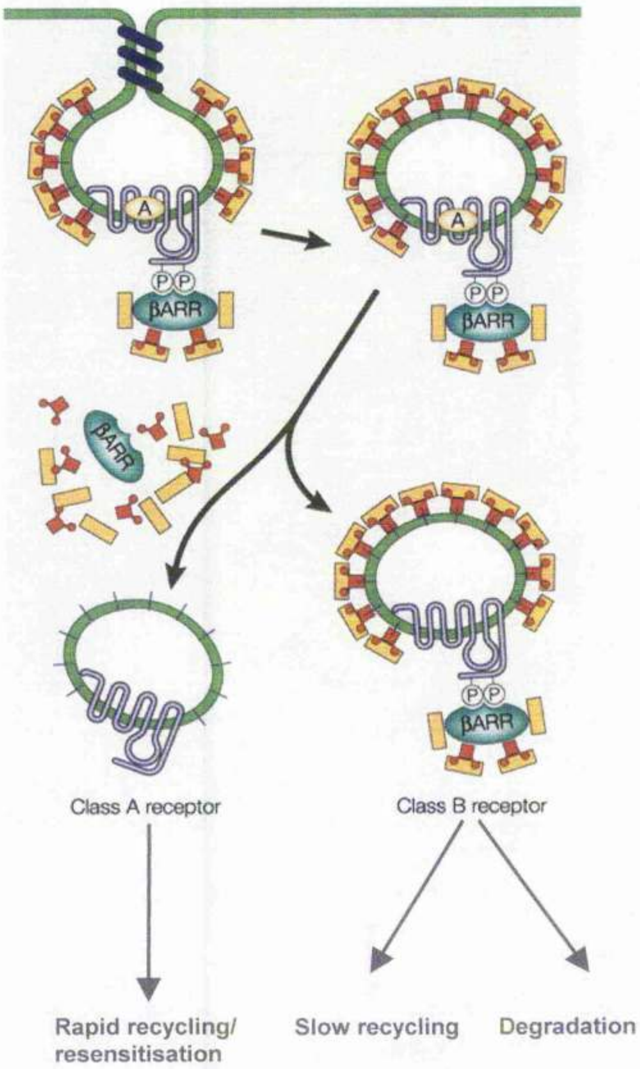
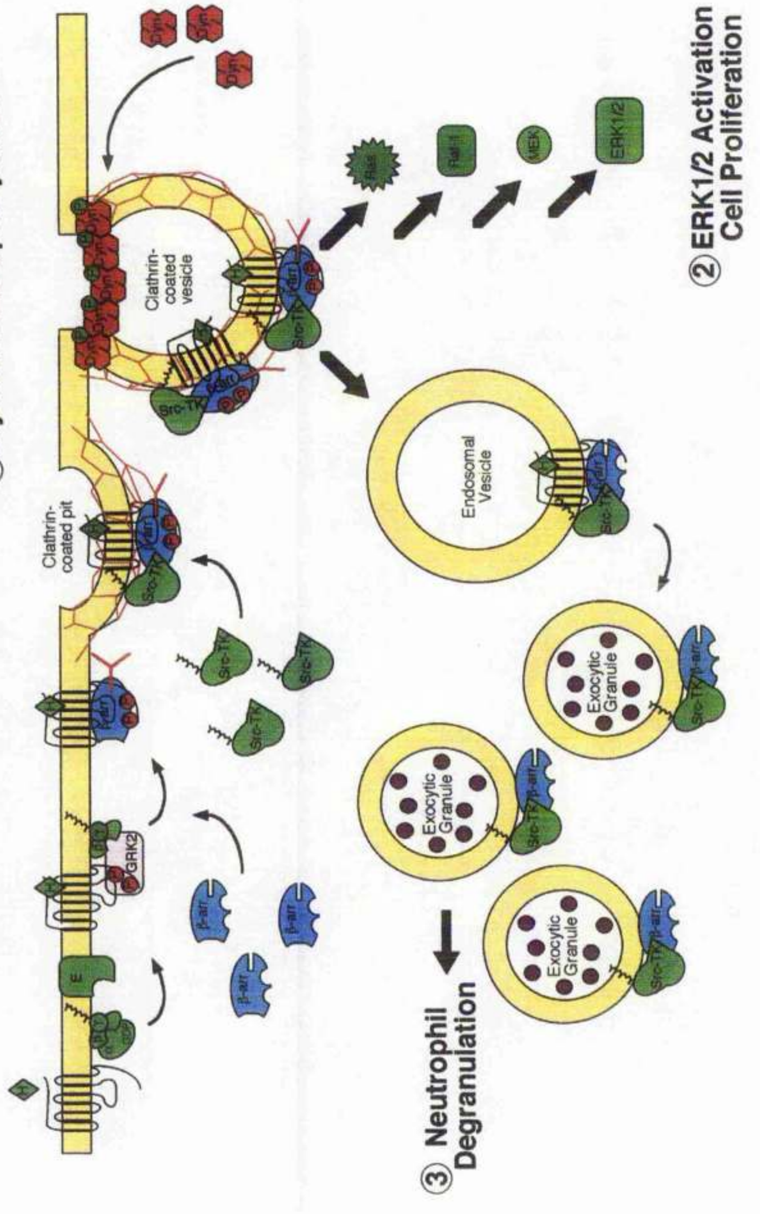

A
P P
βARR
A
P P
βARR
βARR
P P
βARR
Class A receptor
Class B receptor
Rapid recycling/
resensitisation
Slow recycling
Degradation

Figure 1.8: β-arrestin-dependent recruitment of Src kinases

Binding of β-arrestins to the agonist-stimulated receptors leads to concomittant recruitment of Src family tyrosine kinases such as c-Src, Hck, and c-Fgr to the β-arrestin-GPCR complex. The signalling events that involve β-arrestin-dependent recruitment of Src include the regulation of clathrin-dependent β_2-adrenergic receptor endocytosis by tyrosine phosphorylation of dynamin (1), Ras-dependent activation of the ERK 1 and 2 MAPK cascade and stimulation of cell proliferation by the β_2-adrenergic and neurokinin NK1 receptors (2), and stimulation of chemokine CXCR1 receptor-mediated neutrophil degranulation (3) (source: Luttrell and Lefkowitz, 2002).

Figure 1.8



① Dynamin Phosphorylation
② ERK1/2 Activation
Cell Proliferation
③ Neutrophil Degranulation
Clathrin-coated pit
Clathrin-coated vesicle
Endosomal Vesicle
Exocytic Granule
Exocytic Granule
Exocytic Granule
Exocytic Granule
GRK2
β-arr
Src-TK
Dyn
Ras
Raf-1
MEK
ERK1/2
E

Figure 1.9: Role of β-arrestins in the activation and targetting of MAPK

After agonist stimulation of the receptor β-arrestin acts as a scaffold to trigger the assembly of a MAPK activation complex. This pool of β-arrestin-bound ERK1/2 is subsequently activated. The receptor-β–arrestin-ERK1/2 complexes are localised to endosomal vesicles. Formation of these vesicles prevents nuclear translocation of activated ERK1/2 and therefore does not result in stimulation of cell proliferation. Therefore activation of ERK1/2 by β-arrestin scaffolds might cause the phosphorylation of plasma membrane, cytosolic or cytoskeletal ERK1/2 substrates, or it might lead to transcriptional activation through the ERK-dependent stimulation of other kinases. The model shows β-arrestin scaffolding of the ERK1 and 2 MAPK cascade, based upon data obtained with the protease-activated receptor 2 and the angiotensin II type 1A receptor. A similar mechanism has been proposed for the regulation of the JNK3 MAPK cascade by the angiotensin II type 1A receptor (source: Luttrell and Lefkowitz, 2002).

Figure 1.9

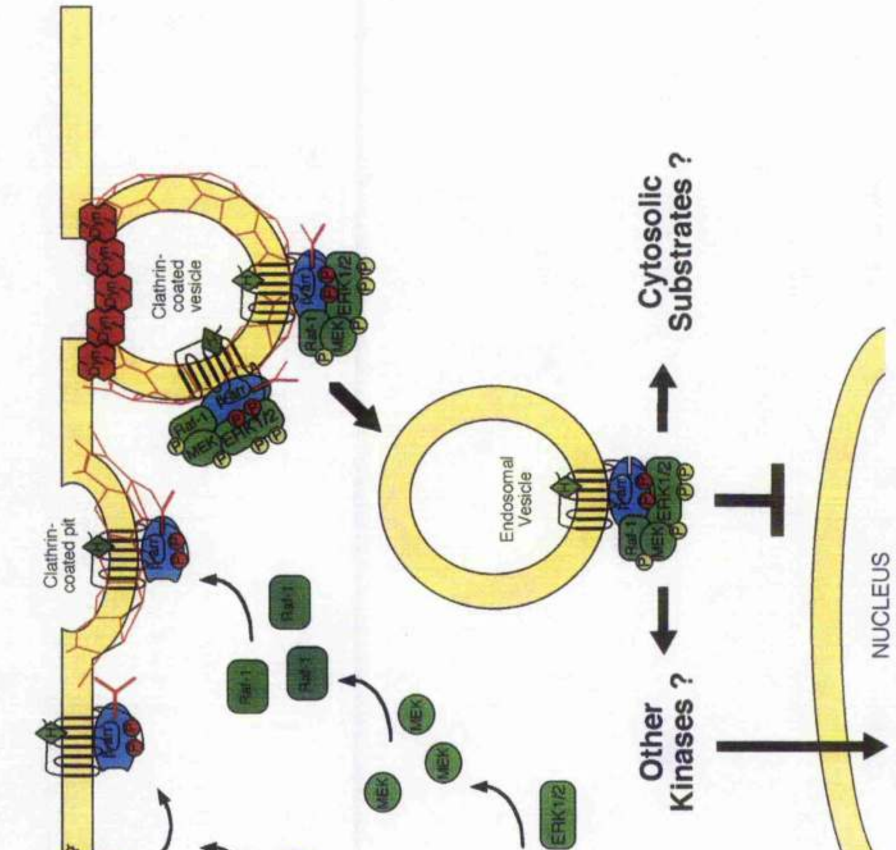

H
α
β
γ
GDP
E
GRK2
β-arr
βarr
P
Clathrin-coated pit
Raf-1
MEK
ERK1/2
Dyn
Clathrin-coated vesicle
Endosomal Vesicle
Cytosolic Substrates ?
Other Kinases ?
NUCLEUS

Figure 1.10: Schematic overview of MAPK modules

The MAPK module consists of a MAPKKK, MAPKK and a MAPK. MAPKKKs respond to a variety of extracellular signals such as growth factors, differentiation factors and stress. The activated MAPKKKs then activate one or more MAPKKs. In contrast the MAPKKs are relatively specific for their target MAPKs. Once activated MAPKs can then phosphorylate transcription factors (for example ATF-2, Chop, c-Jun, c-Myc, DPc4, Elk-1, Ets-1, Max, MEF2C, NFAT4, Sap1a, STATs, Tal, p53) other kinases (MAPK-activated protein (MAPKAP) kinase, $p90^{rsk}$ S6 kinase), upstream regulators like the epidermal growth factor receptor (EGFR) and other regulatory peptides such as phospholipase A2. These downstream targets then control cellular responses including growth, differentiation, and apoptosis.

Figure 1.10

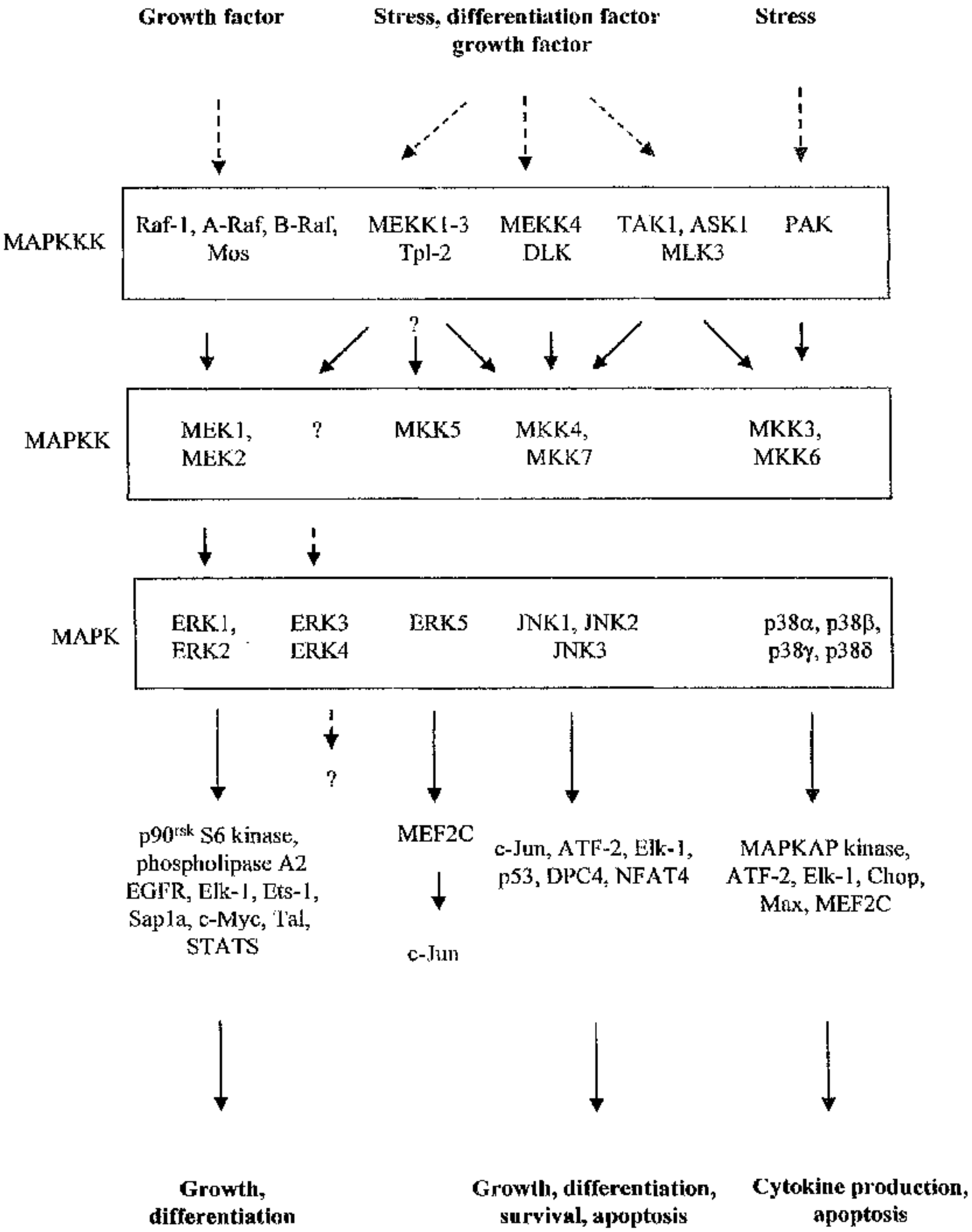

Growth factor
Stress, differentiation factor growth factor
Stress
MAPKKK
Raf-1, A-Raf, B-Raf, Mos
MEKK1-3 Tpl-2
MEKK4 DLK
TAK1, ASK1 MLK3
PAK
?
MAPKK
MEK1, MEK2
?
MKK5
MKK4, MKK7
MKK3, MKK6
MAPK
ERK1, ERK2
ERK3 ERK4
ERK5
JNK1, JNK2 JNK3
p38α, p38β, p38γ, p38δ
?
p90rsk S6 kinase, phospholipase A2 EGFR, Elk-1, Ets-1, Sap1a, c-Myc, Tal, STATS
MEF2C
c-Jun
c-Jun, ATF-2, Elk-1, p53, DPC4, NFAT4
MAPKAP kinase, ATF-2, Elk-1, Chop, Max, MEF2C
Growth, differentiation
Growth, differentiation, survival, apoptosis
Cytokine production, apoptosis

Figure 1.11: Components of the ERK MAPK pathway

MAPKKKs, MAPKKs, and MAPKs that can be components of the pathway. RTK, related tyrosine kinase;

Figure 1.11

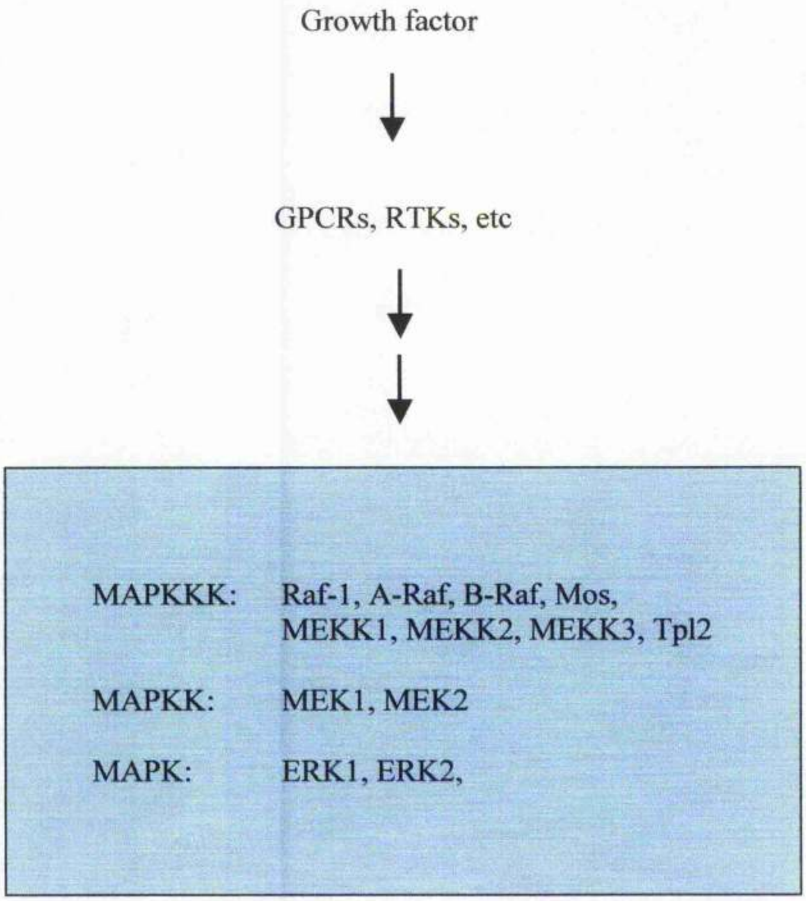
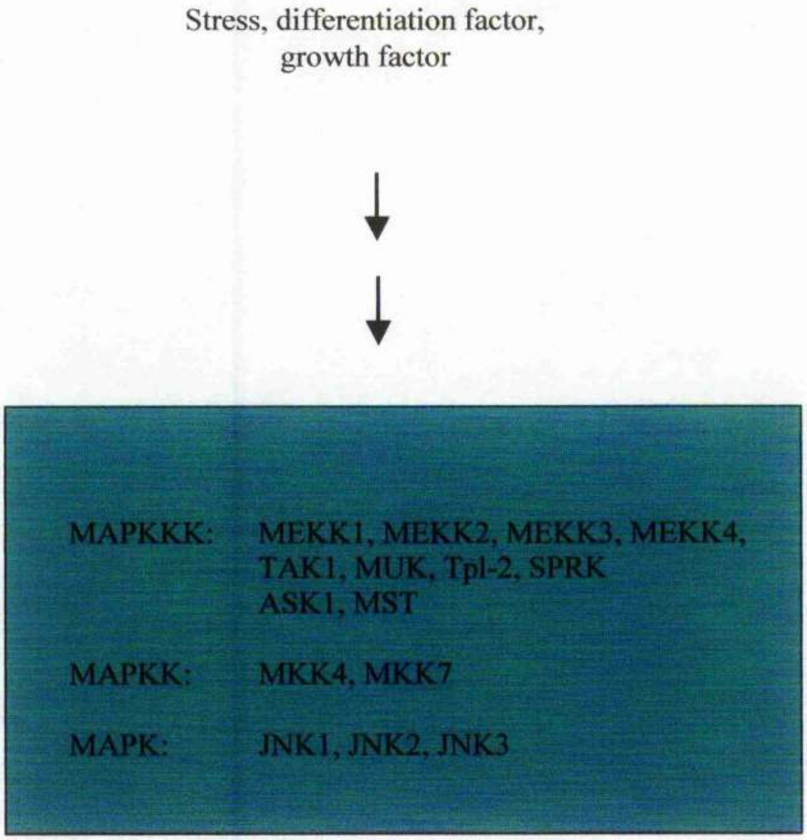

Growth factor
GPCRs, RTKs, etc
MAPKKK: Raf-1, A-Raf, B-Raf, Mos, MEKK1, MEKK2, MEKK3, Tpl2
MAPKK: MEK1, MEK2
MAPK: ERK1, ERK2,

Figure 1.12: Components of the JNK MAPK pathway

MAPKKKs, MAPKKs, and MAPKs that can be components of the pathway

Figure 1.12


Stress, differentiation factor,
growth factor

MAPKKK: MEKK1, MEKK2, MEKK3, MEKK4, TAK1, MUK, Tpl-2, SPRK ASK1, MST

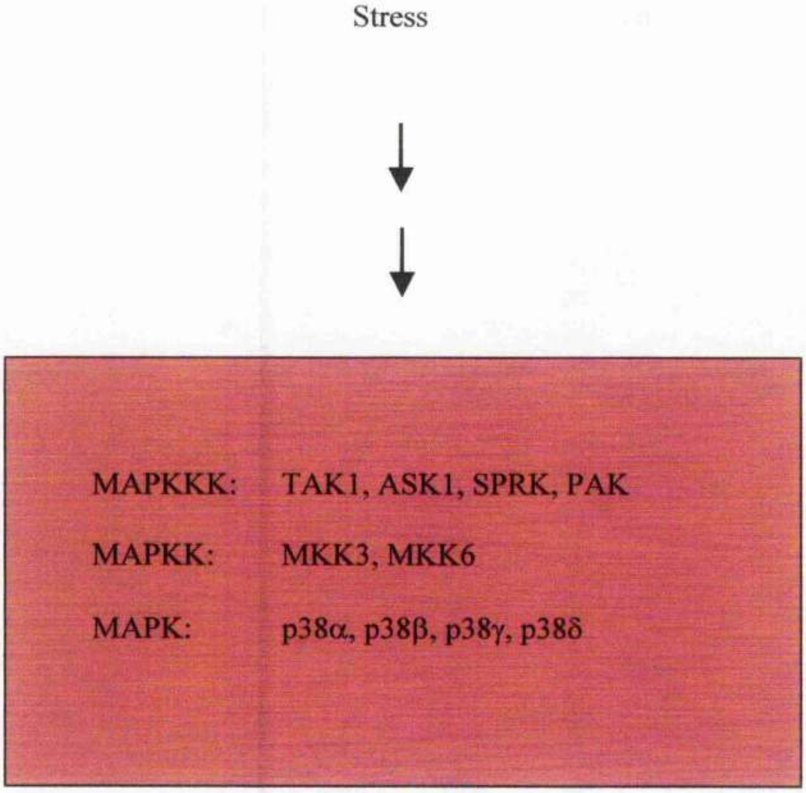
MAPKK: MKK4, MKK7

MAPK: JNK1, JNK2, JNK3

Figure 1.13: Components of the p38 MAPK pathway

MAPKKKs, MAPKKs, and MAPKs that can be components of the pathway

Figure 1.13


Stress
MAPKKK: TAK1, ASK1, SPRK, PAK
MAPKK: MKK3, MKK6
MAPK: p38α, p38β, p38γ, p38δ

Chapter 2

Material and Methods

2.1 Materials

All reagents employed were of the highest grade available and were obtained from the following suppliers.

2.1.1 General reagents, enzymes and kits

Amersham Pharmacia Biotech UK Ltd., Little Chalfont, Buckinghamshire, UK
Glutathione Sepharose™ 4B beads.

BDH, Lutterworth, Leicestershire, UK
Sodium chloride, potassium hydroxide, potassium chloride, glacial acetic acid, ethanol, methanol, isopropanol, microscope slides, 22 mm coverslips.

Calbiochem, CN Biosciences UK, Nottingham, UK
H89, GF109203X, geneticin sulphate (G418), BAPTA/AM, A23187.

Duchefa, Haarlem, The Netherlands
Yeast extract, tryptone, agar.

Fisher Scientific UK Ltd., Loughborough, Leicestershire, UK
Ammonium sulphate, glycine, HEPES, sucrose, SDS, potassium acetate, potassium di-hydrogen orthophosphate, calcium chloride, HCl, manganese chloride.

Millipore, Watford, UK
Immobilon Polyvinylidenfluoride (PVDF) membrane

ThermoBioSciences GmbH, Ulm Germany
Oligonucleotides for PCR reactions

Invitrogen Ltd., Paisley, UK
NuPage ® Novex pre-cast bis-tris gels, Xcell Sureblock™ mini-cell gel tank,
XCell II™ blot module, MOPS running buffer.

Konica Europe, Hohenbrunn, Germany

X-ray film

New England BioLabs, Hitchin, Hertfordshire, UK

Restriction endonucleases.

Pierce, Perbio Science UK Ltd., Tattenhall, Cheshire, UK

Supersignal® West pico chemoluminescent substrate

Promega UK Ltd., Southampton, UK

Restriction endonucleases, *Pfu* polymerase, Wizard^{TM} Plus SV Miniprep kit.

Qiagen, Crawley West Sussex, UK

QIAquick gel extraction kit, PCR purification kit.

Roche Diagnostics Ltd., Lewes, East Sussex, UK

Complete^{TM} protease inhibitor cocktail tablets, 1 kb DNA ladder, T4 DNA ligase, bovine serum albumine (fraction V), hygromycin.

Sigma-Aldrich Company Ltd., Poole, Dorset, UK

Agarose, sodium hydroxide, DTT, di-sodium orthophosphate, Tris, EDTA, EGTA, ATP, bromophenol blue, rubidium chloride, NP-40, Triton X-100, DMSO, glycerol, Tween 20, ethylene glycol, paraformaldehyde, ampicillin, DMEM (powder), Protein G-Sepharose, ethidium bromide, concanavalin A, gelatine (bovine, 2% solution), bovine serum albumin (essentially globulin-free), MOPS, forskolin, nystatin, filipin III, sodium tartrate, 8- bromo-cGMP, Ficoll (Type 400), xylene cyanol FF, BCA, cupric sulfate, sodium carbonate, sodium hydrogen carbonate, sodium deoxycholate, sodium flouride, magnesium sulfate heptahydrate, PMSF, β-glycerol phosphate, benzamidine, apigenin, thyrotropin-releasing hormone.

Whatman International Ltd., Maidstone, UK

3 mm-filter paper

2.1.2 Tissue culture plastic ware and reagents

Costar, Cambridge, MA., USA

15 ml and 50 ml centrifuge tubes, 5 ml, 10 ml and 25 ml pipettes, 25 cm^2 and 75 cm^2 tissue culture flasks, 60 mm and 100 mm dishes, 6 and 24 well plates, cryovials and cell scrapers.

Gibco BRL, Life Technologies Ltd., Paisley, UK

Lipofectamine^{TM} transfection reagent, Optimem-1, L-glutamine (200 mM), NBCS, Versene, DMEM without sodium pyruvate.

Sigma-Aldrich Company LTD., Poole, Dorset, UK

DMEM, 0.25 % trypsin-EDTA, poly-D-lysine, MEM alpha modification with ribonulceosides and deoxyribonucleosides, FBS.

2.1.3 Radiochemicals

PerkinElmer Life Sciences Inc., Boston, MA, USA

[^{32}P] orthophosphoric acid (285.5 Ci/mg), ATP (γ^{32}P) (3000Ci/mmol)

2.1.4 Antisera

Amersham Pharmacia Biotech UK Ltd., Little Chalfont, Buckinghamshire, UK

Goat anti-mouse IgG-HRP conjugate
Donkey anti-rabbit IgG-HRP conjugate
Donkey anti-sheep IgG-HRP conjugate
CypHer 5 Anti-VSV-G antibody

Cell Signaling Technology, Inc., Beverly, MA, USA

Phospho-p44/42 MAP Kinase (Thr 202/Tyr 204) antibody (rabbit polyclonal IgG)
p44/42 MAP Kinase antibody (rabbit polyclonal IgG)
Phospho-p38 MAP Kinase (Thr180/Tyr182) antibody (rabbit polyclonal IgG)
p38 MAP Kinase antibody (rabbit polyclonal IgG)

Molecular Probes, Eugen, Oregon, USA

Alexa ™ 594 goat anti-mouse IgG conjugate

Alexa ™ 488 goat anti-mouse conjugate

Roche Diagnostics Ltd., Lewes, East Sussex, UK

Anti-HA antibody (clone 12CA5)

Anti-VSV-G antibody (clone P5D4)

A sheep polyclonal anti-GFP antibody was generated in house.

2.2 Buffers

2.2.1 General buffers

PBS (10x)

137 mM NaCl

2.7 mM KCl

1.5 mM KH_2PO_4

This was dissolved in deionised water and the pH adjusted to 7.4 with HCl.

RIPA+ (1x)

25 mM HEPES pH 7.5

75 mM NaCl

0.5 % Triton X-100

0.25 % sodium deoxycholate ($C_{24}H_{39}O_4NaH_2O$)

0.05 % SDS

10 mM NaF

5 mM EDTA

10 mM Na_2HPO_4

5 % (w/v) ethylene glycol

TBS (10x)

0.2 M Tris

1.37 M NaCl

This was dissolved in deionised water and the pH adjusted to 7.6 with HCl.

Krebs Ringer Buffer (1x)

120 mM NaCl

25 mM HEPES

4.8 mM KCl

1.2 mM KH_2PO_4

1.2 mM $MgSO_47H_2O$

1.3 mM $CaCl_2$

This was dissolved in deionised water and the pH adjusted to 7.4 with NaOH.

Laemmli Buffer (2x)

12.6 mM Tris

20 % (w/v) glycerol

100 mM DTT

12 % SDS

0.01 % (w/v) Bromophenol Blue

This was dissolved in deionised water and the pH adjusted to 6.8 with HCl.

2.2.2 Molecular Biology Solutions

TAE buffer (50x):

2 M Tris

5.7 % glacial acetic acid

50 mM EDTA

DNA Loading Buffer (6x)

0.25 % bromophenol blue

0.25 % xylene cyanol FF

15 % Ficoll (Type 400)

Liquid Broth (LB)

0.5 % Yeast extract, 1 % tryptone, 1 % NaCl

This was dissolved in deionised water and then autoclaved at 126 °C.

2.3 Molecular Biology Protocols

2.3.1 LB ampicillin agar plates

This has basically the same composition as LB with 1.5 % (w/v) agar added. The solution was autoclaved and then left to cool before adding ampicillin to a final concentration of 50 μg/ml. The still liquid agar was poured into 100 mm dishes and allowed to solidify at room temperature. LB agar plates can be stored at 4 °C for up to 3 weeks without ampicillin losing any of its activity.

2.3.2 Preparation of competent bacteria

The uptake and expression of foreign DNA into *E. Coli* is known as transformation. The *E. Coli* strain, which was DH5α in this case, has to be made competent before transformation in order for the bacteria to be able to take up and express the plasmid vectors containing a particular cDNA insert. Competent *E. Coli* cells are made using the following procedure.

Solution 1

30 mM potassium acetate (CH_3COOK)
10 mM $RbCl_2$
10 mM $CaCl_2$
50 mM $MnCl_2$
15 % (w/v) glycerol
This was dissolved in deionised water and the pH adjusted to 5.8 with 100 mM acetic acid. The solution was filter-sterilised and stored at 4 °C.

Solution 2

10 mM MOPS pH 6.5

75 mM $CaCl_2$

10 mM $RbCl_2$

15 % (w/v) glycerol

This was dissolved in deionised water and the pH adjusted to 6.5 with HCl. The solution was filter-sterilised and stored at 4 °C.

DH5α cells were streaked out on an LB agar plate with no antibiotics and grown overnight at 37 °C. A single colony was picked and cultured in 5 ml LB at 37 °C overnight. The 5 ml culture was then added to 100 ml of fresh LB and grown until the optical density at 550 nm was 0.48. After chilling the culture on ice for 5 min the cells were spun at 3000 rpm (1811 x g) for 20 min at 4 °C in sterile 50 ml tubes. After resuspending each pellet in 20 ml of solution 1 the cells were again chilled on ice for 5 min and then spun as before. Finally each pellet was resuspended in 2ml of solution 2 and chilled on ice for a further 15 min. The cells were aliquoted and stored at -80 °C.

2.3.3 Transformation of competent bacterial cells with plasmid DNA

100 μl of competent cells were incubated on ice with 10-100 ng of plasmid DNA for 15 min. The mix was then heat shocked for 45 sec at 42 °C and placed back on ice for a further 2 min prior to addition of 900 μl of LB. The cells were then allowed to recover by incubation at 37 °C for 1 hr in a shaking incubator. 200 μl of this mix was spread out on a LB agar ampicillin plate and the plates incubated at 37 °C overnight. Transformed colonies were selected and cultured overnight at 37 °C in 5 ml LB containing 50 μg/ml ampicillin.

2.3.4 Preparation of plasmid DNA

2.3.4.1 Miniprep

Plasmid cDNA was prepared using the Promega Wizard^{TM} Plus SV Miniprep purification system. A 5ml culture of transformed cells was grown overnight at 37 °C as described before. 1.5-3 ml of the culture was spun at 13000 rpm and the pellet resuspended in 250 μl of resuspension solution (50 mM Tris-HCl pH 7.5, 10 mM EDTA, 100 μg/ml RNase), followed by lysis with 250 μl of lysis solution (0.2 M NaOH, 1 % SDS). The resulting lysate was neutralised with 350 μl of neutralisation solution (4.09 M guanidine hydrochloride, 0.76 M potassium acetate, 2.12 M glacial acetic acid, pH 4.2) to precipitate any unwanted chromosomal DNA. This was removed by centrifugation and the resulting supernatant was transferred to a DNA purification column. The column was washed twice (once with 750 μl and then with 250 μl) with column wash solution (60 mM potassium acetate, 10 mM Tris-HCl pH 7.5, 60 % ethanol). Finally the cDNA was eluted from the column with 50 μl of sterile water.

2.3.4.2 Maxiprep

To achieve a larger scale of purified DNA the Qiagen Plasmid Maxi system was used. A 5 ml culture of transformed bacteria was grown as before only this time the culture was used to inoculate a 100 ml LB culture. After shaking for 16 hr at 37 °C, the culture was spun at 4000 rpm (3220 x g) and 4 °C for 30 min. The bacterial pellet was resuspended in 10 ml of chilled buffer P1 (50 mM Tris-HCl pH 8.0, 10 mM EDTA, 100 μg/ml RNase A) and lysed with 10 ml buffer P2 (200 mM NaOH, 1 % SDS). The lysate was neutralised with 10 ml of cooled buffer P3 (3.0 M potassium acetate pH 5.5). While the sample was spinning at 4000 rpm (3220 x g), the Qiagen-tip 500 column was equilibrated with 10 ml of buffer QBT (750 mM NaCl, 50 mM MOPS pH 7.0, 15 % isopropanol). The supernatant was poured into the column, the column washed with 60 ml of buffer QC (1.0 M NaCl, 50 mM MOPS pH 7.0, 15 % isopropanol) and the DNA eluted by adding 15 ml of buffer QN (1.25 M NaCl, 50 mM Tris-HCl pH 8.5, 15 % isopropanol). The DNA was precipitated by adding 10.5 ml of isopropanol and the mixture centrifuged at 15000 rpm (27216 x g) for 30 min in a cooled centrifuge. The resulting pellet was washed with 5 ml of 70 % ethanol and then allowed to air dry. Finally the DNA was re-dissolved in 0.5-1.0 ml of sterile water.

2.3.5 DNA quantification

The concentration of DNA in a given sample was determined by measuring the absorbance at 260 nm (A_{260}) of a 1:50 dilution of the DNA sample. It was assumed that 1 absorbance unit was equivalent to 50 μg/ml of double stranded DNA. The purity of DNA was determined by measuring the absorbance at 280 nm (A_{280}). A ratio of approximately 1.7 of A_{260}/A_{280} was considered to be sufficiently pure for use.

2.3.6 Digestion of DNA with restriction endonucleases

To subclone DNA fragments into plasmid vectors, the DNA needed to be digested by restriction endonucleases. The digests were set up using the conditions recommended by the manufacturer. Briefly, 1 μg of DNA was digested in 10 μl of a buffered solution containing 1 unit of the appropriate enzyme for a minimum of 2 h at 37°C.

2.3.7 DNA gel electrophoresis

Digested DNA fragments were separated and analysed using agarose gel electrophoresis. Samples were prepared by addition of 6x loading buffer to a 1x final concentration. DNA fragments between 0.4 and 5 kb were separated on a 1 % (w/v) agarose gel containing 0.5x TAE buffer and 2.5 mg/ml ethidium bromide. The gels were run at 100 mA in horizontal gel tanks, containing 0.5x TAE buffer. To analyse the separated DNA fragments ultraviolet light was used. The size of the bands was assessed by comparison with a 1 kb ladder. To excise fragments for gel purification a sterile scalpel blade was used.

2.3.8 DNA purification from agarose gels

To purify DNA fragments from agarose gels the Qiagen QIAquick gel extraction kit was used. The excised DNA fragments were dissolved in QIAquick buffer QG followed by addition of isopropanol. The solution was loaded onto a QIAquick purification column, washed with an ethanol based wash solution (PE) and finally the DNA eluted from the column with sterile water.

2.3.9 DNA ligations

T4 DNA ligase was used to ligate vector DNA with a desired cDNA insert or inserts. For each ligation, a vector:insert ratio of 1:2 was used. Reactions were performed in a total volume of 10 μl containing enzyme buffer and 1 unit of T4 DNA ligase. The mixture was incubated for 3-5 h at room temperature. The ligation mixtures were transformed into competent bacteria as described in 2.3.2.

2.3.10 Polymerase chain reaction (PCR)

PCR reactions were routinely performed in a total volume of 100 μl containing 20 ng of DNA template 0.2 mM dNTPs (dATP, dCTP, dGTP, dTTP), 0.2 μg of sense and antisense primers, 1 x thermophilic buffer (20 mM Tris-HCl pH 8.2, 10 mM KCl, 6 mM $(NH_4)_2SO_4$, 2 mM $MgCl_2$, 0-1 % Triton X-100, 10 μg/ml BSA) and 2 units of *Pfu* DNA polymerase. The reactions were carried out on an Eppendorf Mastercycler gradient. The reaction mixtures were initially heated to 95 °C for 5 min before the start of the first cycle.

PCR Cycles:

Denaturation	Annealing	Extension	Cycle
95 °C, 1 min	60 °C, 1 min	72 °C, 2min	30
95 °C, 1 min	60 °C, 1 min	72 °C, 10min	1

2.4 Construction of orexin 1 receptor cDNAs

All primers are listed in Figure 2.1

2.4.1 N-terminal tagged constructs

A human *HindIII*-FLAG-orexin 1 receptor-*XhoI* in pcDNA3 had been generated previously in the laboratory and was used as a template to synthesise the N-terminal HA- or VSV-G-tagged constructs. To generate the N-terminal tagged forms of the receptor, advantage was taken of the fact that the orexin 1 receptor has an *AflII* site at aa 330-331, therefore making it unnecessary to amplify the whole receptor cDNA. FLAG-orexin 1 receptor was amplified from the N-terminal FLAG region to the *AflII* site using the *HindIII* sense primers VSV-ox1R or HA-ox1R and the antisense primer ox1R 3. This replaced the FLAG-tag with either a VSV- or an HA tag. The PCR product as well as the FLAG-orexin 1 receptor were digested with *HindIII* and *AflII* and the original insert removed by gel extraction as described in 2.3.8. The resulting PCR fragment was ligated into the vector.

2.4.2 C-terminal truncations

The FLAG-orexin 1 receptor 394 and 378 truncations had been previously generated in the laboratory. Human orexin 1 receptor in pcDNA3 was amplified by PCR using the forward primer ox1R 1 and the *XhoI* reverse primer ox1R389 or ox1R374. This introduced a stop codon in the position immediately following the desired new C-terminus. The PCR products, the FLAG-orexin 1 receptor 394 and 378 constructs and the HA-orexin 1 receptor were digested with *AflII* and *XhoI* and the PCR fragments inserted into the vector as before.

All C-terminal tails of the generated truncation mutants are listed in Figure 2.2.

2.4.3 C-terminal mutations

2.4.3.1 Cluster mutants

Cluster C1 (aa 418-422)

This mutant was generated by amplifying the orexin 1 receptor in pcDNA3 with the sense primer ox1R 1 and the *XhoI* antisense primer ox1RC1. This mutated Thr 418, 421 and 422 and Ser 419 to Ala. The PCR product and HA- or VSV-G-orexin 1 receptor in pcDNA3 were digested with *AflII* and *XhoI* and the PCR fragments inserted into the vector as previously described.

Cluster C2 (aa 393-396) and Cluster C1C2

These were generated using a method called overlap PCR as described in Figure 2.3. In the first round of PCR orexin 1 receptor in pcDNA3 was amplified with the forward primer ox1R 1 and the reverse primer ox1R 3. At the same time, but in separate reactions the orexin 1 receptor DNA was also amplified with the sense primer ox1RC2 and the *XhoI* antisense primer ox1R 5 or ox1RC1 to synthesise the C2 or C1C2 mutant, respectively. In the next round of PCR, the different PCR products were used as templates to generate a PCR product with a N-terminal *AflII* site and a C-terminal *XhoI* site and also the desired mutations using the forward primer ox1R 1 and the *XhoI* reverse primer ox1R 5 or ox1RC1. This mutated the Ser/Thr residues within these regions to Ala. The PCR products and HA- or VSV-G-orexin 1 receptor in pcDNA3 were digested with *AflII* and *XhoI* and the PCR fragments inserted into the vectors as before.

All C-termini of the cluster mutants are given in Figure 2.2.

2.4.3.2 Single and double point mutants within Cluster C1

Orexin 1 receptor in pcDNA3 was amplified with the sense primer ox1R 1 and one of the following *XhoI* antisense primers: ox1RT418A, ox1RS419A, ox1RT421A, ox1RT422A, ox1RT418/S419A, ox1RT418/T421A, ox1RT418/T422A, ox1RS419/T421A, ox1RS419/T422A or ox1RT421/T422A. This mutated the indicated Ser and/or Thr residues to Ala. The resulting PCR products and HA- or VSV-G-orexin 1 receptor in pcDNA3 were digested with *AflII* and *XhoI* and the PCR fragments inserted into the vectors as described in 2.4.1.

All sequences of the C-terminus of the single and double point mutants are described in Figure 2.2.

2.4.4 i2 loop mutants

These constructs were generated using the overlap PCR strategy as previously described. Orexin 1 receptor in pcDNA3 was amplified with the *HindIII* forward primer VSV-ox1R and the reverse primers ox1RI148E-L152D, ox1RI148E, or ox1RL152D. At the same time the template was amplified with the sense primers ox1RI148E-L152D, ox1RI148E, ox1RL152D, and the antisense primer ox1R 3. In the next round the two different PCR products were utilised as templates and amplified with the *HindIII* forward primer VSV-ox1R and the reverse primer ox1R 3. The PCR products and orexin 1 receptor in pcDNA3 were digested with *HindIII* and *AflII* and the resulting PCR fragments inserted into the vector as before.

All sequences of the i2 loop mutants are listed in Figure 2.4.

2.4.5 HA-orexin 1 receptor C1/C2-eYFP

A *HindIII*-orexin 1 receptor-*NotI*-eYFP-*XhoI* construct in pcDNA3 had been previoulsy generated in the laboratory. To generate orexin 1 receptor C1-eYFP, HA-orexin 1 receptor DNA was amplified by PCR with the forward primer ox1R 1 and the *NotI* reverse primer ox1RC1 NotI (noTAG). In case of orexin 1 receptor C2-eYFP, HA-orexin 1 receptor C2 DNA was used as template and amplified by PCR with the forward primer ox1R 1 and the *NotI* reverse primer ox1R NotI (noTAG). This removed the stop codon of the orexin 1 receptor mutants. The PCR product was digested with *AflII* and *NotI*. Since the eYFP sequence contained an *AflII* restriction site, it was necessary to digest the HA-orexin 1 receptor with *HindIII* and *AflII* and the orexin 1 receptor-eYFP construct with *HindIII* and *Not*I. In a two way ligation the *AflII-NotI* PCR fragment and the *HindIII-AflII* receptor fragment were ligated in frame into the vector containing eYFP as described before.

2.5 GST fusion protein preparation

2.5.1 Preparation of protein

Colonies of transformed XL 10 Gold *E. Coli* bacteria were picked and grown overnight in 20 ml of LB broth containing 100 μg/ml of ampicillin. The next morning this culture was added in a 1:30 dilution to 200 ml of LB broth containing 100 μg/ml of ampicillin. This culture was grown until an OD_{600} of 0.2 was reached and then induced with 0.5 mM IPTG for another 3 h. Afterwards the culture was spun for 15 min at 6000 rpm (5524 x g) and and 4 °C to pellet the cells. 1 ml samples that were taken prior to IPTG addition and after the 3 h incubation, were spun at 14000 rpm (20817 x g) and the pellet resuspended in 100 μl of Laemmli buffer.

The pellet of the large culture was resuspended in 9 ml GST extraction buffer and frozen on dry ice. The sample was quickly thawed and sonicated three times for 30 sec each at 60 kHz using a probe sonicator. 1ml of 10 % Triton X-100 was added and the sample rotated for 40 min at 4 °C. The lysate was centrifuged for 15 min at 6000 rpm (5524 x g) and 4 °C and the supernatant transferred to a sterile 50 ml tube containing 300 μl of washed (three times with TE-buffer) Glutathione Sepharose™ 4B beads. The sample was spun on a rotary wheel for 1 hr at 4 °C. The mixture was centrifuged for 5 min at 900 rpm (163 x g) at 4°C and the glutathione Sepharose® 4B gel washed three times with TE-buffer. After the last washing step the pellet was resuspended in 300 μl TE-buffer and stored at 4°C.

TE-buffer
10 mM Tris (pH 7.4)
0.1 mM EDTA

GST extraction buffer

500 mM HEPES (pH 7.4)	2 ml
4 M NaCl	1.7 ml
0.5 M β-glycerol phosphate	2.5 ml
1M Na pyrophosphate	0.1 ml
0.25 M EDTA	0.4ml
10 % (v/v) glycerol	5 ml
1 M DTT	0.25 ml
100 μg/ml benzamidine	0.5 ml
0.2 M PMSF	0.5 ml

2.5.2 SDS polyacrylamide gel electrophoresis (SDS-PAGE)

The samples were diluted in Laemmli-buffer and boiled for 5 min at 95 °C and resolved on NuPage® Novex pre-cast bis tris gels from Invitrogen. The NuPage® system is based upon a bis-tris-HCl buffered (pH 6.4) polyacrylamide gel, containing a separating gel that operates at pH 7.0. Gels with a polyacrylamide concentration of 4-12 % achieved the best separation of the proteins of interest and hence were mostly used. To run the gels NuPage® MOPS SDS running buffer was used. The gels were run at 200 V in the Xcell Sureblock™ mini-cell gel tank, also supplied by Invitrogen.

2.6 Routine cell culture

2.6.1 Cell growth

The cell lines used in this study were Human Embryonic Kidney T (HEK293T) cells, Mouse Embryonic Fibroblasts (MEF), Chinese Hamster Ovary (CHO) cells. Each cell line was grown in monolayers in 75 cm^2 tissue culture flasks. The cells were incubated in a humified atmosphere of 95 % air/5 % CO_2 at 37 °C.

Cell line	Medium	Supplements
HEK293T	DMEM	2 mM L-glutamine, 10% NBCS
MEF	DMEM	2 mM L-glutamine, 10% FBS,
CHO	MEM α modification with ribonucleosides	2 mM L-glutamine, 10 % NBCS

2.6.2 Cell subculture

Cells were grown till confluency and passaged using a sterile 0.25 % trypsin-EDTA solution. The medium was aspirated from the cells and 2 ml of trypsin solution was added. After all the cells had detached from the surface of the tissue culture flask, 8 ml of medium was added to stop the reaction. The cells were resuspended by mixing them gently and the cell suspension was split into flasks or dishes as required.

2.6.3 Coating of plates with poly-D-lysine or gelatine

The poly-D-lysine was diluted with sterile water to make a 1 mg/ml stock solution. The gelatine was already in solution and just warmed prior to use. Tissue culture plates or coverslips were washed twice with either the poly-D-lysine solution or gelatine and then left to dry before cells were added.

2.6.4 Transient transfections

Lipofectamine™ was used according to the manufacturer's instructions to transiently transfect plasmid DNA into HEK293T or CHO cells.

For transfection of cells in 10 cm dishes, the cells were grown to 60-80 % confluency and 5-10 μg of DNA at a concentration of 0.1 μg/μl in sterile water was used for each dish. A typical transfection was as follows:

	Tube 1	Tube 2
DNA	50-100 μl	-
Optimem-1	500-550 μl	570 μl
Lipofectamine™	-	30 μl

The DNA and Lipofectamine^{TM} solutions were mixed and incubated for 30 min at room temperature. In the meantime the medium on the cells was removed and the cells incubated in Optimem-1 for 30 min at 37 °C. Then 4.8 ml of Optimem-1 was added to the DNA-Lipofectamine^{TM} mixture and, after aspirating Optimem-1 from the cells, the mixture was added gently to the cells. After an incubation of 3-5 h at 37 °C, the transfection solution was removed and 10 ml of media added. The next day the cells were either used for experiments directly or split into 6 well plates and used the following day.

For transfection of cells on coverslips in 6 well plates, the same protocol was followed with the exception that 1 μg of DNA/well was used. Also the ratio of Lipofectmine^{TM}:Optimem-1 was not 1:19 but 1:29.

	Tube 1	Tube 2
DNA	10 μl	-
Optimem-1	90 μl	96.7 μl
Lipofectamine^{TM}	-	3.3 μl

The two solutions were mixed and incubated as described before. 800 μl of Optimem-1 was added to the mixture and the resulting solution added to the appropriate well. After 3-5 h, the solution was aspirated and replaced by 2 ml of medium. 24 h after the transfection the cells were fixed and and viewed using confocal microscopy.

2.6.5 Transient transfections using the Amaxa Nucleofactor^{TM}

This was performed according to the manufacturer's instructions. The cells were detached from the flask with trypsin and $5x10^5$ to $2x10^6$ of cells were spun at 1000 rpm (201 x g) for 5 min. The pellet was resuspended in 100 μl of pre-warmed Nucleofactor^{TM} Solution and mixed with 5 μg of DNA. The sample was transferred into an Amaxa certified cuvette and the cuvette inserted into the machine. After nucleofecting the cells with the appropriate program, 500 μl of medium was added to the cells. The cells were transferred to a single well of a 6 well plate and used 24 h after transfection.

	Solution	Program
CHO cells	T	H14
MEF cells	V	T20

2.7 Protein Biochemistry

2.7.1 Protein determination by BCA assay

To determine the protein concentration in cell lysates bincinchoninic acid (BCA) and copper sulphate solutions were used. Proteins reduce Cu(II) ions to Cu(I) in a concentration dependent manner. BCA is a highly specific chromogenic reagent for Cu(I), forming a purple complex with an absorbance maximum at 562 nm. The absorbance is directly proportional to the protein concentration. The protein concentration was determined using known concentrations of BSA solutions as standard (0.2-2 mg/ml).

Reagent A	Reagent B
1 % (w/v) BCA	4 % $CuSO_4$
2 % (w/v) Na_2CO_3	
0.16 % (w/v) sodium tartrate ($C_4H_4O_6Na_2 2H_2O$)	
0.4 % NaOH	
0.95 % $NaHCO_3$	
pH 11.25	

One part reagent B was added to 49 parts reagent A. 200 μl of this solution was added to 10 μl of each protein sample/standard in a 96 well plate. The absorbance was read after incubation at 37 °C for 30 min.

2.7.2 Preparation of samples for SDS gel electrophoresis

2.7.2.1 Whole cell lysates

Cells from 6 well plates were washed twice with ice cold PBS and then lysed using 500 μl of RIPA+ buffer supplemented with 1 x Complete™ protease inhibitor cocktail solution following the appropriate drug treatment. After incubation of the lysates on a rotating wheel for 1 h at 4 °C, the insoluble material was removed by a 10 min centrifugation at 14000 rpm (20817 x g) and 4 °C. The supernatant was transferred to a fresh tube and the protein determined by BCA assay as described in 2.6.1. The samples were diluted to a concentration of 1 mg/ml, then mixed 1:1 with Laemmli buffer and boiled for 3 min at 95 °C before loading onto SDS-PAGE gels.

2.7.2.2 Immunoprecipitation of samples

They were prepared as described for the whole cell lysates. But instead of diluting the extracts to 1 mg/ml, the extracts were equalised after the BCA assay. The protein of interest was immunoprecipitated from each sample by incubation with 20 μl of protein G-Sepharose and the appropriate antibody (2 μg anti-GFP, 1 μg HA, 1 μg anti-VSV-G) in 100 μl of 2 % BSA (virtually globulin-free) in RIPA+ for at least 1 h at 4 °C. The immune complexes were isolated by centrifugation at 14000 rpm (20817 x g) for 1 min, washed twice with 1 ml RIPA+ containing 0.2 M ammonium sulphate and once with RIPA+ alone. The proteins were eluted from the beads by incubation with 50 μl Laemmli buffer overnight at room temperature. The eluates were then loaded onto SDS-PAGE gels.

2.7.2.3 Co-immunoprecipitation of orexin 1 receptor and β-arrestin-2-GFP

Following addition of agonist, the stimulations were stopped by addition of the membrane permeable and reversible cross-linker dithiobis[succinimidylpropionate] (DSP) (Sigma, Gillingham, Dorset, U.K.) at a final concentration of 2 mM. The cells were then incubated under gentle agitation at room temperature, washed twice with 50 mM Tris-HCl pH 7.4 in PBS to neutralise unreacted DSP, lysed in 0.5 ml of 50 mM HEPES pH 7.4, 50 mM NaCl, 10 % (v/v) glycerol, 0.5 % (v/v) NP-40, 2 mM EDTA, 100 μM Na_3VO_4, supplemented with 1 x Complete^{TM} protease inhibitor cocktail solution and clarified by centrifugation. 25 μl aliquots of whole cell lysates were removed and mixed with an equal volume of 2 x reducing loading buffer. To isolate β-arrestin 2-bound orexin 1 receptor, 500 μg of each lysate was incubated with 20 μl of protein G-Sepharose in 100 μl buffer containing 5 % BSA (virtually globulin-free) and 2 μg anti-GFP antibody. Immunoprecipitation was performed for 12-16 h at 4 °C. Immune precipitates were washed 3 times with glycerol lysis buffer and eluted in 1 x reducing loading buffer for 15 min at 45 °C. Proteins were resolved on SDS-PAGE.

2.7.3 Western blotting

Following SDS-PAGE as described in 2.5.2, the proteins were electrophoretically transferred onto a PVDF membrane at 30 V for 1-2 h in transfer buffer (0.2 M glycine, 25 mM Tris, 20 % (v/v) methanol) using the XCell II^{TM} blot module (Invitrogen). The membranes were blocked with 5 % (w/v) fat free milk in TBS/0.1 % (v/v) Tween 20 for 1 h at room temperature. The membranes were then incubated with the appropriate primary antibody in blocking buffer overnight at 4 °C. After removing the primary antibody and washing the membranes 4 times for 5 min each with TBS/0.1 % (v/v) Tween 20, the blots were treated with the required HRP-conjugated secondary antibody in blocking buffer for 1 h at room temperature. After repeating the washing, the reactive proteins were visualised by enhanced chemiluminescence and exposure onto photosensitive film. For Western blot analysis the following antibody dilutions were used:

1° Antibody	Dilution	2° Antibody	Dilution
Anti-VSV-G	1:5000	Anti-mouse IgG	1:5000
Anti-HA	1:5000	Anti-mouse IgG	1:5000
Anti-MAPK	1:1000	Anti-rabbit IgG	1:2000
Anti-P-MAPK	1:1000	Anti-rabbit IgG	1:2000
Anti-p38	1:1000	Anti-rabbit IgG	1:2000
Anti-P-p38	1:1000	Anti-rabbit IgG	1:2000
Anti-GFP	1:10000	Anti-sheep IgG	1:10000

2.8 Assays

2.8.1 Confocal laser scanning microscopy

Cells were observed using a laser scanning confocal micrsocope (Zeiss LSM 5 Pascal) using a Zeiss Plan-Apo 63 X 1.40 NA oil immersion objective, pinhole of 20 and electronic zoom 1 or 2.5. The GFP and eYFP were excited using a 488 nm argon laser and detected with 505-530 band pass filter. The Alexa^{TM} 594 label, TAMRA-orexin A ligand and RFP were excited using a 543 nm helium/neon laser and detected with a 560 long-pass filter. To visualise the CypHer 5-labelled anti-VSV-G antibody, the cells were excited using a 633 helium/neon laser and detected with a 590 long-pass filter. The pictures were manipulated with Universal Imaging MetaMorph software.

2.8.1.1 Fixed cell work

Receptor-eYFP and β-arrestin-RFP

Cells transiently transfected and grown on glass coverslips were washed 3 times with PBS and fixed for 10 min at room temperature using 4 % paraformaldehyde in PBS/5 %

sucrose, pH 7.4. After a further three washes with PBS, coverslips were mounted on microscope slides with 40 % glycerol in PBS.

Immunostaining for VSV/HA-orexin 1 receptor

Immunostaining was performed essentially according to the protocol of Cao *et al.* (1999). Cells were plated onto coverslips and transfected after 24 h with the appropriate constructs. After a further 24 h, the medium was changed for 20 mM HEPES/DMEM containing 2.5 μg/ml of anti-HA/VSV-G- antibody and incubated for 40 min at 37 °C in 5 % CO_2. Where required, to give a final concentration of 0.5 μM agonist, 20 mM HEPES/DMEM containing orexin A was added and incubated for 30 min at 37 °C in 5 % CO_2. Coverslips were washed 3 times with PBS and then cells fixed with 4 % paraformaldehyde in PBS/5 % sucrose for 10 min at room temperature followed by three more PBS washes. Cells were then permeabilised in 0.15 % Triton X-100/3 % nonfat milk/PBS (TM buffer) for 10 min at room temperature. The coverslips were subsequently incubated with an Alexa^{TM} 594 goat anti-mouse secondary antibody at a dilution of 1:400 (5 μg/ml), upside down on Nescofilm, for 1 hr at room temperature; washed twice in TM buffer and 3 times with PBS. Finally, coverslips were mounted onto microscope slides with 40 % glycerol in PBS.

Immunostaining for VSV-G-orexin 1 receptor using the CypHer 5 labelled antibody

The antibody labelling was carried out basically as described in Adie *et al.* (2002). Cells were seeded onto coverslips the day before use and transfected with the different constructs. 24 h later the cells were washed twice with Krebs-Ringer buffer (KRB) at room temperature and then incubated with CypHer 5-labelled anti-VSV-G antibody at a concentration of 20 μg/ml for 30 min at room temperature in the presence of the different inhibitors. After this time orexin A was added to the cells and incubated for 30 min at
37 °C in 5 % CO_2. Coverslips were washed 3 times with KRB and fixed with 4 % paraformaldehyde in PBS/5 % sucrose for 10 min at room temperature followed by 3 more washes with KRB. Then the coverslips were mounted onto microscope slides with 40 % glycerol in PBS.

2.8.1.2 Live cell work

When examining live cells, cells grown on coverslips were kept in a Sodium/HEPES buffer (130 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 20 mM HEPES, pH 7.4).

The temperature was maintained at 37 °C throughout the duration of the experiment. For agonist stimulation 0.5 μM orexin A was added in the Sodium/HEPES buffer.

2.8.2 *In vivo* phosphorylation assays

Agonist dependent phosphorylation of the different receptor constructs was determined by *in vivo* phosphorylation assays. CHO cells stably or transiently expressing the orexin receptor constructs were split onto poly-D-lysine coated 6 well plates. The following day the growth medium was removed from the cells and replaced by phosphate-free DMEM. After a 1 h incubation at 37 °C, the medium was changed to medium supplemented with 0.2 mCi/ml [^{32}P] orthophosphate for 90 min. Cells were then treated with 0.5 μM orexin A for periods ranging from 30 sec to 10 min, or with 50 μM forskolin, 1 μM PMA, 10 μM A23187 or 100 μM 8-bromocyclic GMP for 5 min. To assay kinase inhibition, cells were treated with 10 μM H89, 5 μM GF109203X or 1 μM BAPTA/AM for 30 min prior to agonist exposure. To terminate the reactions the cells were placed on ice and washed 3 times with ice cold PBS. Then the cells were solubilised for receptor immunoprecipiation with the anti-HA or –GFP antibody as detailed in 2.6.2.2. After separation of the immunoprecipitated receptor constructs by SDS-PAGE, the gels were dried and the labelled proteins visualised by autoradiography. The resulting bands were densitometrically quantified.

2.8.3 JNK-MAPK assay

HEK293T cells were plated in 6 well plates and 24 h later transiently transfected with the different cDNAs as described in 2.5.4. Another 24 h later, the cells were washed once with KRB and then serum starved for 1 h at 37 °C. After stimulating the cells with 0.5 μM orexin A, 500 μl of lysis buffer (20 mM Tris-HCl (pH 7.6), 0.5 % NP-40, 250 mM NaCl,
3 mM EDTA, 3 mM EGTA, 1 mM PMSF, 1 mM DTT) was added to the cells, the cells lysed for 5 min on ice on a shaking platform and finally scraped off. The lysate was cleared by a centrifugation step of 5 min at 14000 rpm (20817 x g) at 4 °C. The supernatant was transferred to a fresh tube and the protein determined by BCA assay as described in 2.6.1. Equal amount of protein was added to 20 μl of GST-cJUN/glutathione beads and the samples rotated for 1 h at 4 °C. The samples were then washed twice with 200 μl lysis buffer, twice with 200 μl kinase buffer (20 mM HEPES (pH 7.4), 20 mM β-

glycerophosphate, 10 mM $MgCl_2$, 1 mM DTT, 250 μM sodium orthovanadate) and resuspended in 40 μl kinase reaction mix (560 μl kinase buffer, 5.6 μl 2 mM cold ATP, 2.8 μl hot ATP (3000 Ci/mmol)). After incubating the samples for 20 min at 37 °C, the samples were centrifugated for 1 min at 14000 rpm (20817 x g), the supernatant removed, the beads resuspended in 30 μl Laemmli buffer and heated for 5 min at 95 °C before loading onto SDS-PAGE gels. Finally the gels were stained with Coomassie-blue, dried and the labelled proteins visualised by autoradiography.

2.8.4 WST-1 cell proliferation assay

This colometric assay for the quantification of cell proliferation is based on the cleavage of the tetrazolium salt WST-1 to formazan by mitochondrial dehydrogenases in viable cells. An expansion in the cell number leads to an increase in the overall activity of mitochondrial dehydrogenases, which is directly correlated to the amount of formazan dye formed.

HEK293T cells were transfected with the different cDNAs as described in section 2.6.4, 24 h later cells were seeded into 96 well plates at a density of 0.5 x 10^4 cells/well in 100 μl medium containing the indicated stimuli and incubated at 37 °C. Another 24 h later the WST-1 reagent was added at a dilution of 1:10 and the cells incubated for 1-4 h at 37 °C before measuring the absorbance at 450 nm. The light absorbance of the medium containing all factors but no cells was determined and subtracted from the absorption readings with cells.

Figure 2.1: Primers used to generate the various cDNA fragments using PCR

Figure 2.1

Primer	Sequence	Restriction site	Direction
ox1R389	CAT ATT CTC GAG TCA AGG GGC CTT CAG AGA G	*Xho*I	antisense
ox1R374	CAT TCT CGA GTC AGG AGA AGG CAG CCT TAA AC	*Xho*I	antisense
ox1R 3	CAA GGA CAA GGA CTT GTG GGC GGC GGC GGC GCG GGG ACT AGG GGC CTT C		antisense
VSV-ox1R	GAT AAG CTT AAA GCC ACC ATG TAC ACC GAT ATC GAA ATG AAC CGC CTT GGT AAG GAG CCC TCA GCC ACC CCA G	*Hind*III	sense
HA-ox1R	ATA TAA GCT TAA AAT GTA TCC CTA CGA CGT CCC CGA TTA TGC GGA GCC CTC AGC CAC CCC AG	*Hind*III	sense
ox1R 1	AAG TGA AGC AGA TGC GTG CAC GGA GGA AGA CAG		sense
ox1RC1	GTT ATT CTC GAG TCA GGG CAG CAC TGC GGC GAC GGC GGC GAG CAC CAC ATG CTC AGA GAT TTT GG	*Xho*I	antisense
ox1RC2	GAA GGC CCC TAG TCC CCG CGC CGC CGC CGC CCA CAA GTC CTT GTC CTT G		sense
ox1R 5	GTT ATT CTC GAG TCA GGG CAG CAC TGT GGT GAC GCT GGT G	*Xho*I	antisense
ox1RT418A	GTT ATT CTC GAG TCA GGG CAG CAC TGT GGT GAC GCT GGC GAG CAC CAC ATG CTC AGA GAT TTT GG	*Xho*I	antisense
ox1RS419A	GTT ATT CTC GAG TCA GGG CAG CAC TGT GGT GAC GGC GGT GAG CAC CAC ATG CTC AGA GAT TTT GG	*Xho*I	antisense
ox1RT421A	GTT ATT CTC GAG TCA GGG CAG CAC TGT GGC GAC GCT GGT GAG CAC CAC ATG CTC AGA GAT TTT GG	*Xho*I	antisense
ox1RT422A	GTT ATT CTC GAG TCA GGG CAG CAC TGC GGT GAC GCT GGT GAG CAC CAC ATG CTC AGA GAT TTT GG	*Xho*I	antisense
ox1RT418/T421A	GTT ATT CTC GAG TCA GGG CAG CAC TGT GGC GAC GCT GGC GAG CAC CAC ATG CTC AGA GAT TTT GG	*Xho*I	antisense
ox1RT418/T422A	GTT ATT CTC GAG TCA GGG CAG CAC TGC GGT GAC GCT GGC GAG CAC CAC ATG CTC AGA GAT TTT GG	*Xho*I	antisense
ox1RS419/T421A	GTT ATT CTC GAG TCA GGG CAG CAC TGT GGC GAC GGC GGT GAG CAC CAC ATG CTC AGA GAT TTT GG	*Xho*I	antisense
ox1RS419/T422A	GTT ATT CTC GAG TCA GGG CAG CAC TGC GGT GAC GGC GGT GAG CAC CAC ATG CTC AGA GAT TTT GG	*Xho*I	antisense
ox1RT421/T422A	GTT ATT CTC GAG TCA GGG CAG CAC TGC GGC GAC GCT GGT GAG CAC CAC ATG CTC AGA GAT TTT GG	*Xho*I	antisense
ox1RT418/S419A	GTT ATT CTC GAG TCA GGG CAG CAC TGT GGT GAC GGC GGC GAG CAC CAC ATG CTC AGA GAT TTT GG	*Xho*I	antisense
ox1RI148E-L152D F	GAC CGC TGG TAT GCC GAG TGC CAC CCA GAT TTG TTC AAG AGC ACA GCC		sense
ox1RI148E-L152D R	GGC TGT GCT CTT GAA CAA ATC TGG GTG GCA CTC GGC ATA CCA GCG GTC		antisense

Figure 2.1 continued

Primer	Sequence	Restriction site	Direction
ox1RH148E F	GAC CGC TGG TAT GCC GAG TGC CAC CCA CTA TTG TTC AAG AGC ACA GCC		sense
ox1RH148E R	GGC TGT GCT CTT GAA CAA TAG TGG GTG GCA CTC GGC ATA CCA GCG GTC		antisense
ox1RL152D F	GAC CGC TGG TAT GCC ATC TGC CAC CCA GAT TTG TTC AAG AGC ACA GCC		sense
ox1RL152D R	GGC TGT GCT CTT GAA CAA ATC TGG GTG GCA GAT GGC ATA CCA GCG GTC		antisense
ox1RC1 NotI (noTAG)	GTT ATT GCG GCC GCG GGC AGC ACT GCG GCG ACG GCG GCG AG	*Not*I	antisense
ox1R NotI (noTAG)	GTT ATT GCG GCC GCG GGC AGC ACT GTG GTG ACG CTG GTG	*Not*I	antisense

Figure 2.2: **List of the different C-terminal constructs of the orexin 1 receptor**

The orexin 1 receptor constructs were generated by PCR as described in section 2.4.

Figure 2.2

362 SGKFREQFKAAFSCCLPGLGPCGSLKAPSPRSSASHKSLSLQSRCSISKISEHVVL
TSVTTVLP **425 full length**

362 SGKFREQFKAAFSCCLPGLGPCGSLKAPSPR SS **394 stop**

362 SGKFREQFKAAFSCCLPGLGPCGSLKAP **389 stop**

362 SGKFREQFKAAFSCCLP **378 stop**

362 SGKFREQFKAAFS **374 stop**

362 SGKFREQFKAAFSCCLPGLGPCGSLKAPSPRSSASHKSLSLQSRCSISKISEHVVL
AAV**AA**VLP **C1 mutation**

362 SGKFREQFKAAFSCCLPGLGPCGSLKAPSPR**AAAA**HKSLSLQSRCSISKISEHVVL
TSVTTVLP **C2 mutation**

362 SGKFREQFKAAFSCCLPGLGPCGSLKAPSPR**AAAA**HKSLSLQSRCSISKISEHVVL
AAV**AA**VLP **C1C2 mutation**

362 SGKFREQFKAAFSCCLPGLGPCGSLKAPSPRSSASHKSLSLQSRCSISKISEHVVL
ASVTTVLP **T418A mutation**

362 SGKFREQFKAAFSCCLPGLGPCGSLKAPSPRSSASHKSLSLQSRCSISKISEHVVL
T**A**VTTVLP **S419A mutation**

362 SGKFREQFKAAFSCCLPGLGPCGSLKAPSPRSSASHKSLSLQSRCSISKISEHVVL
TSV**A**TVLP **T421A mutation**

362 SGKFREQFKAAFSCCLPGLGPCGSLKAPSPRSSASHKSLSLQSRCSISKISEHVVL
TSVT**A**VLP **T422A mutation**

362 SGKFREQFKAAFSCCLPGLGPCGSLKAPSPRSSASHKSLSLQSRCSISKISEHVVL
AAVTTVLP **T418/S419A mutation**

362 SGKFREQFKAAFSCCLPGLGPCGSLKAPSPRSSASHKSLSLQSRCSISKISEHVVL
ASV**A**TVLP **T418/T421A mutation**

362 SGKFREQFKAAFSCCLPGLGPCGSLKAPSPRSSASHKSLSLQSRCSISKISEHVVL
ASVT**A**VLP **T418/T422A mutation**

362 SGKFREQFKAAFSCCLPGLGPCGSLKAPSPRSSASHKSLSLQSRCSISKISEHVVL
T**A**V**A**TVLP **S419/T421A mutation**

362 SGKFREQFKAAFSCCLPGLGPCGSLKAPSPRSSASHKSLSLQSRCSISKISEHVVL
T**A**VT**A**VLP **S419/T422A mutation**

362 SGKFREQFKAAFSCCLPGLGPCGSLKAPSPRSSASHKSLSLQSRCSISKISEHVVL
TSV**AA**VLP **T421/T422A mutation**

Figure 2.3: Schematic representation of the overlap PCR strategy

For details see text

Figure 2.3

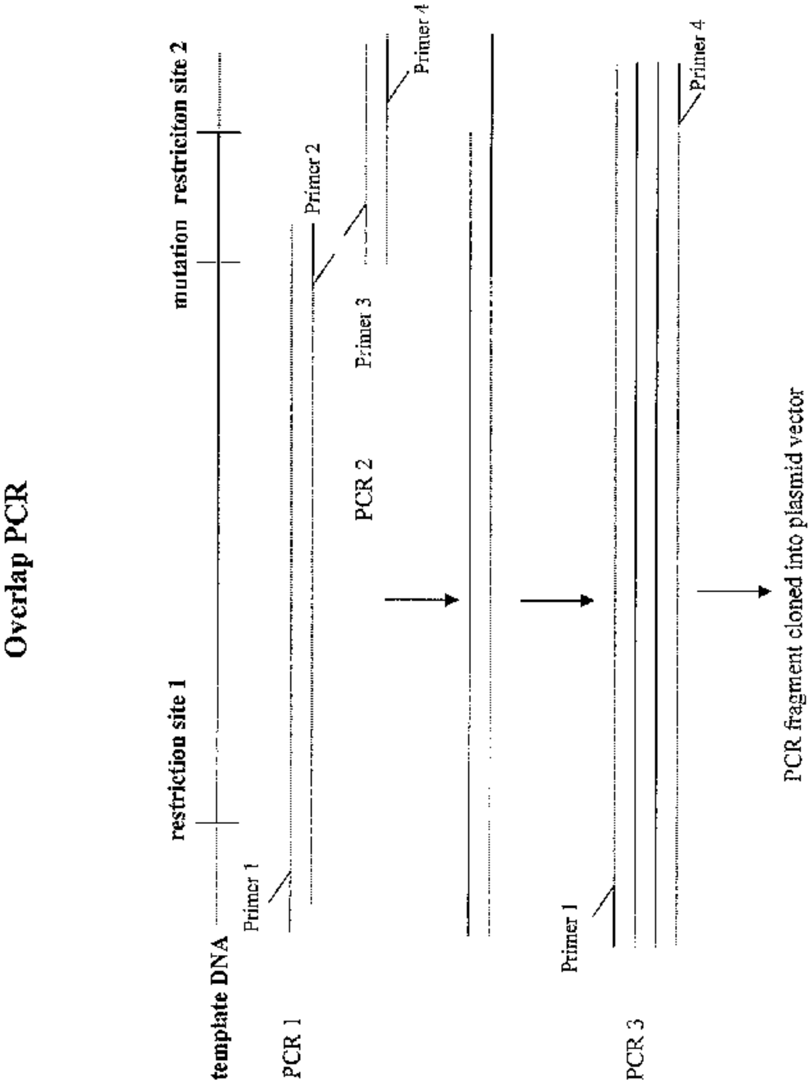

Overlap PCR
template DNA
restriction site 1
mutation
restriciton site 2
PCR 1
Primer 1
Primer 2
PCR 2
Primer 3
Primer 4
PCR 3
Primer 1
Primer 4
PCR fragment cloned into plasmid vector

Figure 2.4: Overview of the different i2 loop mutants of the orexin 1 receptor

The orexin 1 receptor i2loop mutants were generated by PCR as described in section 2.4.

Figure 2.4

DRWYAICHPLLFKSTARRAFGS	**i 2 loop**
DRWYA**E**CHP**D**LFKSTARRAFGS	**I148E-L152D**
DRWYA**E**CHPLLFKSTARRAFGS	**I148E**
DRWYAICHP**D**LFKSTARRAFGS	**L152D**

Chapter 3

Regulation of Orexin 1 Receptor Internalisation by β-Arrestins

3.1 Introduction

Once GPCRs are activated by agonist, their responsiveness to further stimulation becomes modulated (Ferguson and Caron 1998). Three distinct mechanisms have been identified so far which serve to attenuate the response of GPCRs to stimulation: phosphorylation, internalisation and down-regulation. Many GPCRs are desensitised within seconds to minutes after ligand binding. This process is mediated by phosphorylation of the receptor by G protein-coupled receptor kinases (GRKs) and/or the second messenger kinases, PKA and PKC. This phosphorylation promotes binding of β-arrestin to the receptor. The consequence of this interaction is a reduction in coupling of the receptor to the G-protein. β-arrestins also serve as adaptors to target the desensitised receptor to clathrin-coated vesicles for internalisation. The clathrin-coated vesicle is pinched off from the plasma membrane by dynamin, a GTPase (Van der Bliek *et al.*, 1993). The non-receptor tyrosine kinase c-Src plays an important role in this process since tyrosine phosphorylation of dynamin by this kinase is required for dynamin mediated GTP hydrolysis and consequently for receptor internalisation (Ahn *et al.*, 1999; Ahn *et al.*, 2002). Finally prolonged receptor stimulation leads to an overall decrease in receptor number by targetting receptors to lysosomes.

Two classes of GPCRs can be distinguished according to their internalisation properties (Oakely *et al.*, 1999; Pierce and Lefkowitz, 2001). Class A receptors like the β_2-adrenergic receptor bind preferentially β-arrestin 2 over β-arrestin 1. The receptor dissociates from β-arrestin at or near the plasma membrane and internalises alone into endosomes. Class B receptors like the vasopressin V2 receptor bind β-arrestin 1 and β-arrestin 2 equally well. Also the receptor and β-arrestin co-internalise into endocytic vesicles. The C-terminal tail of the GPCR and more precisely clusters of Ser and Thr residues within this region seem to be important to determine into which class a certain GPCR fits (Oakley *et al.*, 2001). Switching the C-terminus of the β_2-adrenergic receptor with the C-terminus of the vasopressin V2 receptor created a receptor-chimera with the internalisation properties of the vasopressin receptor and vice versa. Also these clusters of Ser and Thr residues have only been identified in class B receptors so far. The human orexin 1 receptor appears to belong to the class B since it interacts with high affinity with β-arrestin 1 and 2 and also co-internalises with both β-arrestin 1-GFP and β-arrestin 2-GFP into intracellular vesicles following addition of orexin A (Evans *et al.*, 2001).

GPCRs can also internalise independently of clathrin via caveolae (Dupree *et al.,* 1993). Caveolae are detergent-insoluble plasmalemmal vesicles enriched in cholesterol, sphingolipids and caveolin, a structural coat protein. They are small, flask shaped specialised microdomains formed through oligomerisation of caveolin (Schnitzer *et al.,* 1995; Rothberg *et al.,* 1992). These dynamic structures are able to internalise and this process is regulated by vesicle budding, fission, docking and fusion.

In this chapter a detailed analysis of the mechanism of orexin 1 receptor internalisation was performed. Firstly differently tagged forms of the wild type orexin 1 receptor were created and their interaction with β-arrestin 2 directly monitored by confocal microscopy. After examining internalisation of the wild type receptor C-terminally tagged with eYFP in G_q/G_{11} knock out cells, C-terminal truncated and mutated forms of the orexin 1 receptor were generated and the effects on receptor internalisation and β-arrestin 2 interaction examined using again confocal microscopy. Phosphorylation studies of the different forms of the receptor were also carried out to firstly examine which kinases are involved in agonist-stimulated receptor phosphorylation and secondly to check whether the interaction between the receptor and β-arrestin 2 is governed by agonist-mediated phosphorylation. To investigate an involvement of casein kinase II in the internalisation process, the cells were transfected with the wild type receptor and β-arrestin 2 and pre-treated with apigenin, a casein kinase II inhibitor, before addition of the agonist and the receptor distribution observed under the confocal microscope. Finally, to determine the pathway of internalisation of the wild type receptor and a C-terminal mutation, the different forms of the receptor were either expressed in β-arrestin or Src knock out cells, co-expressed with dominant negative dynamin or the cells pre-treated with inhibitors of internalisation before agonist stimulation and the effects monitored by confocal microscopy.

3.2 Expression and internalisation of the orexin 1 receptor constructs with β-arrestin 2-GFP or -RFP

The various orexin 1 receptor constructs were generated by PCR as described in 2.4. It has been published that the orexin 1 receptor co-internalises with β-arrestin in response to orexin A (Evans *et al.,* 2001). To examine whether the alterations introduced by PCR had any effect on this interaction, the cDNAs were transiently co-expressed with either β-arrestin 2-GFP or -RFP in HEK293T cells and confocal analysis used to visualise the interaction of the orexin 1 receptor and β-arrestin 2. To label the untagged orexin 1 receptor, the cells were stimulated with 0.5 μM 5 and 6-carboxytetramethylrhodamine (TAMRA)-labelled orexin A for 30 min. This made it possible to observe co-internalisation of the receptor, having bound the labelled ligand, and β-arrestin 2-GFP into punctate intracellular vesicles (Figure 3. 1) since merging of the pictures corresponding to TAMRA-orexin A (red) and β-arrestin 2-GFP (green) resulted in the vesicles being stained in yellow, indicating overlapping distributions of the two signals (Figure 3.1). As this form of the agonist is membrane impermeable, the receptor had to be expressed at the plasma membrane prior to addition of the agonist to be labelled.

In unstimulated cells, the immunostaining of the orexin 1 receptor tagged with the HA-epitope sequence at the N-terminus indicated it to be expressed at the plasma membrane and β-arrestin 2-GFP localised in the cytoplasm (Figure 3.2 a). This form of the receptor also internalised in response to addition of 0.5 μM orexin A for 30 min and again co-localised in intracellular vesicles with co-expressed β-arrestin 2-GFP (Figure 3.2 b).

Prior to agonist stimulation the orexin 1 receptor C-terminally tagged with eYFP was located at the plasma membrane and β-arrestin 2-RFP in the cytoplasm (Figure 3.3 a). Addition of 0.5 μM orexin A for 30 min also caused internalisation of the orexin 1 receptor-eYFP (Figure 3.3 b). Co-expression of this construct with β-arrestin 2 tagged with RFP from *Anemonia sulcata* at the C-terminus again indicated the interaction and intracellular co-localisation of these two proteins following agonist stimulation (Figure 3.3 b).

The N-terminally tagged VSV-G orexin 1 receptor was highlighted with a novel, pH-sensitive, cyanine dye termed CypHer-5 linked to an anti-VSV-G antibody. The advantage of using this dye was that only internalised receptors were highlighted, as this dye only fluoresces when in an acidic environment (pKa = 6.1). Stimulation of the receptor with 0.5 μM orexin A for 30 min caused red fluorescence in an intracellular punctate pattern (Figure 3.4 b). β-arrestin 2-GFP also internalised into intracelluar vesicles upon agonist stimulation (Figure 3.4 b). These results confirmed that the receptor and β-arrestin 2-GFP were co-internalised into acidic endosomes because merging of the individual red and green signal resulted in vesicles being stained in yellow. It was not possible to visualise the receptor at the plasma membrane (Figure 3.4 a), but since the antibody-conjugated dye cannot cross the plasma membrane, it can be concluded that this form of the receptor was trafficked correctly to the plasma membrane.

3.3 Internalisation of the orexin 1 receptor in G_q/G_{11} double knockout cells

To test whether internalisation of the orexin 1 receptor is G_q/G_{11} dependent, the receptor C-terminally tagged with eYFP was transiently expressed in EF88 cells. These cells are derived from fibroblasts isolated from the embryo of a G_q/G_{11} double knockout mouse (Stevens *et al.*, 2001; Liu *et al.*, 2002). The orexin 1 receptor-eYFP fusion protein was expressed at the plasma membrane in the absence of agonist (Figure 3.5 a). Addition of 0.5 μM orexin A for 30 min caused internalisation of the receptor (Figure 3.5 b). Lack of expression of G_q/G_{11} therefore did not prevent agonist-induced internalisation of the orexin 1 receptor-eYFP construct in these cells confirming that agonist-induced internalisation of the orexin 1 receptor is independent of G_q/G_{11} signalling.

3.4 Expression and internalisation of the orexin 1 receptor C-tail mutants with β-arrestin 2-GFP

To determine whether the C-terminus of the orexin 1 receptor is important for binding β-arrestin 2 as described for the δ- and κ-opioid receptors (Cen *et al.*, 2001) or the angiotensin II type 1A receptor (Oakley *et al.*, 2001), a PCR based strategy was used to generate orexin 1 receptor C-tail truncations by introducing a stop-codon at the desired position in the C-terminus of the receptor as described in 2.4 and Figure 2.1. These constructs were also HA-epitope tagged at the N-terminus (2.4). To show that each cDNA construct was translated into a truncated protein, HEK293T cells were transiently transfected with the different truncation constructs and the protein expression determined by Western blot analysis. Immunodetection of the different truncations via the N-terminal HA-tag confirmed that each cDNA construct was translated into a truncated protein of the expected size (Figure 3.6).

Confocal analysis was used to visualise the location of the immunostained orexin 1 receptor truncations and β-arrestin 2-GFP in transiently transfected HEK293T cells. Prior to agonist stimulation, the immunostained receptor truncations were expressed at the plasma membrane whereas β-arrestin 2-GFP was localised in the cytoplasm (Figures 3.7 a, c, e, g). Stimulating the cells for 30 min with 0.5 μM orexin A caused each of the truncated forms to internalise (Figures 3.7 b, d, f, h). However co-expressed β-arrestin 2-GFP interacted only very transiently with the truncated forms of the receptor since the cellular distribution of β-arrestin 2-GFP was basically unchanged by addition of orexin A (Figures

3.7 b, d, f, h). For that reason the internalised receptor was labelled red on a green background and no yellow staining could be observed indicating that there was no detectable co-internalisation of the truncated forms of the receptor and β-arrestin 2.

To further define the region of the orexin 1 receptor C-terminus which is involved in the interaction with β-arrestin 2, two clusters of hydroxy amino acids located at positions 393-396 (cluster C2) and 418-422 (cluster C1) were mutated to Ala residues individually or together (C1C2) by PCR (2.4 and Figure 2.1) since such clusters of hydroxy amino acids have been suggested to be important binding sites for β-arrestins (Oakley *et al.,* 2001). These mutants also carried a N-terminal HA-tag. Each of the cluster mutants was transiently co-transfected with β-arrestin 2-GFP in HEK293T cells and visualised by confocal microscopy. In unstimulated cells, all three cluster mutants were expressed at the plasma membrane and β-arrestin 2-GFP in the cytoplasm (Figures 3.8 a, c, e). Mutation of the three Ser residues in cluster C2 had no effect on the interaction of the receptor with β-arrestin 2-GFP. This mutant behaved essentially as the wild type as it co-internalised with β-arrestin 2-GFP into punctate vesicles after being stimulated with 0.5 μM orexin A for 30 min as indicated by the yellow staining (Figure 3.8 b). By contrast, alteration of all four hydroxy amino acids in cluster C1 to Ala greatly reduced translocation of β-arrestin 2-GFP from the cytoplasm to the plasma membrane. The receptor internalised without β-arrestin-2-GFP since the receptor was stained in red on a green background (Figure 3.8 d). The form of the receptor carrying both sets of mutations (cluster C1C2 mutant) showed the same staining pattern as the cluster C1 mutant after addition of agonist (0.5 μM orexin A, 30 min). Therefore this mutant was also unable to co-internalise with β-arrestin 2-GFP in response to orexin A (Figure 3.8 f).

To investigate whether a particular hydroxy amino acid in cluster C1 is important for co-internalisation of the orexin 1 receptor and β-arrestin 2, each of these four amino acids was individually modified to Ala by PCR (2.4 and Figure 2.1). Again these constructs were tagged with the HA-epitope at the N-terminus. The expression pattern of these constructs transiently co-transfected with β-arrestin 2-GFP into HEK293T cells was analysed by confocal microscopy. Without agonist stimulation all four immunostained receptor mutants were localised at the plasma membrane whereas β-arrestin 2-GFP was expressed in the cytoplasm (Figures 3.9 a, c, e, g,). Agonist-mediated co-internalisation with β-arrestin 2-GFP was not abolished by any of these single point mutations because stimulation with 0.5 μM orexin A for 30 min resulted in yellow staining of intracellular punctate vesicles

(Figures 3.9 b, d, f, h). Since mutating any single hydroxy amino acid in cluster C1 had no effect on the interaction of the receptor and β-arrestin 2, further mutations carrying all of the possible combinations in which two of the four hydroxy amino acids in this cluster were mutated to Ala and also having a N-terminal HA-tag were produced by PCR (2.4 and Figure 2.1). Again in the absence of agonist these mutants were expressed at the plasma membrane and β-arrestin 2-GFP in the cytoplasm when transiently transfected in HEK293T cells as assessed by confocal microscopy (Figures 3.10 a, c, e, g, i, k). In contrast to the single point mutants, changing any two residues within cluster C1 eliminated detectable co-internalisation of β-arrestin 2-GFP and any of these forms of the receptor in response to 0.5 μM orexin A for 30 min (Figures 3.10 b, d, f, h, j, l). For all the double mutants, cells in the field were observed in which a small amount of β-arrestin 2-GFP moved to the plasma membrane (Figures 3.10 b, d, f, h, j, l). However, unlike in equivalent experiments with the wild type orexin 1 receptor the majority of the β-arrestin 2-GFP seemed to remain in the cytoplasm.

3.5 Internalisation studies of the wild type orexin 1 receptor and the cluster C1 mutant

The agonist-stimulated sequestration of the HA-tagged receptors transiently co-transfected with β-arrestin 2-GFP in HEK293T cells was monitored by visualisation of the immunostained receptor using the confocal microscope. Cells grown on glass coverslips were incubated with 0.5 μM orexin A for time points ranging from 0-15 min and then labelled with anti-HA antibody and fixed before examination by confocal microscopy. The wild type receptor was expressed at the plasma membrane whereas β-arrestin 2 displayed a cytosolic expression pattern in unstimulated cells (Figure 3.11). After stimulation with orexin A for 2 min, the receptor was distributed at the plasma membrane in a punctate pattern and β-arrestin 2 translocated to the same vesicles at the plasma membrane containing the receptor, since these vesicles were stained in yellow. This re-distribution reflects the recruitment of β-arrestin 2 to the agonist-occupied orexin 1 receptor and the accumulation of receptor-β-arrestin complexes in clathrin-coated pits. After 5 min most of the receptor-β-arrestin 2 complexes could be seen in intracellular vesicles and this was even more pronounced after 10 min of agonist stimulation (Figure 3.11). Exposure of the receptor to agonist for 10 min seemed to already result in maximum receptor sequestration, since most of the HA-labelled receptors were removed from the plasma membrane at this point and also no difference in receptor internalisation and β-arrestin co-localisation could be detected between the 10 min and the 15 min time point (Figure 3.11). The observed overlapping pattern of redistribution of the receptor and β-arrestin 2-GFP is indicative of the stable formation of receptor-β-arrestin 2 complexes that remain associated during endocytosis. A similar result concerning the time course of receptor internalisation could be obtained for the cluster C1 mutant. However β-arrestin 2 did not translocate to the receptor but instead stayed in the cytosol as the receptor was stained red on a green background throughout the course of the experiment, suggesting that the receptor β-arrestin complex dissociates at or near the plasma membrane (Figure 3.11).

3.6 The C1 mutant binds β-arrestin 2 less well than the wild type orexin 1 receptor

To prove biochemically that the cluster C1 mutant binds β-arrestin 2 with lower affinity compared to the wild type receptor, the ability of each form of the receptor to recruit β-arrestin 2 upon agonist stimulation was investigated in co-immunoprecipitation studies using the reversible, membrane permeable, cross-linker dithiobis[succinimidylpropionate] (DSP). Therefore β-arrestin 2-GFP was co-expressed in HEK293T cells with VSV-G tagged forms of either the wild type or the cluster C1 mutation of the orexin 1 receptor. β-arrestin 2-GFP was immunoprecipitated with anti-GFP antiserum, samples resolved by SDS-PAGE and probed for the presence of forms of the receptor by immunoblotting to detect the VSV-G epitope. As shown in Figure 3.12 the wild type receptor as well as the cluster C1 mutant could be co-immunoprecipitated with β-arrestin 2-GFP even without prior stimulation with orexin A. However orexin A (0.5 μM, 30 min) induced an increase of the wild type orexin 1 receptor in the β-arrestin 2-GFP immunoprecipitates, whereas no increase of the cluster C1 could be detected under the same conditions.

3.7 Phosphorylation of the wild type orexin 1 receptor and the cluster mutants

Since alteration of hydroxy-amino acids in the C-terminus of the orexin 1 receptor abolished co-internalisation of the receptor with β-arrestin 2, it seemed appropriate to investigate whether phosphorylation of the orexin 1 receptor regulates the interaction between the receptor and β-arrestin 2. The first step was to examine whether the wild type form of the receptor becomes phosphorylated upon agonist stimulation. Therefore a stable

CHO cell line stably expressing the wild type HA-orexin 1 receptor which had been generated previously at GlaxoSmithKline was used. Figure 3.13 shows the phosphorylation of the receptor over time. The receptor seemed to be already maximally phosphorylated after stimulation with 0.5 μM orexin A for 1 min as the level of phosphorylation did not increase any further for the duration of the experiment. In the absence of agonist, a basal level of phosphorylation was detectable. The mechanism of phosphorylation was further investigated using either protein kinase inhibitors (10 μM H89, 5 μM GF109203X, 10 μM BAPTA/AM) or activators (50 μM forskolin, 10 μM A23187, 5 μM PMA, 100 μM 8-bromo-cGMP). BAPTA/AM, GF109203X and H89 modestly decreased the agonist-mediated receptor phosphorylation (Figure 3.14). On the other hand forskolin, A23187 and PMA were found to induce phosphorylation of the receptor whereas 8-bromo-cGMP had no effect on the level of phosphorylation (Figure 3.14). Taken together these results indicate that agonist-mediated phosphorylation of the receptor may, in part, be mediated by PKC and PKA, whereas PKG does not seem to be involved. To determine whether there was a difference in phosphorylation levels between the different receptor constructs, CHO cells were transiently transfected with C-terminally eYFP-tagged wild type, cluster C1 or C2 mutant forms of the receptor using the Amaxa system (2.5.5) to achieve high transfection efficiencies. After equalising the protein concentration for the immunoprecipitation step, a small amount of protein was loaded on a SDS-PAGE gel and the receptor expression of each construct determined by Western blot analysis (Figure 3.15 a). Figure 3.15 b depicts the level of phosphorylation for each construct before and after stimulation with 0.5 μM orexin A for 5 min. In the absence of agonist, a basal level of phosphorylation was evident for each form of the receptor and after agonist stimulation each receptor construct became phosphorylated to a similar extent. The average fold induction for each construct was approximately 2.1 ± 0.6 for the wild type receptor, 1.7 ± 0.34 for the cluster C1 mutant and 1.6 ± 0.44 for the cluster C2 mutant (Figure 15 c) indicating that additional sites are involved in agonist-mediated phosphorylation of the orexin 1 receptor.

3.8 Involvement of casein kinase II in receptor internalisation

The data from section 3.7 indicated that second messenger kinase activation was involved in agonist-mediated phosphorylation of the wild type receptor, but that additional kinases also played a role in this process. Casein kinase II phosphorylation of three consensus sites within the C-terminal tail of the thyrotropin-releasing hormone receptor was shown to mediate agonist-stimulated coupling of β-arrestin to the receptor (Hanyaloglu *et al.,* 2001). To examine the possible involvement of casein kinase II-mediated phosphorylation in the internalisation of the orexin 1 receptor, HEK293T cells were transiently transfected with HA-orexin 1 receptor and β-arrestin 2-GFP. Following incubation with 80 μM apigenin, a casein kinase II inhibitor, for 30 min the cells were treated with agonist (0.5 μM orexin A for 30 min) and the distribution of the receptor and β-arrestin 2 visualised by confocal microscopy. In unstimulated cells the receptor was expressed at the plasma membrane and β-arrestin 2-GFP displayed a diffuse cytosolic distribution (Figure 3.16 a). In response to agonist, the receptor and β-arrestin 2 co-internalised into endocytic vesicles as indicated by the yellow staining (Figure 3.16 b). Pre-treatment of the cells with apigenin did not abolish co-internalisation (Figure 3.16 c), suggesting that casein kinase II does not play an important role in agonist-mediated phosphorylation and sequestration of the orexin 1 receptor.

The thyrotropin-releasing hormone receptor was used as positive control. The receptor tagged with VSV-G at the N-terminus was co-transfected with β-arrestin 2-GFP into HEK293T cells and the receptor labelled by immunostaining with anti-VSV-G antibody. The receptor was expressed at the plasma membrane and internalised in response to treatment with 1 μM thyrotropin-releasing hormone into intracellular vesicles (Figure 3.17 a, b). An overlapping pattern of redistribution of β-arrestin 2-GFP from the cytosol to intracellular vesicles was observed since the vesicles exhibited a yellow colour (Figure 3.17 a, b). In contrast to the orexin 1 receptor, incubation of the cells with 80 μM apigenin completely inhibited agonist-mediated receptor internalisation but had no effect on the recruitment of β-arrestin 2 to the activated receptor as the plasma membrane was labelled yellow (Figure 3.17 c). In contrast to the orexin 1 receptor where casein kinase II does not seem to be involved in internalisation, casein kinase II appears to be important for internalisation of the thyrotropin-releasing hormone receptor but not for coupling of β-arrestin to the stimulated receptor.

3.9 Internalisation of the orexin 1 receptor wild type and the cluster C1 mutant in β-arrestin knock out cells

To investigate if there was a difference in the dependence of the wild type receptor and the cluster C1 mutant on β-arrestin for internalisation both constructs, fused to eYFP at the C-terminus as described in 2.4, were transiently transfected into either wild type or β-arrestin 1 and 2 double knock out mouse embryonic fibroblast (MEF) (Kohout *et al.*, 2001). Confocal microscopy revealed that in unstimulated wild type or knock out cells both receptor constructs were expressed at the plasma membrane (Figures 3.18 a, b, c, d). After treatment of the cells with 0.5 μM orexin A for 30 min the wild type receptor as well as the cluster mutant was internalised into punctate vesicles in the MEF wild type cells (Figures 3.18 a, b). By contrast addition of orexin A did not cause internalisation of either receptor form in the β-arrestin null cells (Figures 3.18 c, d). To show that the double knock out cells were fully functional, β-arrestin 2-RFP was transiently co-transfected with either the wild type or C1 eYFP-tagged forms of the orexin 1 receptor. Prior to agonist stimulation both forms of the receptor were expressed at the plasma membrane and β-arrestin 2-RFP in the cytoplasm (Figure 3.19 a, c). Each form of the receptor internalised in response to challenge with 0.5 μM orexin A for 30 min (Figure 3.19 b, d), demonstrating that reconstitution with β-arrestin 2 was sufficient to restore receptor internalisation. In the case of the wild type receptor, β-arrestin 2 co-internalised into intracellular vesicles, whereas there was no discernable movement of β-arrestin 2 when co-expressed with the cluster C1 form of the receptor. Hence the wild type orexin 1 receptor as well as the cluster C1 mutant internalise in a β-arrestin-dependent manner.

3.10 Effects of inhibitors of clathrin-mediated endocytosis or caveolae on agonist mediated internalisation of the orexin 1 receptor constructs

Internalisation of receptors via the clathrin-mediated pathway can be blocked in different ways. The plant lectin concanvalin A binds to carbohydrate moieties on the cell surface thereby interfering with receptor endocytosis but not with ligand binding or receptor signalling (Pippig *et al.*, 1995; Luttrell *et al.*, 1997). Hypertonic sucrose impaires clathrin-mediated internalisation by causing abnormal clathrin polymerisation into empty micro-cages on the plasma membrane (Heuser and Anderson, 1989). Since caveolae are characterised by high levels of cholesterol, their function can be disrupted by cholesterol chelating agents such as nystatin and filipin (Okamoto *et al.*, 2000) or methyl-β-cyclodextrin (MβCD) (Foster *et al.*, 2003).

To explore the pathway of internalisation of the wild type and cluster C1 mutant of the orexin 1 receptor forms of these receptors N-terminally modified by PCR to express the VSV-G epitope tag (2.4 and Figure 2.1) were transiently co-transfected with β-arrestin 2-

GFP in HEK293T cells and the effects of the different inhibitors on internalisation visualised by confocal microscopy. In response to addition of CypHer-5 tagged anti-VSV-G antibody followed by addition of 0.5 μM orexin A for 30 min the wild type orexin 1 receptor internalised and many of the intracellular vesicles containing the receptor appeared yellow due to the presence of both the receptor and β-arrestin 2-GFP (Figure 3.20 a). By contrast, in cells expressing the cluster C1 mutant and β-arrestin 2-GFP, the intracellular vesicles appeared red (Figure 3.20 b). This indicates that these vesicles are indeed acidic, as the CypHer-5 antibody only fluoresces at acidic pH, but that β-arrestin 2-GFP did not co-internalise with the cluster C1 mutant. Pre-treatment of the cells with 0.4 M sucrose for 30 min blocked internalisation of the wild type orexin 1 receptor and the cluster C1 mutant in response to agonist (Figure 3.20 c, d). However, the presence of sucrose did not prevent translocation of β-arrestin 2-GFP into punctate vesicles at the plasma membrane indicating that receptor signalling is not affected by hypertonic sucrose concentrations. The only difference between the wild type and the mutant form of the receptor was in the amount of β-arrestin 2-GFP being translocated to the plasma membrane. Whereas there was hardly any β-arrestin 2-GFP localised in the cytoplasm after activation of the wild type orexin 1 receptor, much of the cellular β-arrestin 2-GFP was not translocated following stimulation of the cluster C1 mutant, again confirming the interaction of β-arrestin 2-GFP with the cluster C1 mutant to be much more transient than with the wild type form of the receptor. The staining also showed that the sites of interaction of both forms of the orexin 1 receptor and β-arrestin 2-GFP at the plasma membrane were not acidic since no red signal could be detected. Treatment of cells expressing these forms of the orexin 1 receptor with 0.25 mg/ml concanavalin A for 30 min prior to addition of the CypHer-5–labelled anti-VSV-G antiserum resulted in a subtly different pattern for the wild type orexin 1 receptor. Although β-arrestin 2-GFP was translocated to the plasma membrane its distribution remained even rather than punctate (Figure 3.20 e). Again, there was no indication that the receptor was able to enter into an acidic environment in the presence of concanavalin A because the CypHer-5 bound to the receptor remained dark and non-fluorescent (Figure 3.20 e). Also, no red fluorescent signal was obtained for the cluster C1 mutant when orexin A was added following pre-treatment with 0.25 mg/ml concanavalin A (Figure 3.20 f). Again a substantially smaller fraction of the β-arrestin 2-GFP was translocated to the plasma membrane compared to the wild type receptor (Figure 3.20 f).

Pre-incubation of cells with either 50 μg/ml nystatin (Figure 3.20 g, h) or 5 μg/ml filipin (Figure 3.20 i, j) for 30 min before stimulation with 0.5 μM orexin A for 30 min had no effect on the interaction of β-arrestin 2-GFP with either form of the receptor or internalisation of the wild type orexin 1 receptor or the cluster C1 mutant. In both cases the staining pattern was the same as observed in cells stimulated with agonist alone. These data suggest that neither form of this receptor internalises to any significant extent in response to orexin A via cholesterol-rich, detergent-insensitive, domains.

3.11 Involvement of c-Src and dynamin in receptor internalisation

The results in 3.10 indicate that the orexin wild type receptor and the cluster C1 mutant internalise in clathrin-coated vesicles. To further compare the pathways of internalisation of the wild type and the C1 mutant either form of the receptor tagged with VSV-G at the N-terminus was expressed alone or co-expressed with N272 dynamin, which lacks the complete GTP-binding domain (Werbonat *et al.,* 2000) in HEK293T cells. 24 h after transfection the cells were stimulated with 0.5 μM orexin A for 30 min at 37 °C. The immunostained receptors were visualised by confocal microscopy. In cells expressing either form of the receptor on its own, the receptor internalised into intracellular vesicles as illustrated by the loss of fluorescence from the plasma membrane and a concomitant increase of the fluorescent signal in intracellular vesicles upon agonist exposure (Figure 3.21 a). In contrast, co-expression of dominant negative dynamin (Figure 3.21 b) completely abolished internalisation of both forms of the receptor since the antibody-stained receptor stayed at the cell surface.

To test the role of Src family kinases in internalisation of the orexin 1 receptor, the eYFP-tagged wild type receptor and the cluster C1 mutant were transiently co-expressed with β-arrestin 2-RFP in Src family kinase knock out (SYF) MEF cells. These cells were derived from Src, Yes and Fyn triple knock out embryos (Klinghoffer *et al.,* 1999). In the absence of agonist the wild type receptor was localised at the plasma membrane and β-arrestin 2-RFP in the cytosol (Figure 3.22 a). Incubation of the cells with 0.5 μM orexin A for 30 min induced internalisation of the receptor into endosomes. β-arrestin 2 translocated to the same vesicles as indicated by the yellow staining (Figure 3.22 b). Similar results were obtained for the cluster C1 mutant (Figure 3.22 c, d). However, in contrast to the wild type receptor, stimulation of the cluster C1 mutant did not result in any detectable movement of β-arrestin 2-RFP from the cytosol to intracellular vesicles (Figure 3.22 d). Therefore the orexin 1 receptor wild type as well as the cluster C1 mutant seem to internalise via clathrin-coated pits in a process depending on dynamin but not c-Src activity.

Figure 3.1: Visualisation of orexin 1 receptor internalisation using fluorescently labelled agonist

HEK293T cells were transfected with the human orexin 1 receptor and β-arrestin 2-GFP. 24 h post-transfection cells were challenged with TAMRA-labelled orexin A (0.5 μM) for 30 min. TAMRA-labelled orexin A was shown to have low nM potency in internalisation experiments. Cells were then fixed and images taken with the confocal microscope. The distribution of the orexin 1 receptor (i), β-arrestin 2-GFP (ii) and a composite of these images (iii) are shown. The images are representative of two further experiments.

Figure 3.1

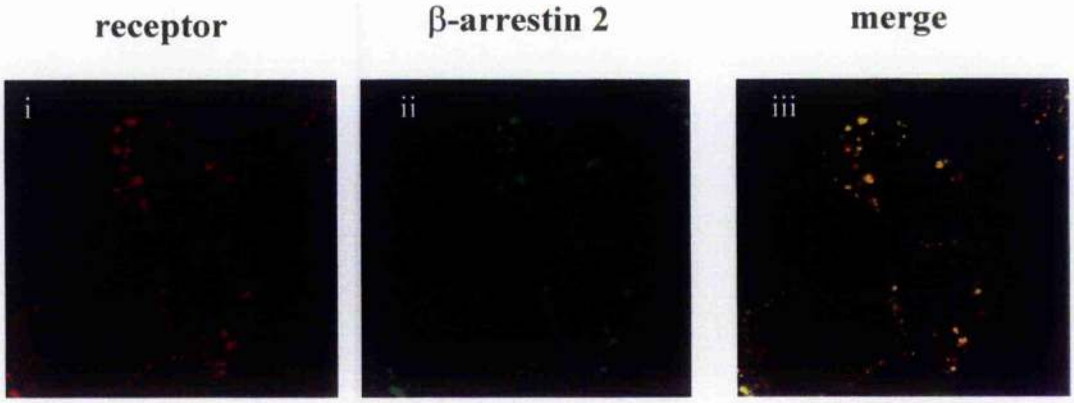

receptor
β-arrestin 2
merge
i
ii
iii

Figure 3.2: N-terminally HA-tagged orexin 1 receptor internalises and co-localises with β-arrestin 2-GFP after agonist stimulation

HEK293T cells transiently transfected with the N-terminally HA tagged orexin 1 receptor and β-arrestin 2-GFP were immunostained with anti-HA antibody prior to agonist stimulation. Images were taken of unstimulated control cells (a) and cells treated with 0.5 μM orexin A for 30 min (b). The distribution of the orexin 1 receptor (i), β-arrestin 2-GFP (ii) and a composite of these images (iii) are shown. The images are representative of two further experiments.

Figure 3.2

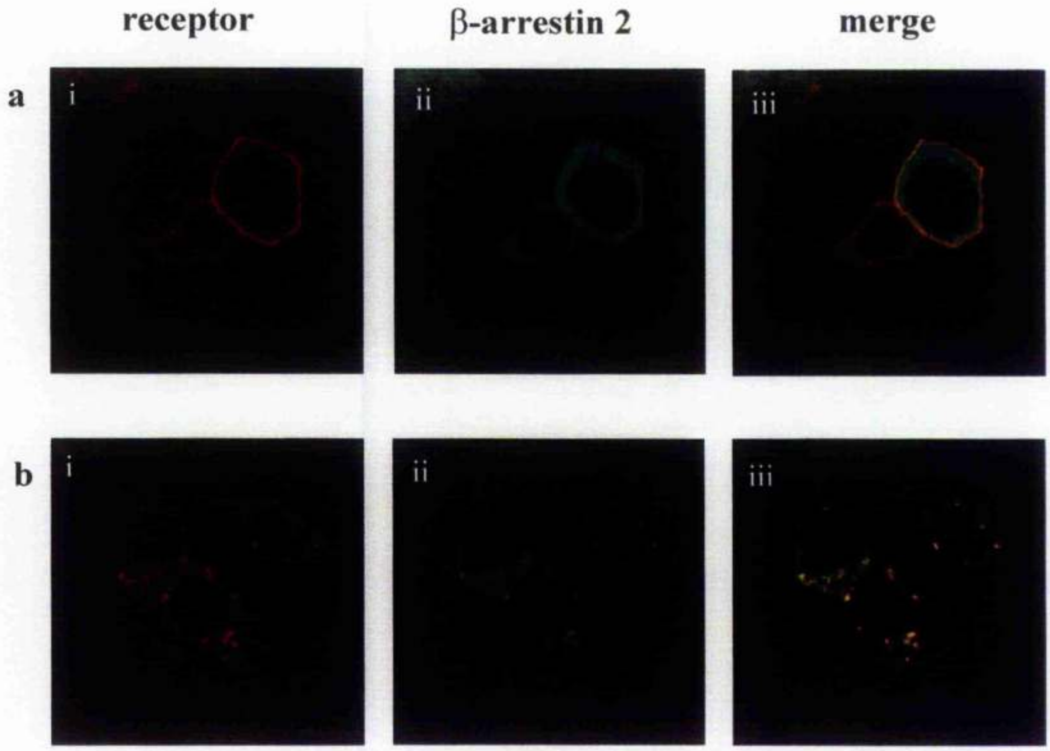

receptor
β-arrestin 2
merge
a
i
ii
iii
b
i
ii
iii

Figure 3.3: Orexin 1 receptor C-terminally tagged with eYFP co-localises with β-arrestin 2-RFP after agonist challenge

Both constructs were transiently expressed in HEK293T cells and the cells fixed 24 h after transfection. The distribution of the orexin 1 receptor-eYFP (i) and β-arrestin 2-RFP (ii) was visualised by confocal microscopy in unstimulated cells (a) and cells challenged with 0.5 μM orexin A for 30 min (b). An overlay of these pictures (iii) is also shown. Similar results were gained in two further experiments.

Figure 3.3

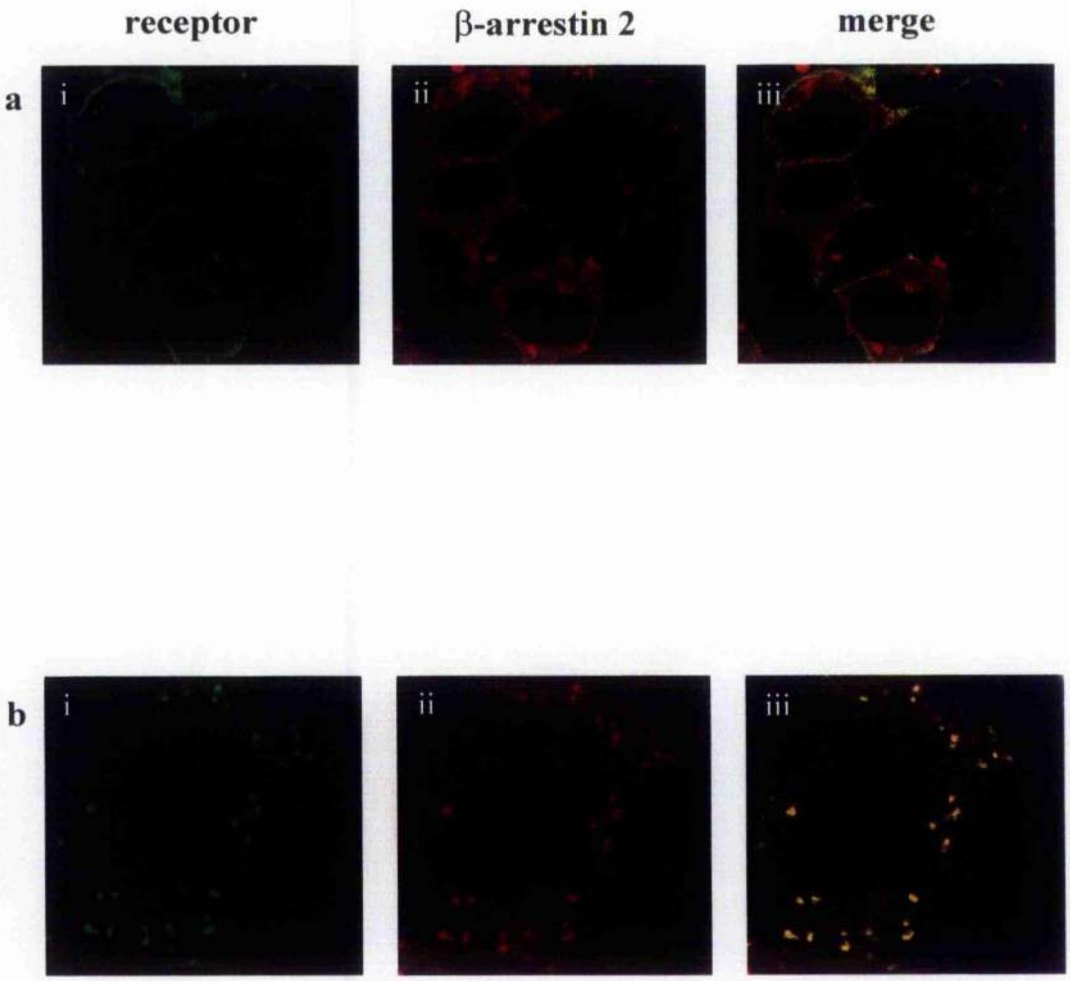

receptor
β-arrestin 2
merge
a
i
ii
iii
b
i
ii
iii

Figure 3.4: N-terminally VSV-G-tagged orexin 1 receptor internalises and co-localises with β-arrestin 2-GFP after agonist stimulation

N-terminally VSV-G-tagged orexin 1 receptor and β-arrestin 2-GFP were co-expressed in HEK293T cells. The surface receptor was immunostained with CypHer-5 labelled anti-VSV-G antibody. Confocal images of unstimulated cells (a) and agonist-treated cells (b) (0.5 μM orexin A for 30 min) were taken. The distribution of the orexin 1 receptor (i), β-arrestin 2-GFP (ii) and a composite of these images (iii) are shown. Similar results were obtained in two further independent experiments.

Figure 3.4

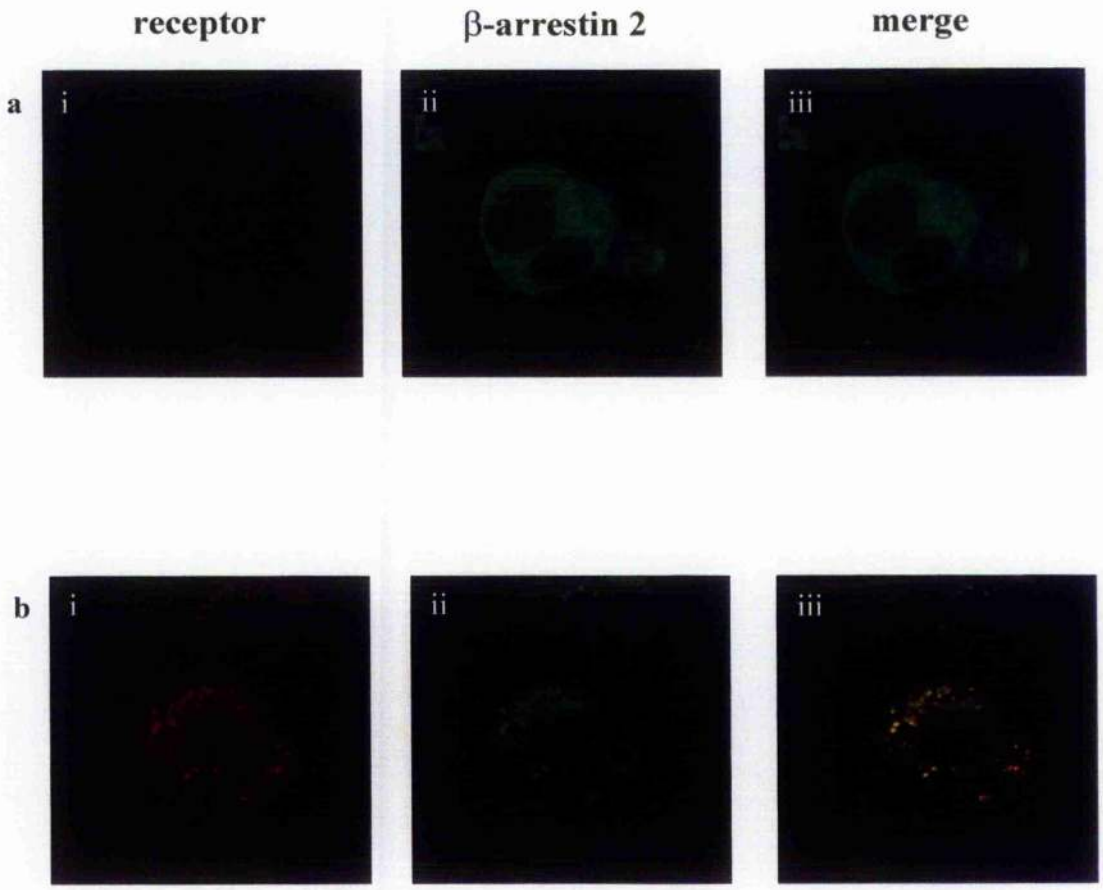

receptor
β-arrestin 2
merge
a
i
ii
iii
b
i
ii
iii

Figure 3.5: The orexin 1 receptor internalises in the absence of G_q/G_{11}

EF88 cells were transiently transfected with the orexin 1 receptor-eYFP. The cells were subsequently challenged with vehicle (a) or orexin A (0.5 μM, 30 min) (b). Receptor distribution was then visualised by confocal microscopy. Similar observations were made in two further experiments.

Figure 3.5

a

b

Figure 3.6: The C-terminally truncated orexin 1 receptor cDNAs are translated into truncated proteins

The HA-orexin 1 receptor wild type and the C-terminal truncations were each expressed in HEK293T cells. 24 h after transfection the cells were lysed. 20 μg protein of each sample was loaded on a 4-12 % Bis Tris gel, the proteins subsequently transferred onto a PVDF membrane and the different forms of the receptor detected by incubating the membranes with anti-HA antibody. A representative blot from three different experiments is shown.

Figure 3.6

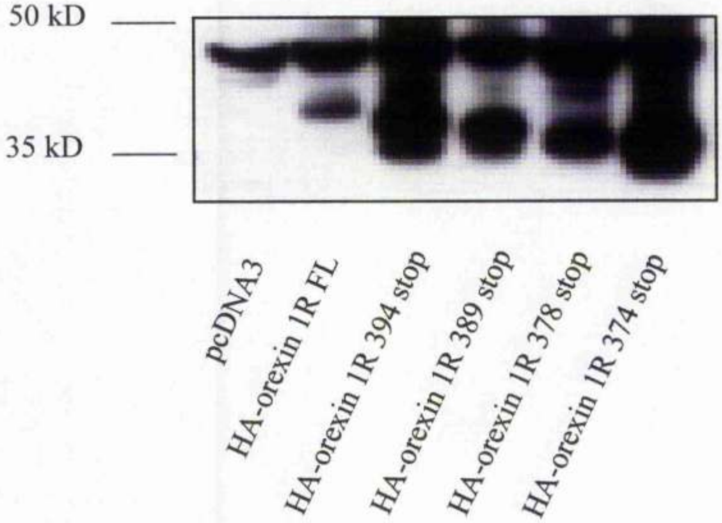

50 kD
35 kD
pcDNA3
HA-orexin 1R FL
HA-orexin 1R 394 stop
HA-orexin 1R 389 stop
HA-orexin 1R 378 stop
HA-orexin 1R 374 stop

Figure 3.7: C-terminal truncation of the orexin 1 receptor prevents interaction with β-arrestin 2 but not agonist-induced internalisation

The C-terminal truncations (N-terminally tagged with HA) of the orexin 1 receptor 394 stop (a, b), 389 stop (c, d), 378 stop (e, f) and 374 stop (g, h) were transiently co-expressed with β-arrestin 2-GFP in HEK293T and after 24 h the receptor visualised by staining with anti-HA antibody. Images were taken of cells prior to stimulation (a, c, e, g) and after stimulation with 0.5 μM orexin A for 30 min (b, d, f, g). The distribution of the orexin 1 receptor (i), β-arrestin 2 (ii) and a composite of these images (iii) are shown. The images are representative of two further experiments.

Figure 3.7

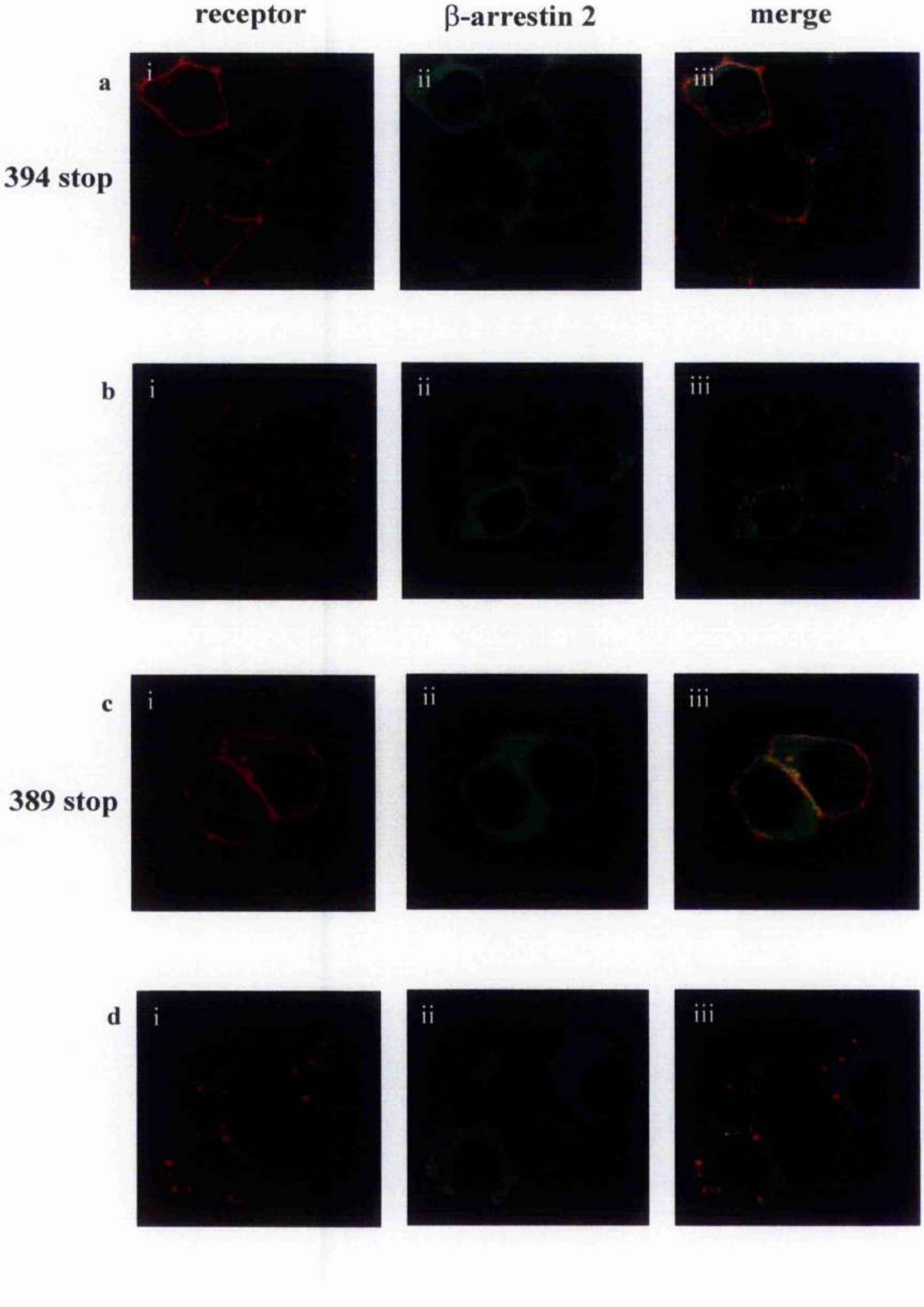

receptor
β-arrestin 2
merge
a
394 stop
i
ii
iii
b
i
ii
iii
c
389 stop
i
ii
iii
d
i
ii
iii

Figure 3.7 continued

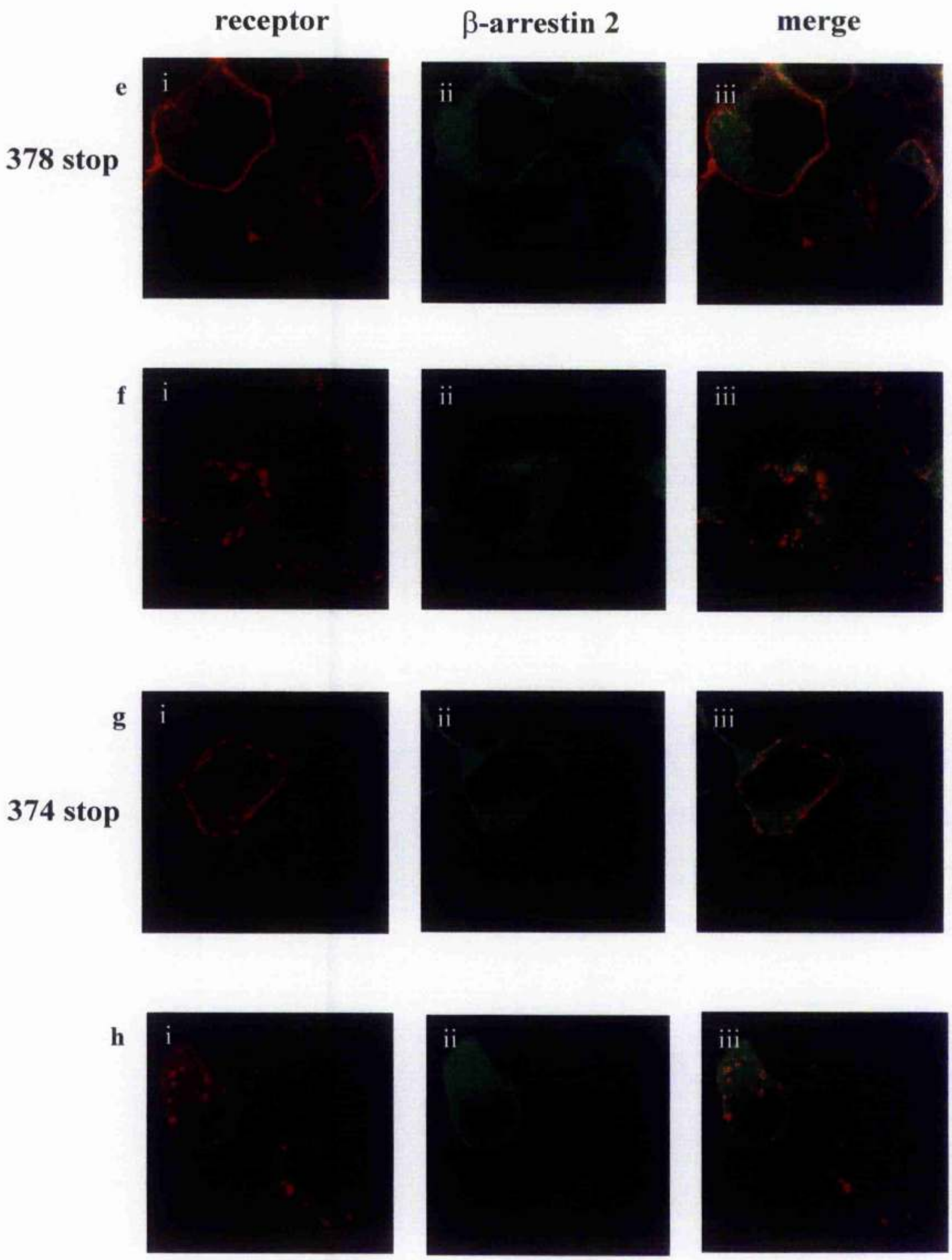

receptor
β-arrestin 2
merge
e
378 stop
i
ii
iii
f
i
ii
iii
g
374 stop
i
ii
iii
h
i
ii
iii

Figure 3.8: A single cluster of hydroxy amino acids within the C-terminus allows co-internalisation of the orexin 1 receptor and β-arrestin 2

The cDNAs for HA-orexin 1 receptor cluster C2 mutant (a, b), cluster C1 mutant (c, d) and cluster C1C2 mutant (e, f) were co-expressed with β-arrestin 2-GFP in HEK293T cells. 24 h after transfection the surface receptors were immunostained with anti-HA antibody. Confocal images were taken of unstimulated cells (a, c, e) and of cells treated with 0.5 μM orexin A for 30 min (b, d, f). Shown are confocal visualisations of the receptor immunofluorescence (i) and the β-arrestin 2-GFP fluorescence (ii). Co-localisation of the receptor with β-arrestin 2 is indicated in the overlay (iii). This result was confirmed in two further experiments.

Figure 3.8

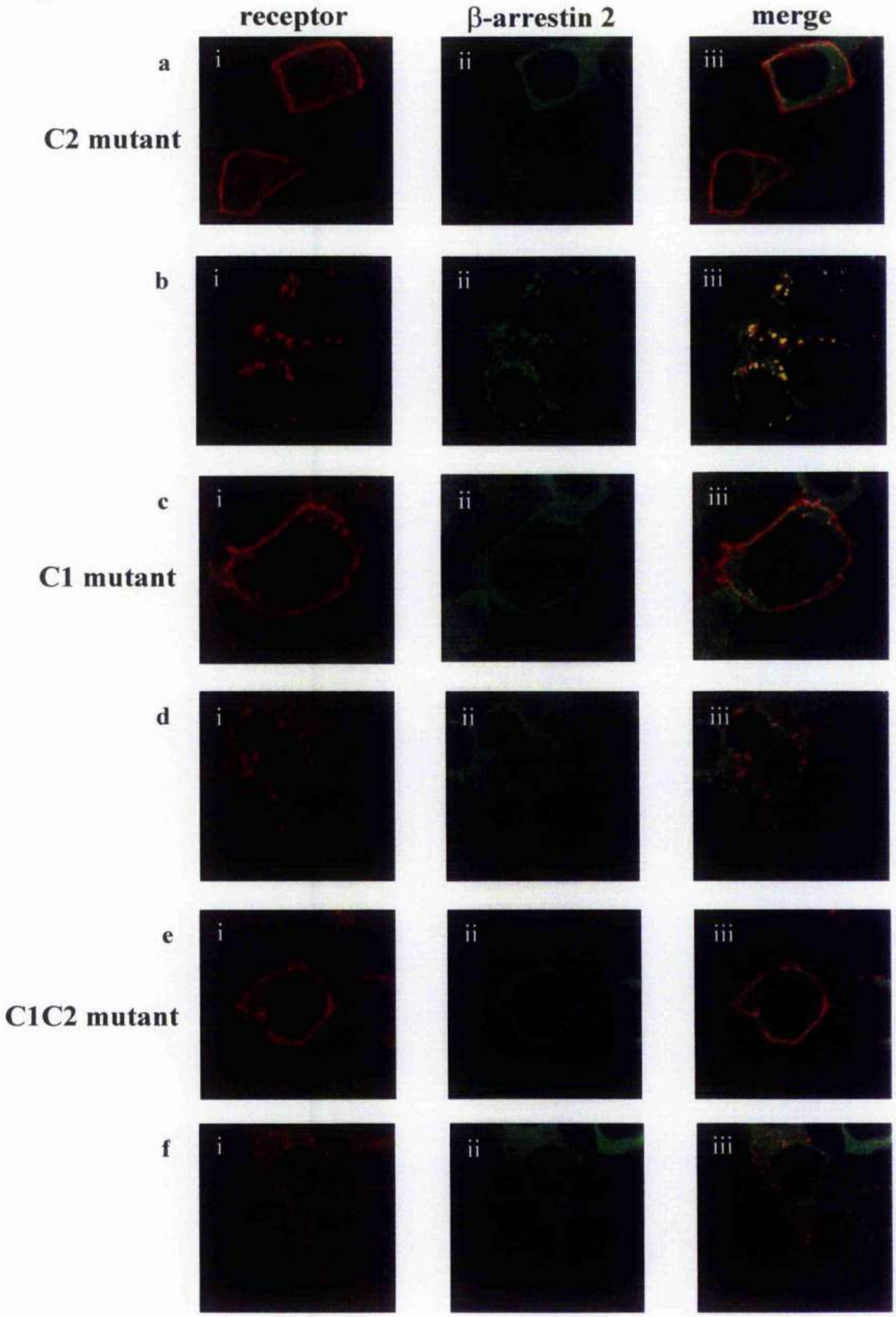

receptor
β-arrestin 2
merge
a
C2 mutant
b
c
C1 mutant
d
e
C1C2 mutant
f
i
ii
iii

Figure 3.9: Mutation of any individual amino acid in cluster C1 has no effect on the co-localisation of the orexin 1 receptor and β-arrestin 2 after agonist challenge

All HA-orexin 1 receptor single point mutants (T418A: a, b; S419A:c, d; T421A:e, f; T422A:g, h) were co-transfected with β-arrestin 2-GFP into HEK293T cells. The different forms of the receptor were labelled with anti-HA antibody and the distribution of the receptor (i) and β-arrestin 2 (ii) and the overlay of the two signals (iii) in cells prior to stimulation (a, c, e, g) and after stimulation (0.5 μM orexin A, 30 min) (b, d, f, h) examined by confocal microscopy. This experiment was repeated twice.

Figure 3.9

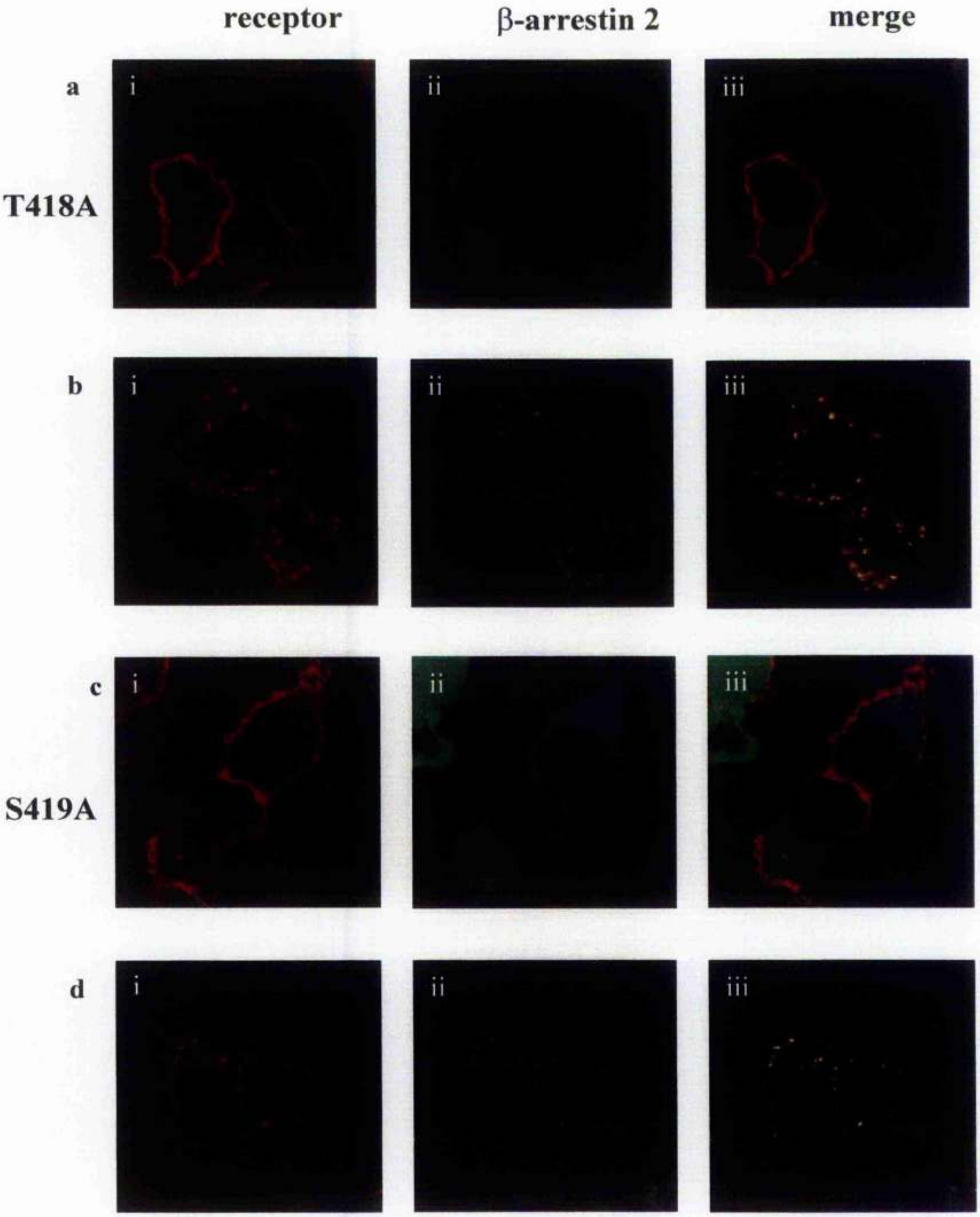

receptor
β-arrestin 2
merge
a
T418A
b
c
S419A
d
i
ii
iii
i
ii
iii
i
ii
iii
i
ii
iii

Figure 3.9 continued

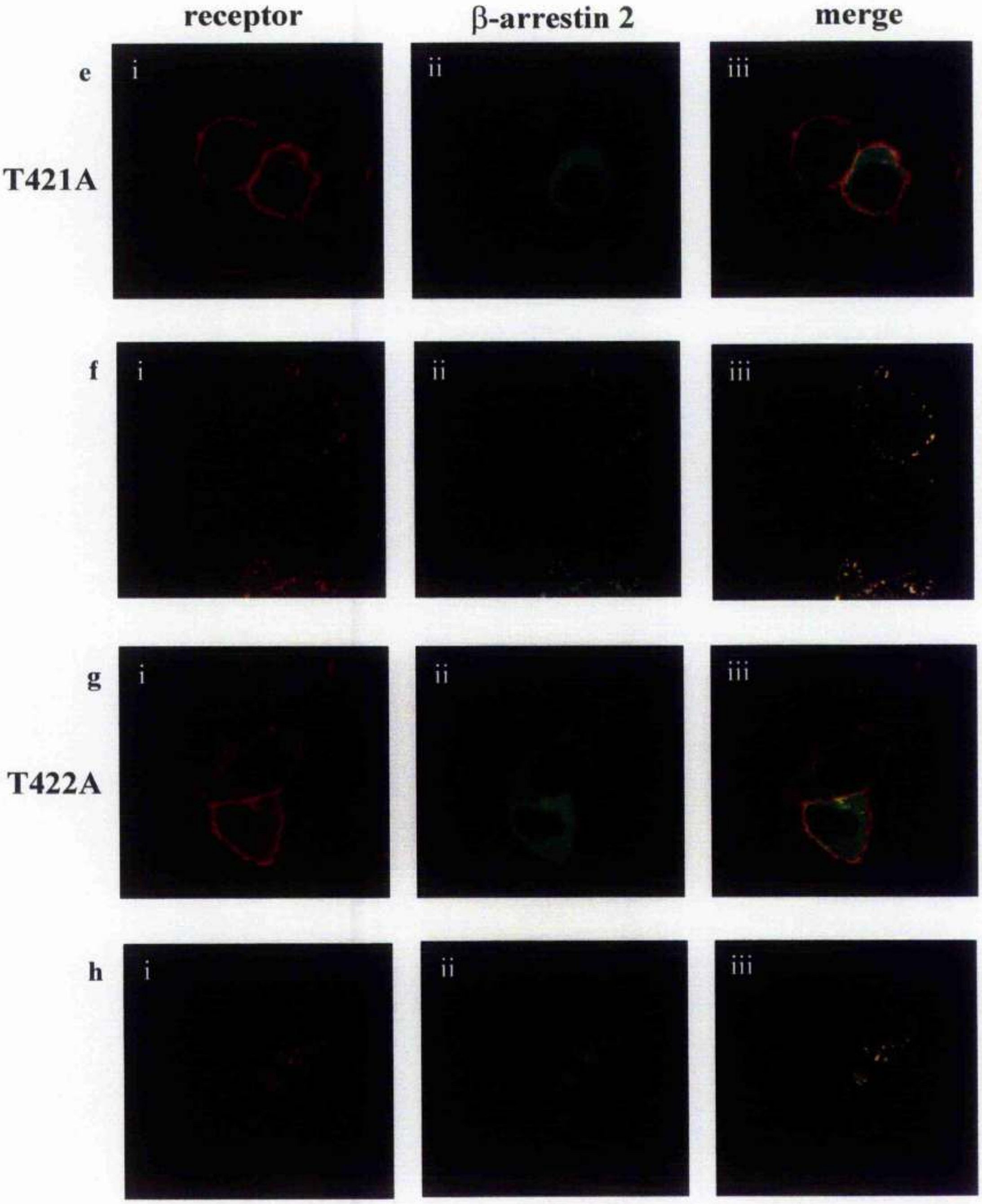

receptor
β-arrestin 2
merge
e
T421A
f
g
T422A
h
i
ii
iii

Figure 3.10: Double point mutations in cluster C1 of the orexin-1 receptor disrupt co-internalisation of the receptor with β-arrestin 2

The six different N-terminally HA-tagged double point mutants of the orexin 1 receptor (T418/S419A: a, b; T418/T421A:c, d; T418/T422A:e, f; S419/T421A:g, h; S419/T422A:i, j; T421/T422A:k, l) were transiently transfected into HEK293T cells with β-arrestin 2-GFP. The different forms of the receptor were visualised by immunostaining with anti-HA antibody. The cells were either treated with vehicle (a, c, e, g, i, k) or with 0.5 μM orexin A for 30 min (b, d, f, h, j, l). The distribution of the orexin 1 receptor (i), β-arrestin 2 (ii) and a composite of these images (iii) are shown. The confocal images shown are representative for a set of three experiments performed.

Figure 3.10

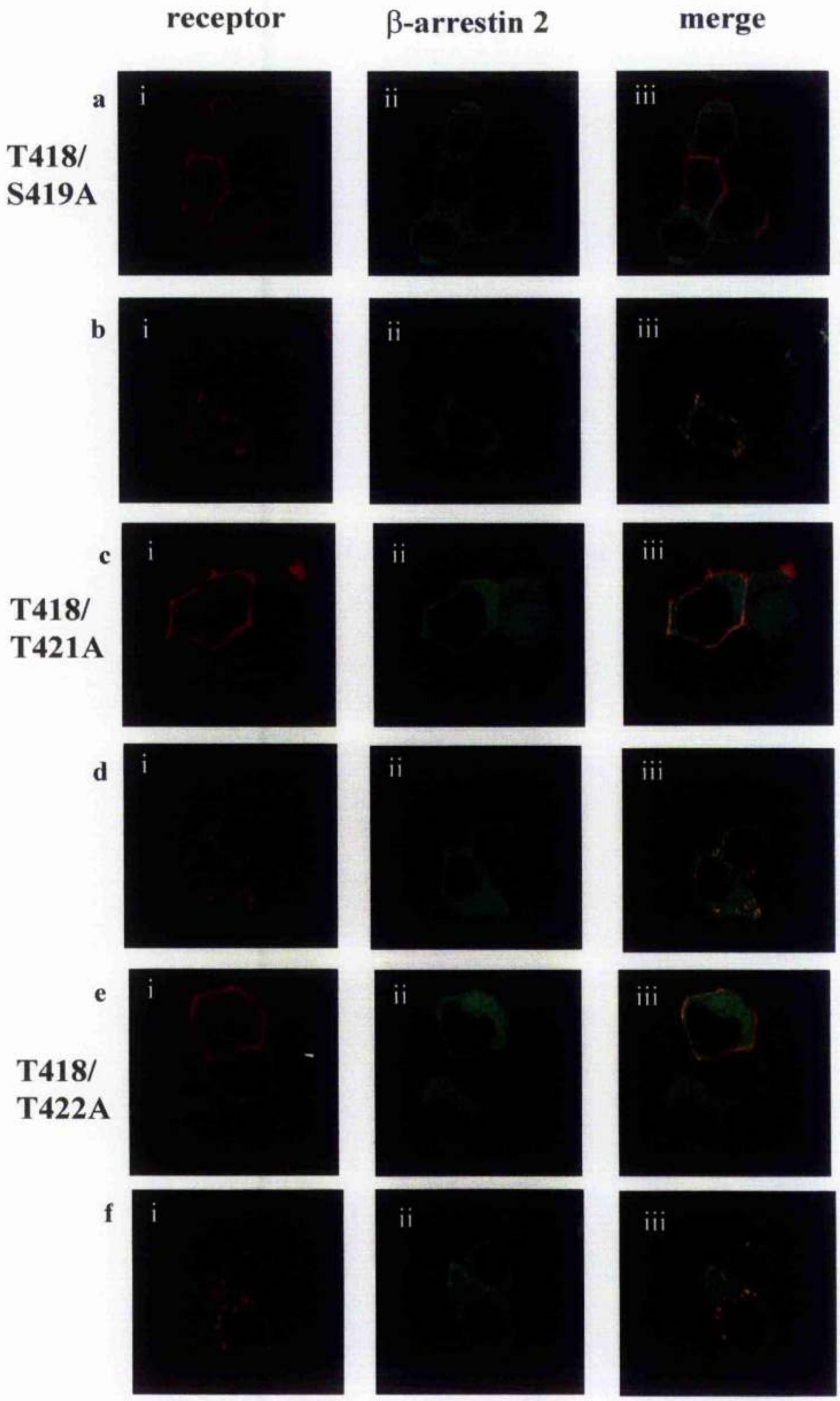

receptor
β-arrestin 2
merge
a
T418/
S419A
b
c
T418/
T421A
d
e
T418/
T422A
f
i
ii
iii

Figure 3.10 continued

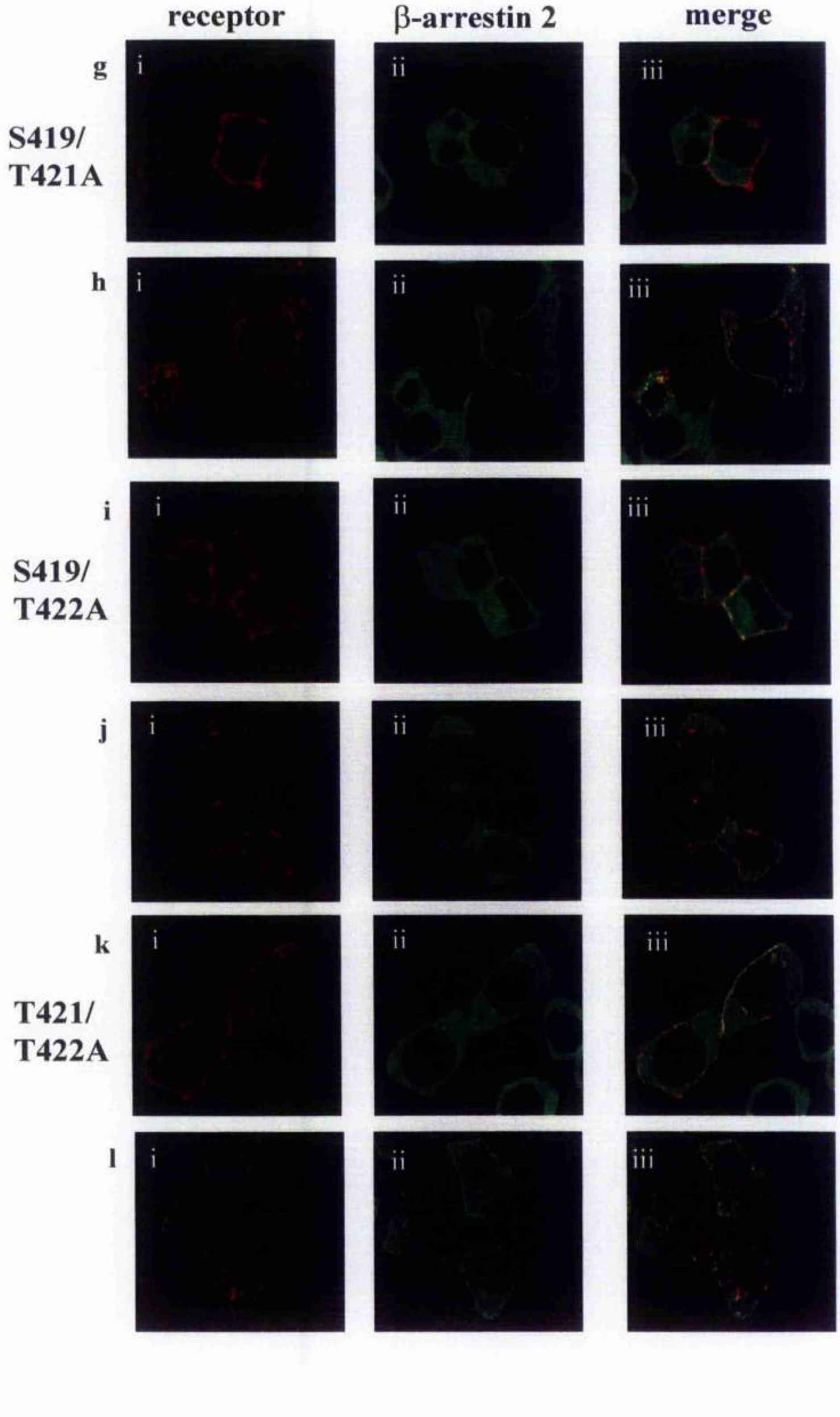

receptor
β-arrestin 2
merge
g
S419/
T421A
h
i
S419/
T422A
j
k
T421/
T422A
l
i
ii
iii

Figure 3.11: Time course of the agonist-mediated internalisation of the orexin 1 receptor wild type and C1 cluster mutant

HEK293T cells were co-transfected with plasmids containing the cDNA for β-arrestin 2-GFP and the cDNA for the HA-wild type or the HA-C1 cluster mutant. The different forms of the receptor were stained with the anti-HA antibody and visualised with the confocal microscope. The cells were stimulated with 0.5 μM orexin A as indicated. The distribution of the orexin 1 receptor (i), β-arrestin 2 (ii) and a composite of these images (iii) are shown. This result was confirmed in two further experiments.

Figure 3.11

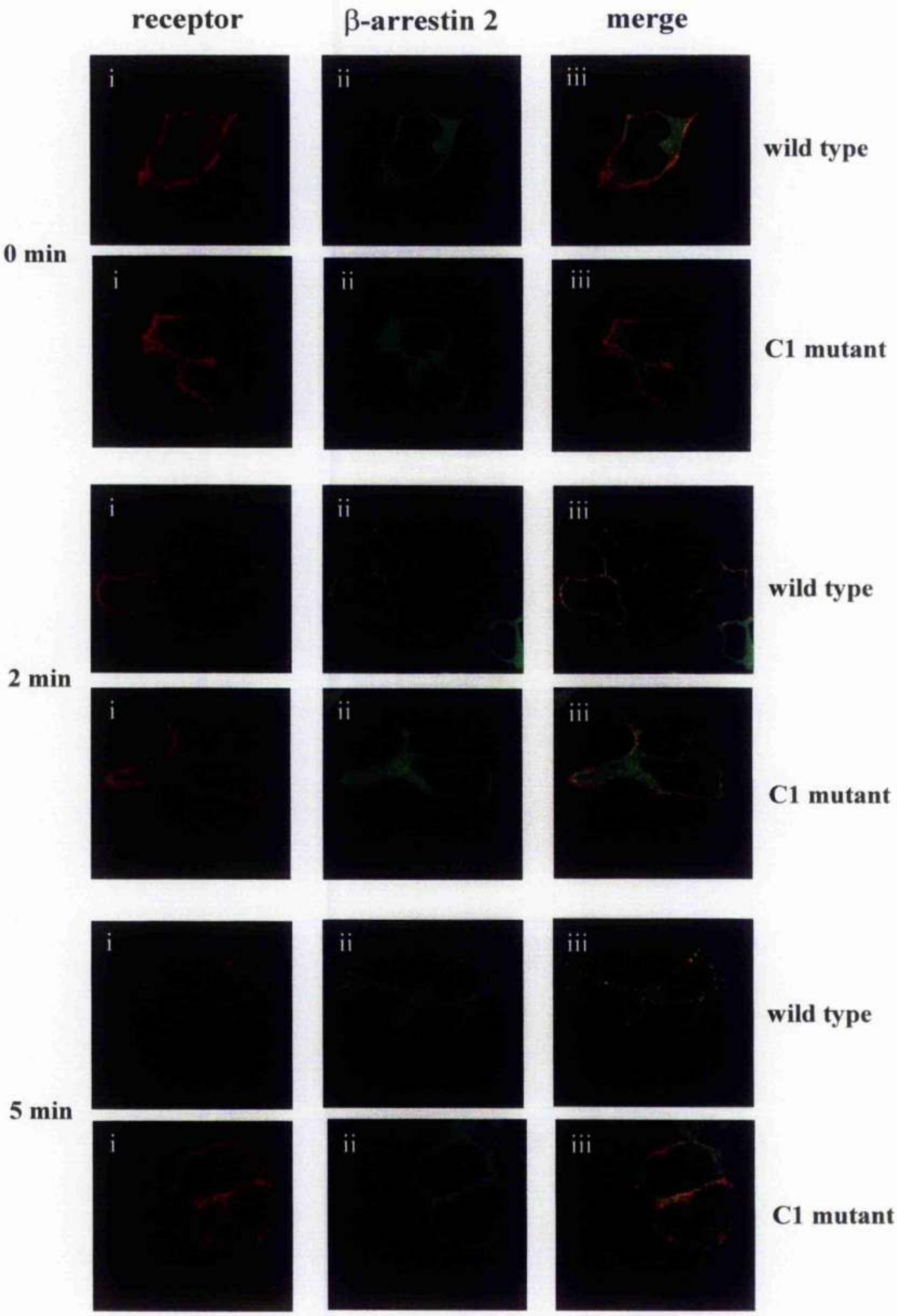

receptor
β-arrestin 2
merge
i
ii
iii
wild type
0 min
C1 mutant
2 min
5 min

Figure 3.11 continued

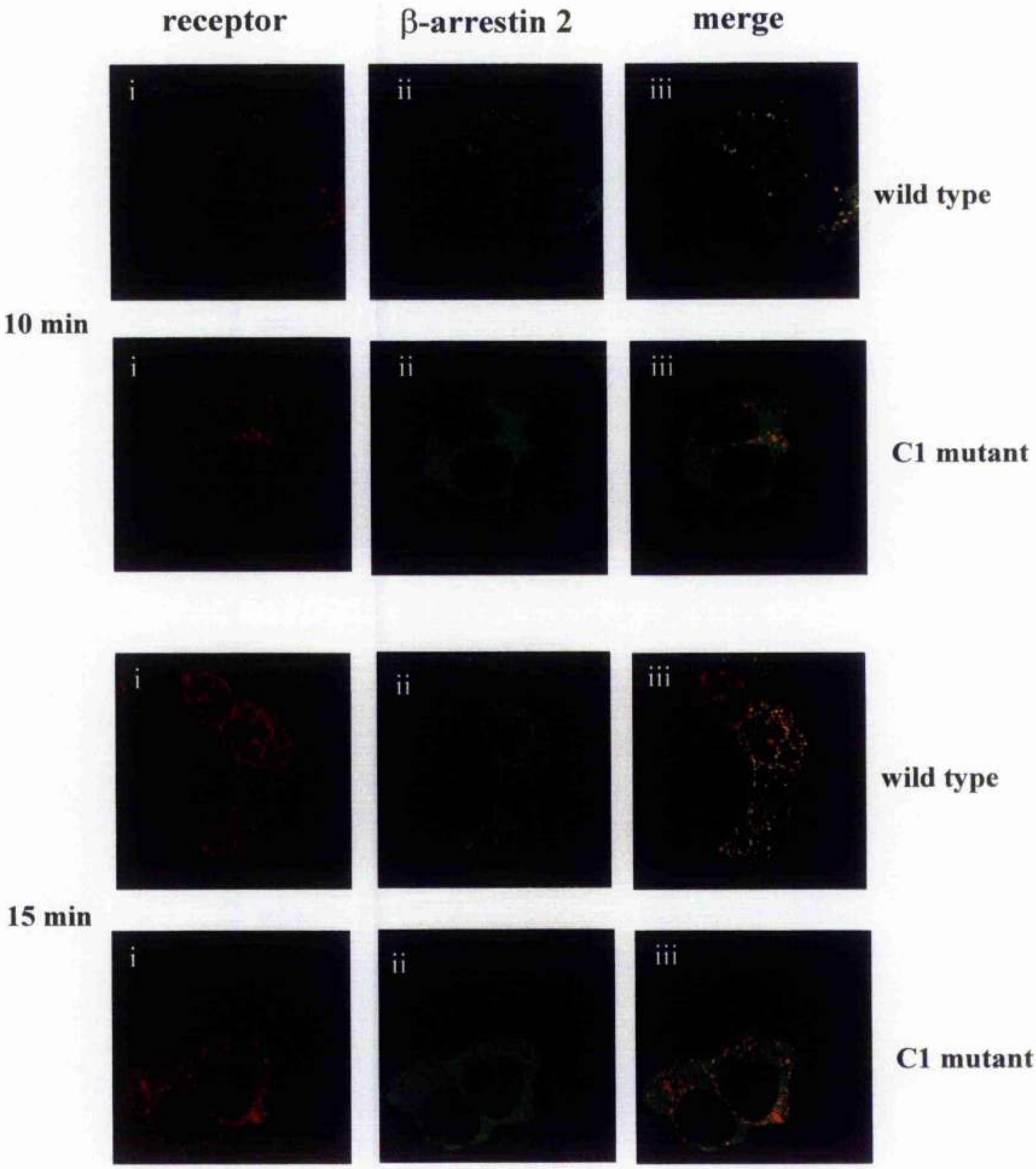

receptor
β-arrestin 2
merge
i
ii
iii
wild type
10 min
i
ii
iii
C1 mutant
i
ii
iii
wild type
15 min
i
ii
iii
C1 mutant

Figure 3.12: β-arrestin 2-GFP co-immunoprecipitation with agonist-activated wild type and cluster C1 mutant

VSV-G-tagged forms of the wild type (WT) and cluster C1 mutant (C1) orexin 1 receptor were transiently co-expressed with β-arrestin 2–GFP in HEK293T cells. 24 h post transfection the cells were stimulated with vehicle or 0.5 μM orexin A for 15 min. Following addition of 2 mM dithiobis[succinimidylpropionate] (DSP), β-arrestin 2–GFP was immunoprecipitated using anti-GFP antiserum and the samples resolved on SDS-PAGE. β-arrestin 2-GFP and forms of the receptor bound were monitored by immunoblotting with anti-GFP and anti-VSV-G antisera respectively. The data shown is representative of two independent experiments.

Figure 3.12

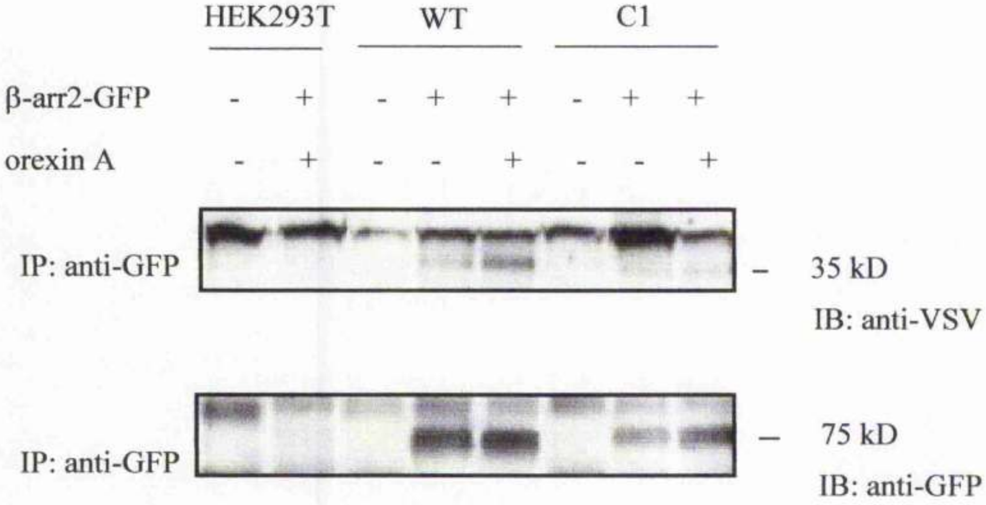

HEK293T
WT
C1
β-arr2-GFP - + - + + - + +
orexin A - + - - + - - +
IP: anti-GFP
35 kD
IB: anti-VSV
IP: anti-GFP
75 kD
IB: anti-GFP

Figure 3. 13: Agonist induced phosphorylation of the orexin 1 receptor

CHO cells stably expressing HA-orexin 1 receptor were labelled with ^{32}P. Following stimulation of the receptor with 0.5 μM orexin A as indicated, the receptors were then immunoprecipitated. Dried gels were analysed by autoradiography. Data shown are representative from a single experiment, which was repeated three times.

Figure 3.13

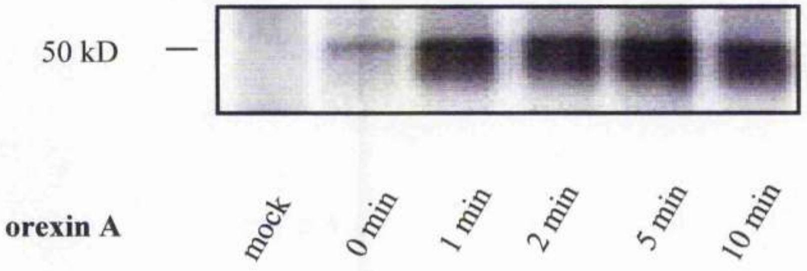

50 kD
orexin A
mock
0 min
1 min
2 min
5 min
10 min

Figure 3. 14: Effects of second messenger kinases on phosphorylation of the orexin 1 receptor

CHO cells stably expressing HA-orexin 1 receptor were pre-treated for 30 min with vehicle, 10 μM H89, 10 μM BAPTA/AM or 5 μM GF109203X prior to stimulation with 0.5 μM orexin A for 5 min. Other samples were incubated with 0.5μM orexin A, 50 μM forskolin, 5 μM PMA, 10 μM A23187 or 100 μM 8-bromo-cGMP for 5 min at 37 °C and the receptors immunoprecipitated with anti-HA antibody. Representative autoradiographs of the immunoprecipitated receptors are shown. Similar results were produced with two further experiments.

Figure 3.14

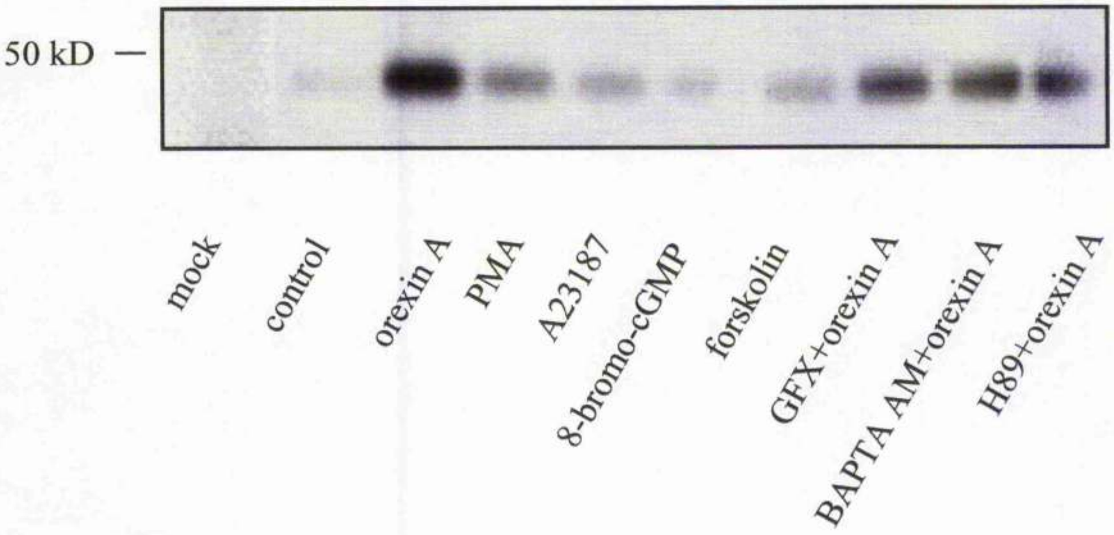

50 kD
mock
control
orexin A
PMA
A23187
8-bromo-cGMP
forskolin
GFX+orexin A
BAPTA AM+orexin A
H89+orexin A

Figure 3.15: Agonist induced phosphorylation of the cluster mutants

CHO cells were transiently transfected with orexin 1 receptor wild type, HA-C1 or -C2 mutant C-terminally tagged with eYFP using the Amaxa nucleofection system. 24 h post-transfection the cells were labelled with ^{32}P prior to agonist stimulation (0.5 μM orexin A, 5 min). The receptors were purified by immunoprecipitation using an anti-GFP antibody and resolved on SDS-PAGE. Representative autoradiographs of the immunoprecipitated receptors are shown (a). To assess the expression level of each receptor by Western Blot analysis equal amounts of cell lysate were loaded on a SDS-PAGE and the PVDF membrane probed with anti-GFP antibody (b). Similar results were obtained in two further experiments. Quantification of receptor phosphorylation is expressed as fold over basal (c). The data shown represent the mean ± SD of 5 independent experiments.

Figure 3.15

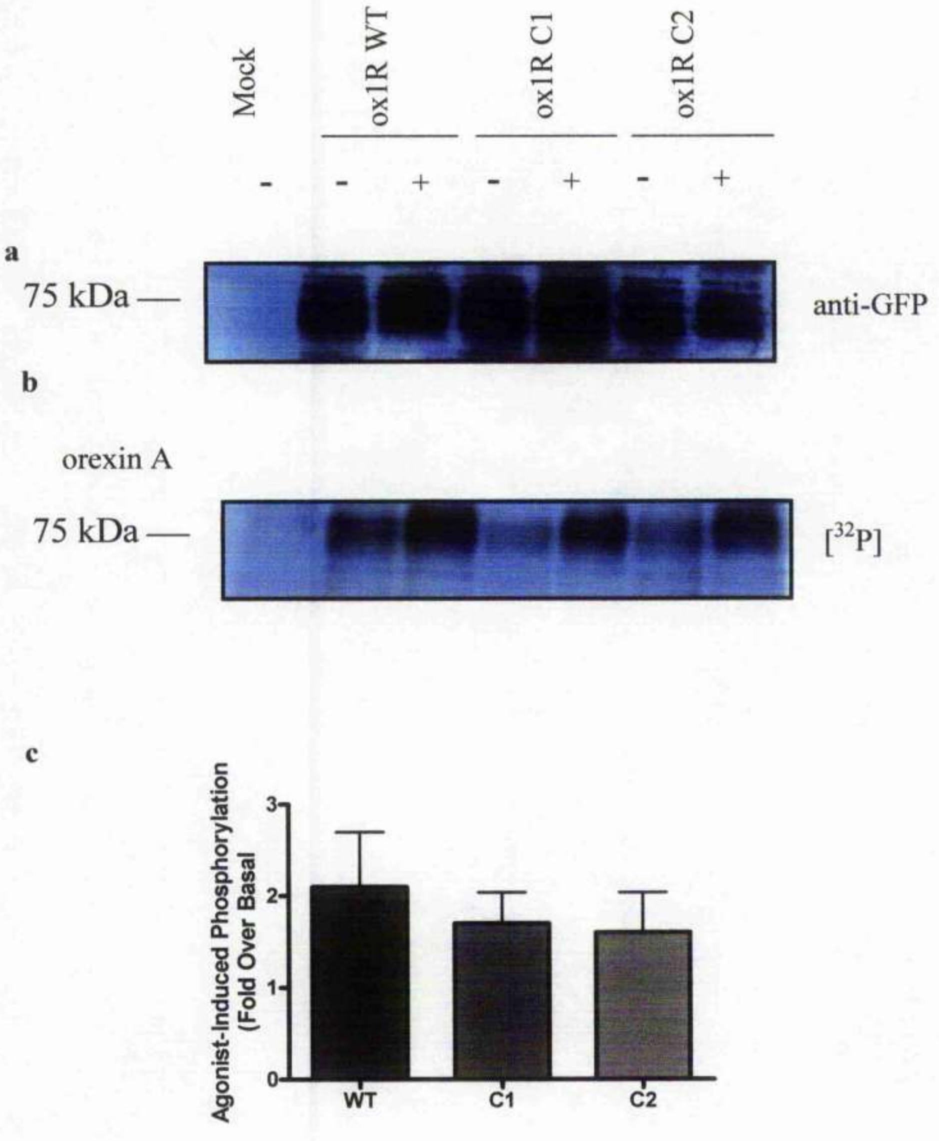

Mock
ox1R WT
ox1R C1
ox1R C2
- - + - + - +
a
75 kDa
anti-GFP
b
orexin A
75 kDa
[^{32}P]
c
Agonist-Induced Phosphorylation (Fold Over Basal)
3
2
1
0
WT
C1
C2

Figure 3.16: Effect of casein kinase II inhibiton on orexin 1 receptor internalisation Transiently transfected HEK293T cells co-expressing β-arrestin 2-GFP and HA-orexin 1 receptor were plated on coverslips and the receptor labelled with anti-HA antibody. The cells were untreated (a) or stimulated with 0.5 μM orexin A for 30 min following incubation of the cells for 30 min with vehicle (b) or 80 μM apigenin (c), fixed and analysed with confocal microscopy. The distribution of the receptor (i), β-arrestin 2 (ii) and a composite of these images (iii) are shown. The confocal images shown are representative for a set of three experiments performed.

Figure 3.16

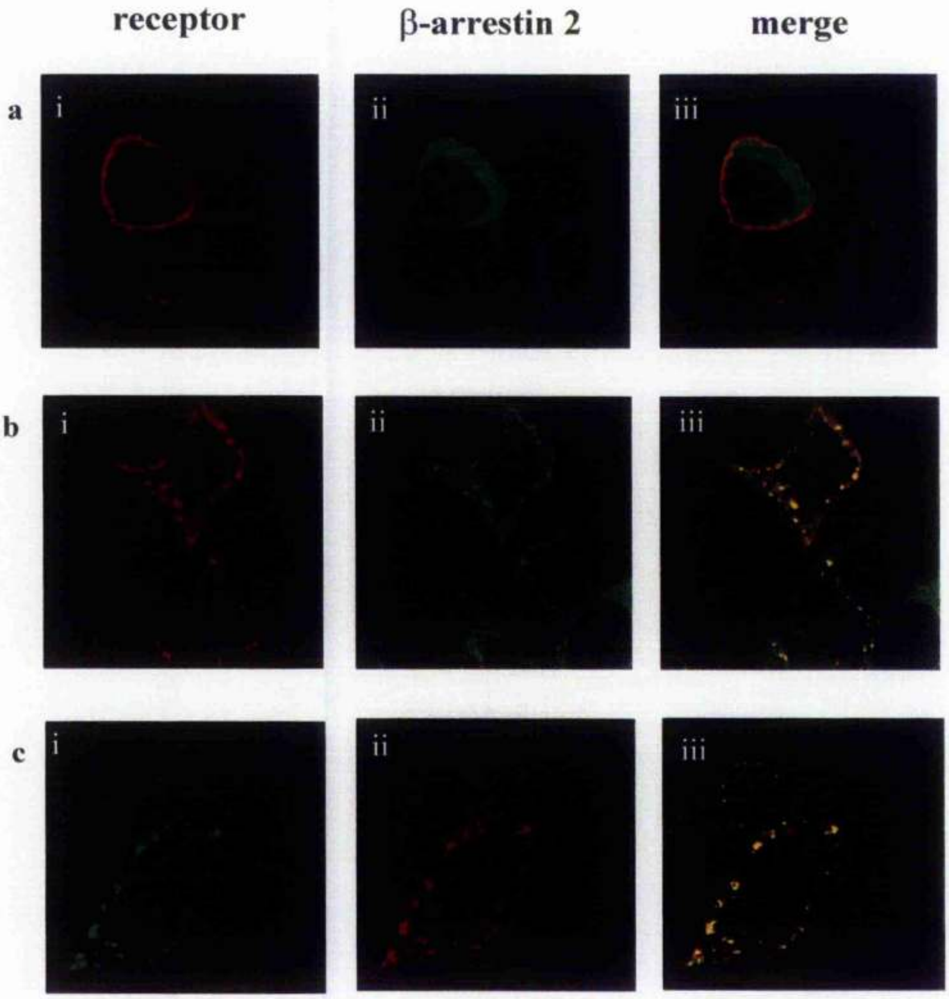

receptor
β-arrestin 2
merge
a
i
ii
iii
b
i
ii
iii
c
i
ii
iii

Figure 3.17: Involvement of casein kinase II in internalisation of the thyrotropin-releasing hormone receptor

The thyrotropin-releasing hormone receptor N-terminally tagged with VSV-G was co-transfected with β-arrestin 2-GFP into HEK293T cells. The receptor was visualised by immunostaining with anti-VSV-G antibody. Confocal images of untreated cells (a), cells treated with 1 μM thyrotropin-releasing hormone for 30 min (b), and of cells incubated with 80 μM apigenin for 30 min prior to addition of thyrotropin-releasing hormone (c) were taken. Shown are visualisations of the receptor immunofluorescence (i) and the β-arrestin 2-GFP fluorescence (ii). Co-localisation of the receptor with β-arrestin 2 is indicated in the overlay (iii). This experiment was repeated twice.

Figure 3.17

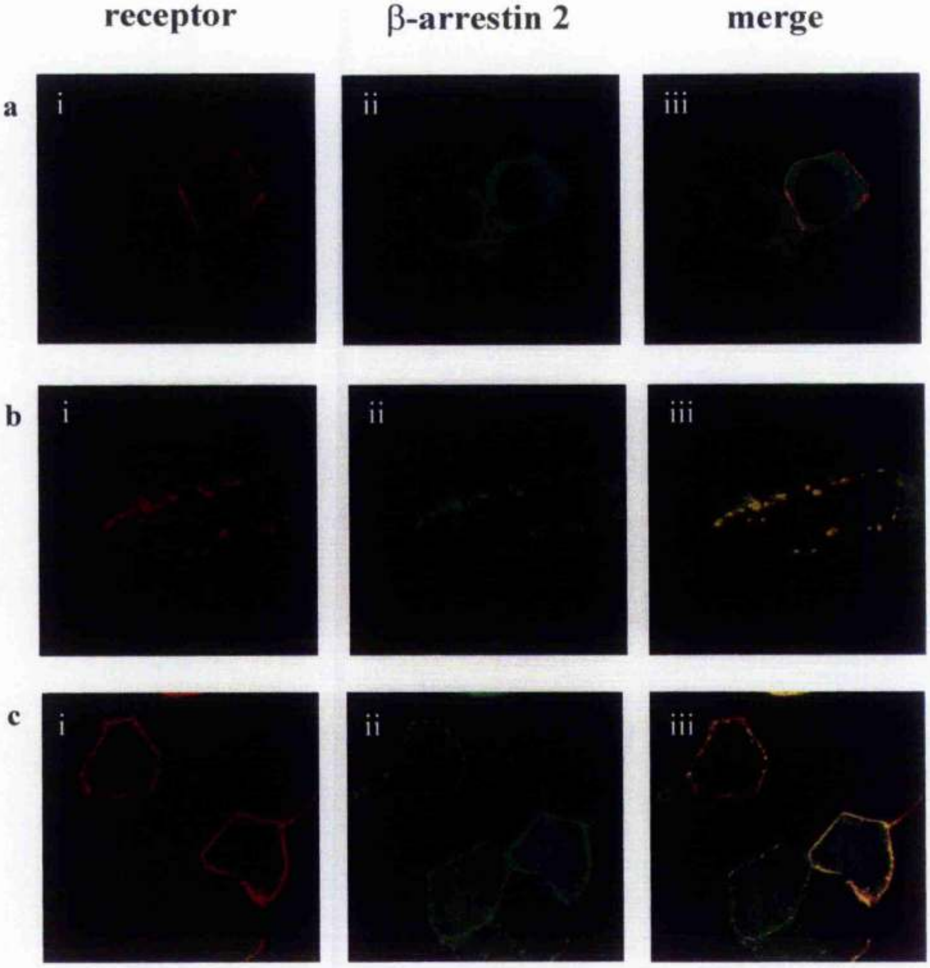

receptor
β-arrestin 2
merge
a
i
ii
iii
b
i
ii
iii
c
i
ii
iii

Figure 3.18: Internalisation of the orexin 1 receptor wild type and the cluster C1 mutant is β-arrestin dependent

The C-terminally eYFP-tagged form of the orexin 1 receptor wild type (a, c) and the HA-C1 mutant (b, d) were individually expressed in wild type MEF cells (a, b) and β-arrestin 1 and 2 knock out MEF cells (c, d). The distribution of the different forms of the receptor were visualised by confocal microscopy prior (i) and after (ii) agonist challenge (0.5 μM orexin A, 30 min). Similar confocal images were obtained in two further experiments.

Figure 3.18

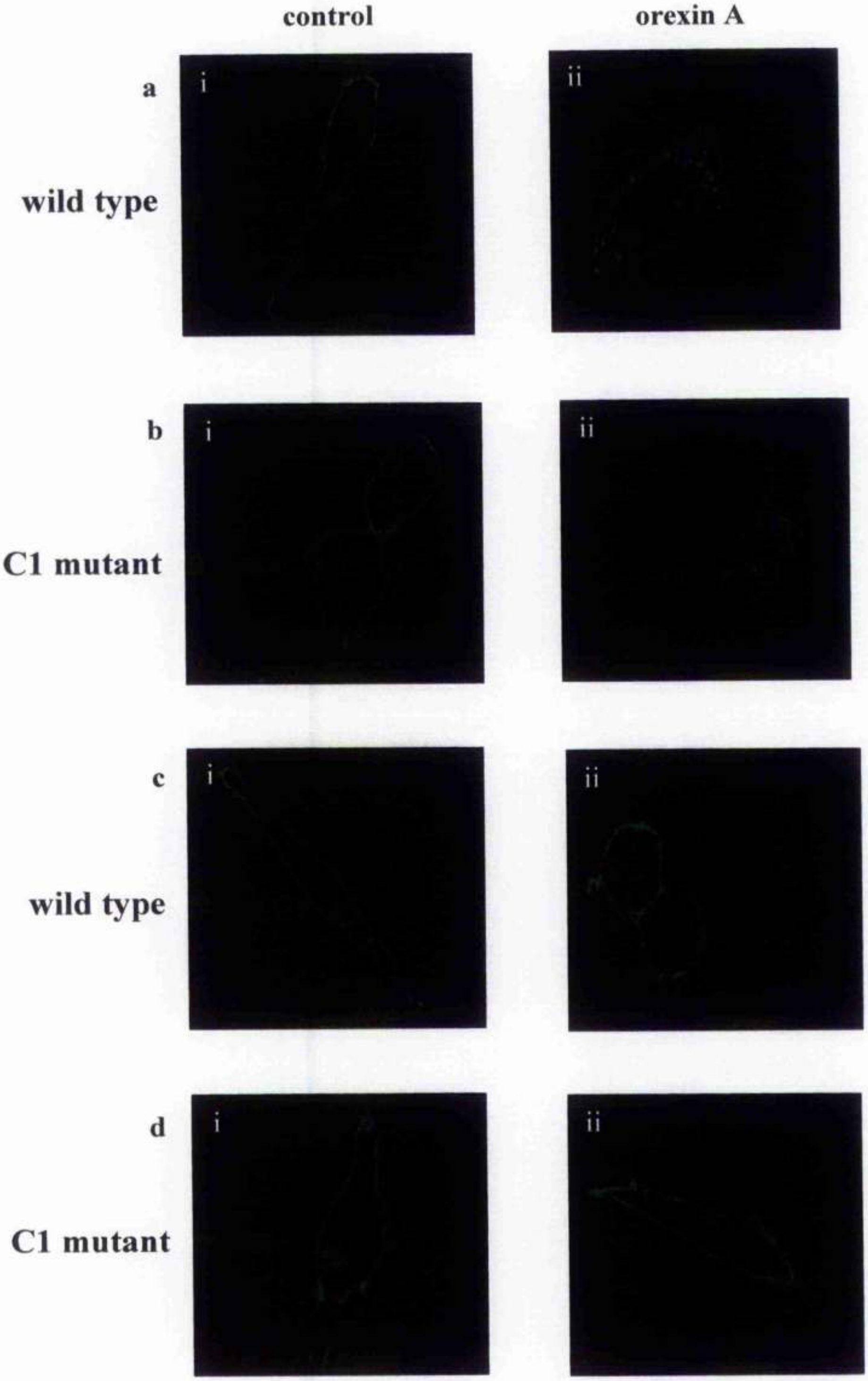

control
orexin A
a
i
ii
wild type
b
i
ii
C1 mutant
c
i
ii
wild type
d
i
ii
C1 mutant

Figure 3.19: Internalisation of the orexin 1 receptor wild type and the cluster C1 mutant in MEF knock out cells can be reconstituted by co-transfecting β-arrestin 2

The orexin 1 receptor-eYFP (i) wild type (a, b) and HA-C1 mutant-eYFP (c, d) were co-transfected with β-arrestin 2-RFP (ii) into β-arrestin 1 and 2 knock out MEF cells. 24 h after transfection confocal images were taken of unstimulated cells (a, c) and cells challenged with 0.5 μM orexin A for 30 min (b, d). A composite of the individual pictures is also displayed (iii). Similar confocal images were obtained in two further experiments.

Figure 3.19

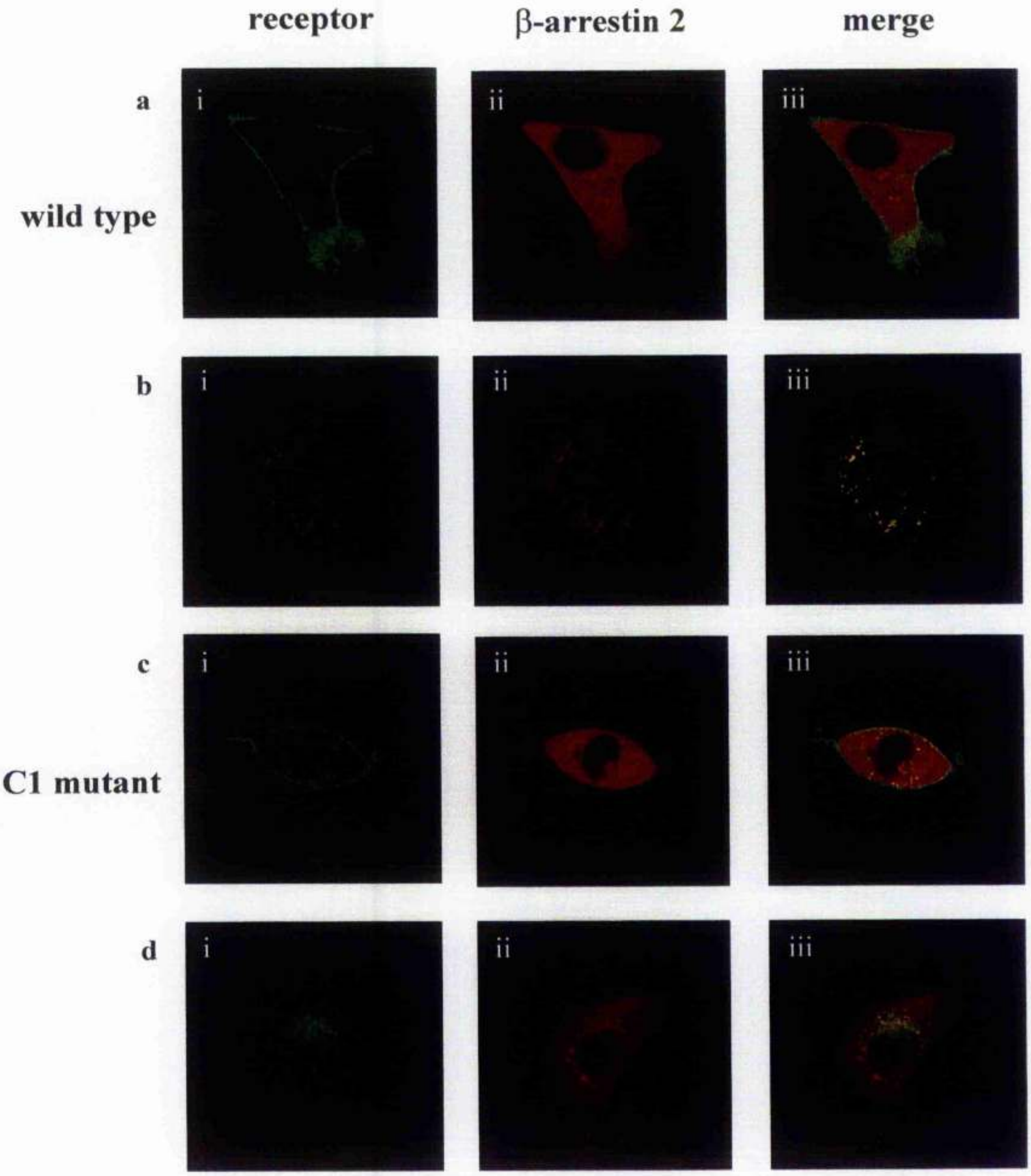

receptor
β-arrestin 2
merge
a
wild type
i
ii
iii
b
i
ii
iii
c
C1 mutant
i
ii
iii
d
i
ii
iii

Figure 3.20: Disruption of co-internalisation with β-arrestin 2 does not alter the pathways of agonist-induced internalisation of the orexin-1 receptor

VSV-G-orexin 1 receptor wild type (a, c, e, g, i) or cluster C1 mutant (b, d, f, h, j) were transfected into HEK293T cells along with β-arrestin 2-GFP. The receptors were then incubated for 30 min with anti-VSV-G-antibody labelled with CypHer-5 in the presence of vehicle (a, b), sucrose (0.4 M) (c, d), concanavalin A (0.25 mg/ml) (e, f), filipin (5 μg/ml) (g, h) or nystatin (50 μg/ml) (i, j). Subsequently orexin A was added (0.5 μM, 30 min) and the cells visualised. The distribution of the orexin 1 receptor (i), β-arrestin 2 (ii) and a composite of these images (iii) are shown. These results were confirmed in two further experiments.

Figure 3.20

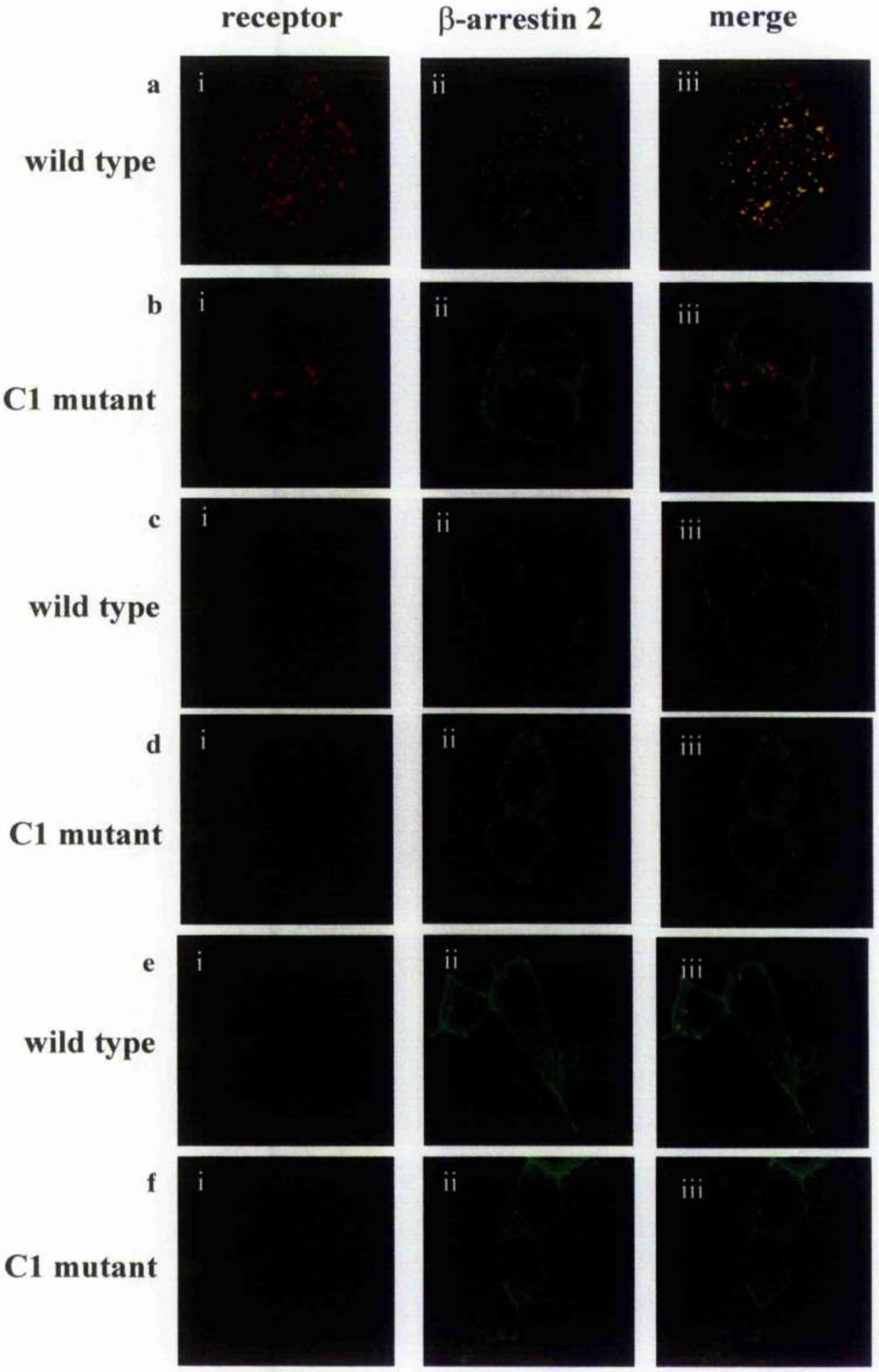

receptor
β-arrestin 2
merge
a
wild type
b
C1 mutant
c
wild type
d
C1 mutant
e
wild type
f
C1 mutant
i
ii
iii

Figure 3.20 continued

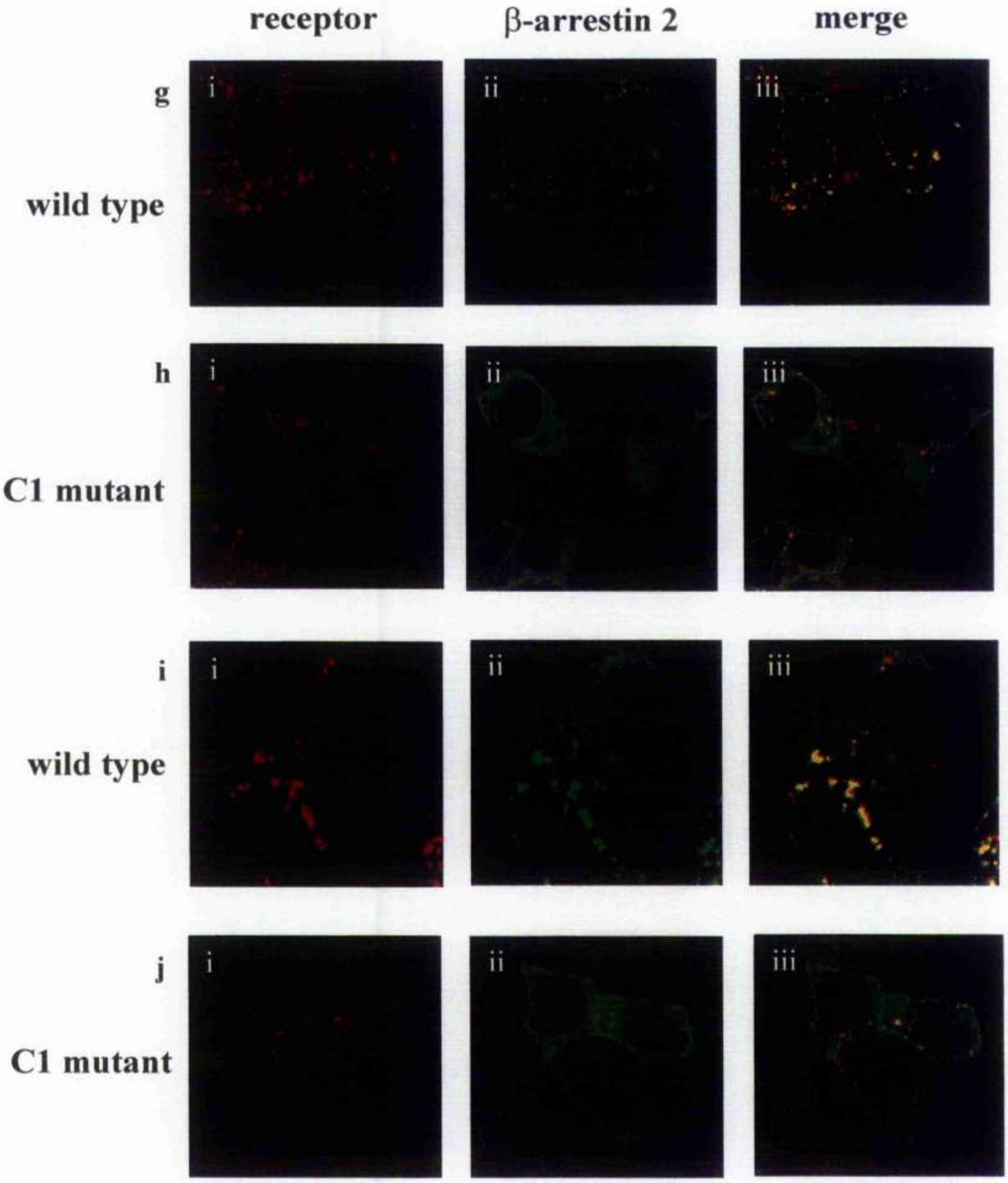

receptor
β-arrestin 2
merge
g
wild type
i
ii
iii
h
C1 mutant
i
ii
iii
i
wild type
i
ii
iii
j
C1 mutant
i
ii
iii

Figure 3.21: Internalisation of the orexin 1 receptor wild type and the cluster C1 mutant is dependent on dynamin

The VSV-G-wild type and the VSV-G-C1 mutant form of the receptor were transiently transfected into HEK293T cells either alone (a), or with dominant negative dynamin (b). 24 h post-transfection the cells were stimulated with 0.5 μM orexin A for 30 min. After immunostaining the receptor with the anti-VSV-G antibody, confocal images were taken to visualise the receptor distribution. Similar results were obtained in two further experiments.

Figure 3.21

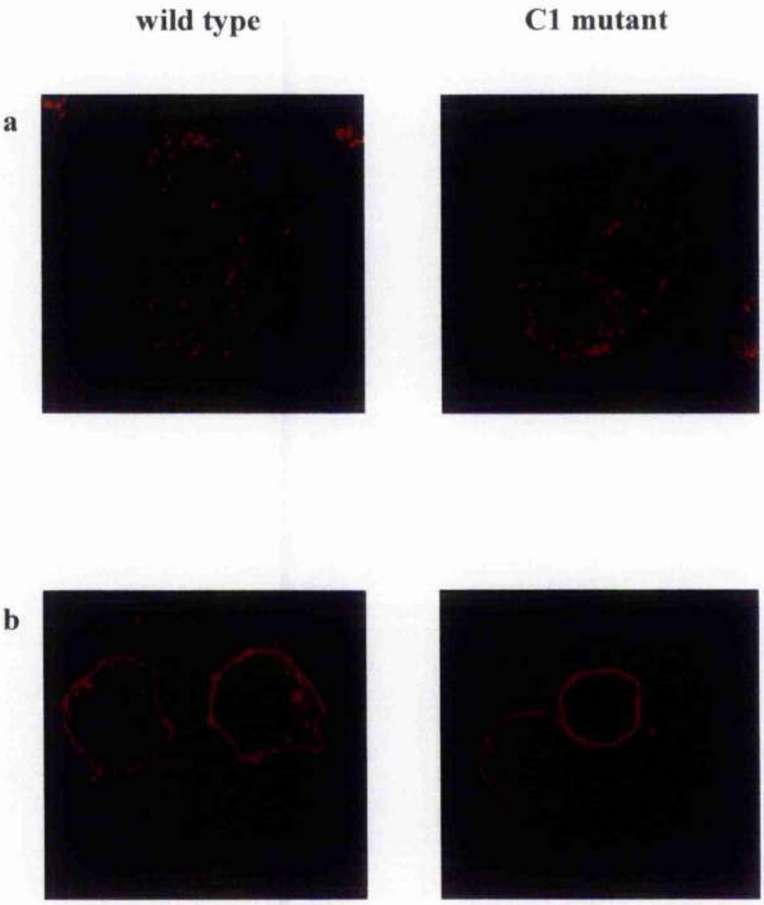
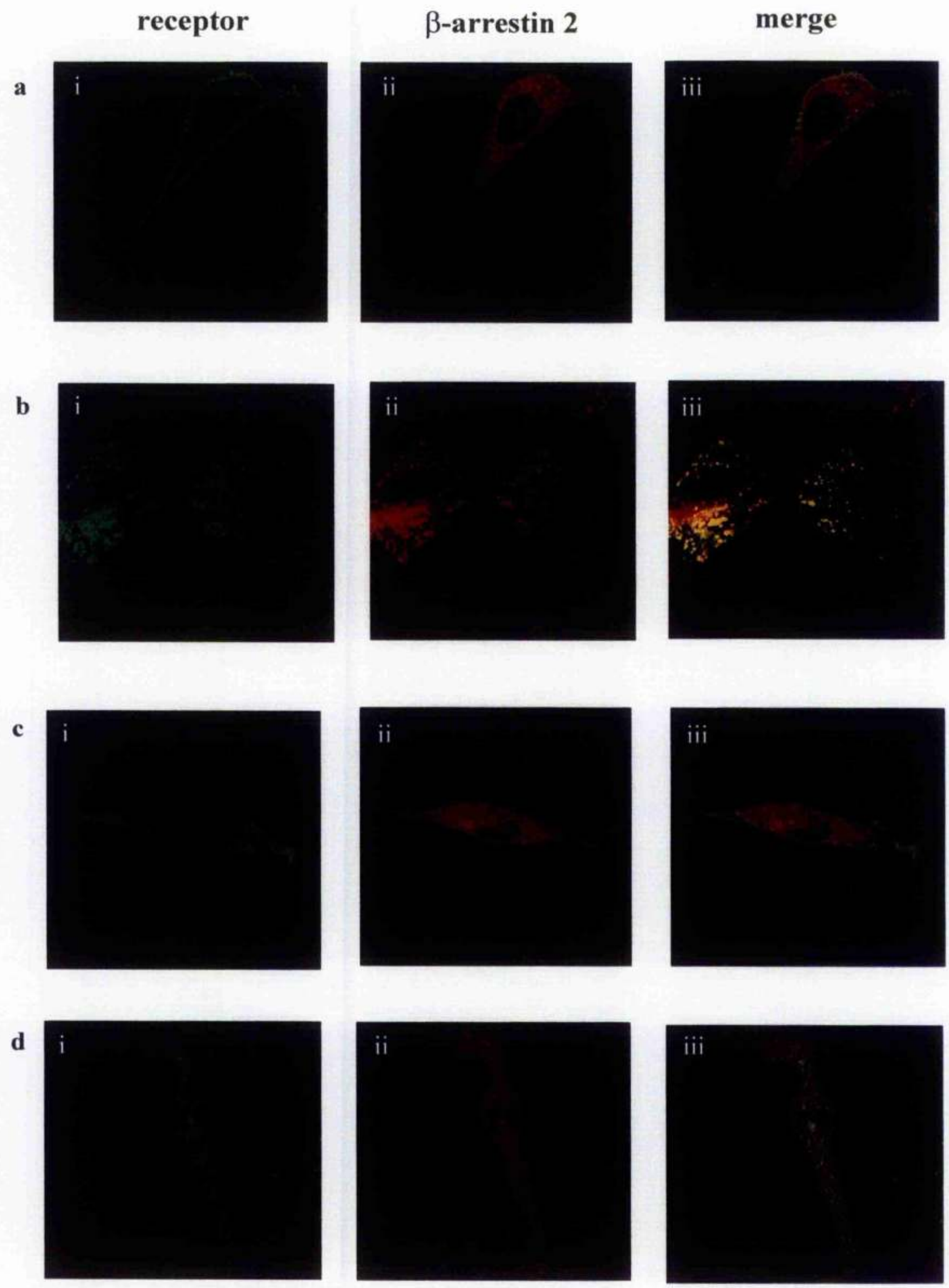

wild type
C1 mutant
a
b

Figure 3.22: c-Src is not required for internalisation of the orexin 1 receptor wild type and the cluster C1 mutant

The orexin 1 receptor-eYFP (i) wild type (a, b) and HA-C1 mutant-eYFP (c, d) were co-transfected with β-arrestin 2-RFP (ii) into SYF cells using the Amaxa nucleofection system. 24 h after transfection the cells were stimulated with vehicle (a, c) or with 0.5 μM orexin A for 30 min (b, d) and confocal images taken. A composite of the individual pictures is also displayed (iii). Similar confocal images were obtained in two further experiments.

Figure 3.22


receptor
β-arrestin 2
merge
a
i
ii
iii
b
i
ii
iii
c
i
ii
iii
d
i
ii
iii

3.12 Discussion

In the present chapter the role of the orexin 1 receptor C-terminus and β-arrestin 2 in the agonist-mediated internalisation process was evaluated. The orexin 1 receptor became phosphorylated and recruited β-arrestin 2 to the plasma membrane upon agonist activation and internalised in a β-arrestin- and clathrin-dependent fashion. The receptor- β-arrestin complex did not dissociate at the plasma membrane and was internalised into endosomes. The molecular determinants that stabilised the receptor- β-arrestin complex appeared to be a motif of three Thr and one Ser residue located at the extreme C-terminus of the orexin 1 receptor as substitution of these residues with Ala resulted in a form of the receptor that bound to β-arrestin 2 with lower affinity compared to the wild type receptor. This form did not co-internalise with β-arrestin 2 into acidic endosomes, but instead dissociated from β-arrestin 2 at or near the plasma membrane. However this cluster C1 mutant still internalised via clathrin-coated pits in a process dependent on β-arrestin.

It was shown that the orexin 1 receptor binds to and co-internalises with β-arrestin 1 and 2 in response to stimulation with orexin A (Evans *et al.,* 2001). Previous studies have indicated that multiple receptor determinants in the cytoplasmic loops and the carboxyl terminus are involved in arrestin binding (Raman *et al.,* 1999; Puig *et al.,* 1995; Nakamura *et al.,* 2000). In agreement with these findings truncation of the C-tail of the orexin 1 receptor diminished the ability of the receptor to tightly bind to and to co-internalise with β-arrestin 2 upon agonist stimulation, but did not prevent orexin A-mediated internalisation of the orexin 1 receptor. It is noteworthy that similar truncations in the C-terminus of the thyrotropin-releasing hormone receptor not only ablated β-arrestin binding, but moreover inhibited receptor internalisation (Groarke *et al.,* 2001; Hanyaloglu *et al.,* 2001). Furthermore, like the parathyroid hormone receptor (Vilardaga *et al.,* 2001), orexin 1 receptor sequestration does not appear to require G protein activation, since the eYFP tagged form of the orexin 1 receptor still endocytosed in response to agonist in $G_{q/11}$ knock out cells.

The orexin 1 receptor contains a significant number of hydroxy amino acids within its C-terminus as is the case for many members of the rhodopsin family of GPCRs. These residues when organised in clusters are reported to provide high affinity binding sites for β-arrestin upon agonist stimulation of class B GPCRs (Oakley *et al.*, 2001).

Although there are two such clusters of Ser/Thr residues present in the C-terminus of the orexin 1 receptor, one is at the extreme C-terminus (C1 aa 418-422) and one further upstream (C2 aa 393-396), only the cluster C1 at the extreme C-terminus seems to provide key interactions for β-arrestin binding, since substitution of the Ser/Thr residues with Ala resulted in a receptor unable to bind to β-arrestin 2 with high affinity and to co-internalise in a complex with β-arrestin 2 in response to agonist challenge. On the other hand concerted mutation of the Ser/Thr residues in cluster C2 did not alter interaction with β-arrestin 2 significantly. However, in contrast to findings indicating a significant degree of conservation in the relative position of these clusters within the C-terminus of the vasopressin V2, the neurotensin 1 and the oxytocin receptors, the cluster C1 of the orexin 1 receptor does not fulfil these criteria (Oakley *et al.*, 2001). Further mutational analysis indicates that no single residue within cluster C1 is the key docking site for β-arrestin, but that β-arrestin binding requires the presence of any three residues within this cluster. Similar results concerning the flexibility in the C-terminal sites that are involved in high-affinity β-arrestin binding were obtained for the CC chemokine receptor 5 and the formyl peptide receptor (Huttenrauch *et al.*, 2002; Bennett *et al.*, 2001). In contrast, interaction between the A_{2B} adenosine receptor and β-arrestin, is mediated by a single Ser residue in the receptor C-terminal tail (Matharu *et al.*, 2001). Since substitution of the residues in the cluster C1 with Ala only abolished high affinity binding of β-arrestin 2 to the receptor, other binding sites that are able to promote a transient interaction between the receptor and β-arrestin must exist. A possible site could be provided by the highly conserved DRY motif that is located at the bottom of transmembrane helix 3 of the rhodopsin-like receptors. This motif has been shown to play a key role in interactions between β-arrestins and the CC chemokine receptor 5 (Huttenrauch *et al.*, 2002) or the N-formyl peptide receptor (Bennett *et al.*, 2000). In case of the D_1 dopamine receptor it is the third intracellular loop that serves as the β-arrestin binding domain (Kim *et al.*, 2004).

The affinity of interactions between GPCRs and β-arrestins also dictates the rate of receptor dephosphorylation, recycling, and resensitisation (Oakley *et al.*, 1999). Thus high affinity binding of β-arrestin to the receptor results in a slow recycling rate. It might therefore be inferred, that, although there was no obvious difference in the time course of internalisation, the cluster C1 mutant recycles and resensitises more quickly than the wild type form. Whether this is true for the orexin 1 receptor still remains to be shown.

The current concept of GPCR internalisation involves agonist-stimulated receptor phosphorylation by both second messenger-dependent protein kinases and GRKs. This in turn promotes binding of arrestins thereby targetting the receptor for endocytosis (Pitcher *et al.,* 1998; Bunemann *et al.,* 1999). Agonist-induced phosphorylation has been demonstrated in numerous GPCRs including the β_2-adrenergic, the m1 and m2 muscarinic acetylcholine, the vasopressin V2 and the AT_1 and AT_2 angiotensin receptors (Haga *et al.,* 1996; Richardson *et al.* 1993; Innamorati *et al.,* 1997; Oppermann *et al.,* 1996). The orexin 1 receptor was rapidly phosphorylated in response to agonist treatment. PKA and PKC are implicated in the phosphorylation process since inhibition of either kinase leads to a small decrease in the phosphorylation signal. In addition, stimulation of either kinase directly, caused receptor phosphorylation, but not to the same extent as treatment of the receptor with the agonist. However, although PKA and PKC seem to be involved in the agonist-mediated phosphorylation of the orexin 1 receptor, other kinases are the major players in this event with GRKs being possible candidates. Other kinases that have been reported to phosphorylate GPCRs in response to agonist include casein kinase 1α, which phosphorylates the m3 muscarinic acetylcholine receptor (Budd *et al.,* 2000), and casein kinase II in case of the thyrotropin-releasing hormone receptor (Hanyaloglu *et al.,* 2001). However inhibition of casein kinase II activity had no effect on β-arrestin binding and internalisation of the orexin 1 receptor, ruling out an involvement of casein kinase II in these events. The ability of different kinases to phosphorylate the orexin 1 receptor could have implications for the signalling and internalisation of the receptor. For example, phosphorylation of the β_2-adrenergic receptor by PKA switches its predominant coupling from G_s to G_i (Zamah *et al.,* 2002). Similar results were reported for the mouse prostacyclin receptor (Lawler *et al.,* 2001). In case of the β_1-adrenergic receptor, PKA- and GRK-mediated phosphorylation can trigger agonist-induced desensitisation and internalisation, but the pathway is primarily determined by the kinase that phosphorylates the receptor i.e., PKA-mediated phosphorylation directs internalisation via a caveolae pathway, whereas GRK-mediated phosphorylation directs it through clathrin coated pits (Rapacciuolo *et al.,* 2003).

The orexin 1 receptor cluster C1 and cluster C2 mutants were also phoshorylated in response to agonist to almost the same extent as the wild type form of the receptor. These findings are in agreement with recent reports concerning the human prostaglandin E_2 receptor, subtype EP4. In this receptor the principal phosphorylation site is distinct from the cluster of Ser/Thr residues essential for agonist-induced recruitment of β-arrestin 1 (Neuschafer-Rube *et al.,* 2004). However, other studies do exist where these clusters of Ser/Thr residues clearly served as primary sites for agonist-dependent receptor phosphorylation. Mutation of these residues in the neurotensin-1 receptor resulted in a mutant form of the receptor that only became phosphorylated to 5 % in response to agonist compared to the wild type receptor (Oakley *et al.*, 2001). Another example is the complement 5a anaphylatoxin receptor. A phosphorylation-deficient mutant of this receptor was still able to bind loosely to β-arrestin 2, but phosphorylation of two Ser residues in the C-terminus was neccessary to promote firm association with β-arrestins (Braun *et al.*, 2003). Similar observations were made for the parathyroid hormone receptor (Vilardaga *et al.*, 2002).

Although receptor phosphorylation and subsequent β-arrestin binding are clearly important for receptor desensitisation and internalisation there is now growing evidence that not all GPCRs utilise this mechanism. Recent reports indicate that not all effects of kinases on GPCR desensitisation involve phosphorylation of the receptor. For example attenuation of the metabotropic glutamate receptor 1 by GRK is phosphorylation-independent (Dhami *et al.*, 2002). It has also been shown, that the parathyroid hormone receptor internalises in a phosphorylation independent manner (Malecz *et al.*, 1998) and in case of the luteneising hormone/choriogonadotropin receptor an Asp residue in the third intracellular loop confers high affinity binding to β-arrestin in the absence of phosphorylation (Mukherjee *et al.*, 2002). Also, the N-formyl peptide receptor still internalised in the absence of β-arrestin in β-arrestin knock out cells (Vines *et al.*, 2003). The m1, m3 and m4 muscarinic acetylcholine receptors are further examples of GPCRs that sequester independently of β-arrestins (Lee *et al.*, 1998).

As mentioned above, ablation of the C-terminus or mutation of the cluster C1 did not prevent agonist-induced internalisation of the orexin 1 receptor. Despite the reduced affinity for β-arrestin 2 of the cluster C1 mutant compared to the wild type receptor, studies using a MEF cell line, which lacks β-arrestin 1 and 2, clearly demonstrated that internalisation of both forms of the receptor was dependent on β-arrestin. Furthermore, the internalisation pathway of the orexin 1 receptor in HEK293T cells seemed not to be affected by the reduced affinity for β-arrestin 2. Treatment with hyperosmotic sucrose to disrupt the clathrin coat at the plasma membrane effectively inhibited endocytosis of the orexin 1 receptor. In contrast, inhibitors of caveolar endocytosis, such as filipin or nystatin, had no major effect on the endocytosis of this receptor and comparable results were obtained for the cluster C1 mutant. It is noteworthy that alternate pathways for internalisation in HEK293 cells exist. Endocytosis of the 5-hydroxytryptmaine 2A receptor is dependent on dynamin, but independent of β-arrestin (Gray *et al.*, 2001; Bhatnagar *et al.*, 2001). Also in the presence of dominant negative inhibitors of clathrin-mediated endocytosis, the angiotensin II type 1A receptor internalises via a pathway that does not depend on β-arrestin and dynamin (Zhang *et al.*, 1996). Furthermore there is strong evidence that β-arrestins target the wild type orexin 1 receptor and the cluster C1 mutant to clathrin-coated pits in a dynamin-dependent manner as co-expression of each form of the receptor with a dominant negative mutant of dynamin resulted in complete inhibition of internalisation of the receptor. However both forms of the receptor still internalised in Src-deficient MEF (SYF) cells. These results are in contrast to previous findings concerning the internalisation of the β_2-adrenergic receptor. Agonist challenge led to rapid recruitment of activated c-Src to the receptor, with β-arrestin 1 serving as adaptor protein. As a consequence, dynamin became phosphorylated and thereby activated in a c-Src-dependent manner and co-expression of a c-Src kinase dead mutant did indeed block dynamin phosphorylation and receptor endocytosis (Miller *et al.*, 2000; Luttrell *et al.*, 1999; Ahn *et al.*, 1999). The different results described above might be due to the fact that the experiments were carried out in different cell lines. Consequently the experiments should be repeated in HEK293 cells using for example, siRNAs to knock out the different proteins. Interestingly, internalisation of the β_2-adrenergic receptor was blocked in SYF cells (Huang *et al.*, 2004).

In conclusion, it could be demonstrated that the orexin 1 receptor internalised in clathrin-coated vesicles in a β-arrestin- and dynamin-dependent, but G protein- and c-Src-independent manner. Also high affinity binding between the receptor and β-arrestin 2 is conferred by a single cluster of Ser/Thr residues in the extreme end of the C-terminus and substituting the residues within this cluster with Ala transforms the receptor from a class B to a class A GPCR.

Chapter 4

Regulation of Orexin 1 Receptor Signalling by β-Arrestin 2

4.1 Introduction

The principal action of GPCRs is to transmit information about the extracellular environment to the interior of the cell. This is achieved through binding of a ligand to the receptor, which induces or stabilises an active conformation of the receptor allowing activation of an associated G protein. As a result of this interaction a receptor can influence a variety of effector systems. Upon activation the G protein α subunit dissociates from the βγ subunit and each subunit may regulate distinct signalling pathways. The G protein α subunits can be divided into four families based upon sequence similarity: αs, which activates adenylyl cyclase and therefore protein kinase A (PKA); αi, which inhibits adenylyl cyclase; αq, which activates phospholipase Cβ (PLCβ) and thus protein kinase C (PKC); and α12, which regulates the Na^+/H^+ antiporter and the Rho-dependent formation of actin stress fibres. The βγ subunits have been shown to be positive regulators of K^+-channels, certain adenylyl cyclase isoforms, PLCβ, phospholipase A2 (PLA2), phosphoinositide 3-kinase, and β-adrenergic receptor kinase (Clapham and Neer, 1993).

A wide variety of GPCRs are also able to activate MAPKs such as ERK1 and 2 and in some cases thereby effect a mitogenic response (van Biesen *et al.*, 1996a; Gutkind, 1998). The mechanism by which GPCRs mediate activation of ERK1 and 2 have been extensively studied and G_i-, G_q-, and G_o-mediated pathways have been described (van Biesen *et al.*, 1996a). In case of the G_i-mediated signals, MAPK activation is generally carried out by the βγ subunits, which results in the activation of a c-Src family tyrosine kinase followed by the subsequent tyrosine phosphorylation of the same downstream adaptor proteins used by receptor tyrosine kinases (Scheme 1) (van Biesen *et al.*, 1996a; Gutkind, 1998).

GPCR → $G_{\beta\gamma}$ → Tyr kinase → Shc → Grb2-mSos → Ras → Raf → MEK → MAPK

Scheme 1

The activation of MAPK by the β_2-adrenergic receptor, which couples to G_s, involves the PKA-dependent switch of β_2-adrenergic receptor coupling to G_i rather than to G_s (Daaka *et al.*, 1997). There are also reports implicating the G protein α subunit in the stimulation of the MAPK pathway. The m1 muscarinic acetylcholine receptor and the platelet-activating receptor were shown to be able to couple to G_o and activate MAPK in a pathway depending on $G_o\alpha$, but not on Ras activation (Van Biesen *et al.*, 1996b). ERK1/2 activation mediated by the calcium-sensing receptor requires $G_{i2}\alpha$-coupling (Holstein *et al.*, 2004). Moreover α subunits of $G_{12/13}$ promote stress fibre formation and cellular transformation through another family of small GTP-binding proteins, the Rho-family (Buhl *et al.*, 1995; Fromm *et al.*, 1997). Recent evidence suggests that GPCRs linked to the G_q family of G proteins can also activate signalling routes through the α subunit that are dependent on the functional activity of Rho (Fromm *et al.*, 1997; Mao *et al.*, 1998).

However, GPCRs can activate MAPK through an additional mechanism that involves β-arrestins and depends on endocytosis (Daaka *et al.*, 1998; Barlic *et al.*, 2000). Activation of the β_2-adrenergic receptor results in rapid translocation of β-arrestin 1 coupled to c-Src to the activated receptor at the plasma membrane (Luttrell *et al.*, 1999). The same could be shown for the $G_{q/11}$-coupled neurokinin-1 receptor (DeFea *et al.*, 2000b). In addition to interacting with c-Src family tyrosine kinases, β-arrestins were recently shown to directly interact with both MAPK and Raf-1 in response to the activation of the protease-activated receptor 2 (DeFea *et al.*, 2000a).

β-arrestins not only play a role as adaptor proteins in the ERK pathway, but also in the other groups of MAPKs: JNKs and p38 protein kinases. McDonald *et al.* (2000) could show that β-arrestin 2 could be co-immunoprecipitated with JNK 3 and that stimulation of the angiotensin II type 1A receptor activated JNK3 and triggered the co-localisation of β-arrestin 2 and active JNK3 to intracellular vesicles. Moreover β-arrestin 2 seems to be critically involved in CXCR4-mediated chemotaxis by increasing activation of the p38 MAPK pathway via ASK1 (Sun *et al.*, 2002).

In the previous chapter it was shown, that internalisation of the orexin 1 receptor was dependent on β-arrestins, dynamin and clathrin and that changing the receptor affinity for β-arrestin had no obvious effect on internalisation. The aim of this chapter was to examine the relative contributions of G proteins and β-arrestins to the activation of the different MAPK pathways. Therefore i2 loop mutants deficient in G protein signalling were generated and firstly the effects of these mutations on β-arrestin binding and internalisation examined by confocal microscopy. Afterwards to assess whether and by what mechanism the orexin 1 receptor stimulates the ERK1/2 MAPK pathway the wild type form of the receptor as well as the i2 loop and the cluster mutants were transiently transfected into HEK293T cells and the activation of ERK1/2 in response to orexin A determined by examining the levels of kinase phosphorylation. In addition, the wild type receptor was expressed in $G_{q/11}$ and also in β-arrestin 1/2 deficient MEF cells to investigate the relative importance of each pathway in orexin A mediated-ERK1/2 activation. To study the involvement of receptor endocytosis in MAPK activation, HEK293T cells transiently expressing wild type orexin 1 receptor were pre-treated with sucrose and concanavalin A before addition of orexin A. Also CHO cells stably expressing the wild type receptor were co-transfected with dominant negative dynamin. To investigate whether Src plays an important role in ERK1/2 activation, the wild type receptor was expressed in Src-deficient cells and the effect on ERK1/2 activation assessed. Also the rate of cell proliferation in response to stimulation of the wild type and the cluster C1 mutant receptor was determined. Finally the implications of receptor stimulation, either of the wild type or the cluster and i2 loop mutants, on JNK and p38 MAPK activation was investigated, by examining the levels of kinase activity or phosphorylation, respectively.

4.2 Internalisation of the i2 loop mutants

Stimulation of GPCRs by ligand binding leads to activation of downstream signalling cascades. To determine the involvement of G proteins and β-arrestins in transmitting the signal from the activated receptor to the downstream targets, G protein activation deficient mutants were generated. It was reported that mutation of hydrophobic residues in the i2 loop of the α_{1b}-adrenergic receptor and the histamine H1 receptor abolishes agonist-mediated signal transduction by eliminating receptor-mediated activation of G proteins (Greasley *et al.*, 2001; Carrillo *et al.*, 2003). Based on these studies the following constructs of the orexin 1 receptor were generated as described in section 2.4.4: orexin 1 receptor-I148E, -L152D and –I148E-L152D. To detect any effect these mutations might have on receptor internalisation, the different forms of the receptor N-terminally tagged with VSV-G were co-expressed with β-arrestin 2-GFP in HEK293T cells and sequestration of the immuno-labelled receptor visualised by confocal microscopy. In the absence of agonist, all three i2 loop mutants were expressed at the plasma membrane whereas β-arrestin 2 exhibited a cytosolic distribution (Figure 4.1). The L152D orexin 1 receptor co-internalised with β-arrestin 2 in response to agonist-stimulation (0.5 μM orexin A for 30 min) since the intracellular vesicles were stained yellow. However neither the I148E nor the I148E-L152D form of the orexin 1 receptor translocated from the plasma membrane into the cell after addition of 0.5 μM orexin A for 30 min. Moreover, there was little indication of interaction with β-arrestin 2 as no movement of β-arrestin 2 was detectable

(Figure 4.1). This suggests that these i2 loop mutants did not internalise because they failed to bind β-arrestin since receptor internalisation was shown to be G protein independent but β-arrestin-dependent (sections 3.3 and 3.9). In addition, calcium signalling studies performed by Laura Ormiston showed that the stimulation of the L152D mutant with 0.5 μM orexin A, as for the wild type receptor, caused elevation of intracellular $[Ca^{2+}]$ levels when co-expressed with $G\alpha_{11}$ in the G_q/G_{11} knock out, EF88 cells. On the other hand the I148E and the I148E-L152D mutant were not able to elevate intracellular $[Ca^{2+}]$ levels (Figure 4.1 a). Hence I148E and I148E-L152D, but not L152D orexin 1 receptors seem to be impaired in G protein activation.

4.3 Activation of the ERK MAPK cascade by the different forms of the orexin 1 receptor

To assess the importance of G protein-coupling and β-arrestin binding in activation of ERK1/2 by the orexin 1 receptor, the wild type receptor, the cluster C1 and the i2 loop mutant forms, N-terminally tagged with VSV-G, were transiently expressed in HEK293T cells. The next day the cells were stimulated with 0.5 μM orexin A for 0-30 min. After lysis of the cells with RIPA buffer, the amount of ERK1/2 phosphorylation was assessed by Western-blotting using phospho-specifc ERK1/2 antibodies. Although the wild type and the cluster C1 mutant were both able to stimulate the production of phosphorylated forms of ERK1/2 in response to orexin A, clear differences in the longevity of the signal were noted. Phosphorylation of ERK1/2 was maintained for a significantly longer period in cells expressing the wild type orexin 1 receptor compared to those expressing the cluster C1 (Figure 4.2). On the other hand the I148E mutant was unable to activate ERK1/2 during the duration of the experiment (Figure 4.2). The cluster C2 mutant and the L152D mutant displayed a similar pattern of ERK1/2 activation compared to the wild type receptor, whereas the activation pattern stimulated by the I148E-L152D resembled the results obtained for the I148E mutant (data not shown).

To determine that the results described above were not due to lack of receptor expression, different amounts of the various receptor constructs tagged with VSV-G at the N-terminus were transiently transfected into HEK293T. 24 h after transfection, the cells were stimulated for 5 min with 0.5 μM orexin A before lysis with RIPA buffer. In the case of the wild type receptor, at low receptor expression levels the amount of detectable receptor was correlated to an increase in orexin A-mediated ERK1/2 activity. However, at a certain receptor concentration, maximal ERK1/2 activation was achieved and irrespective of the expressed amount of receptor, no further gain in ERK1/2 activity could be detected (Figure 4.3). One of the functions of signalling cascades is to amplify the signal from the receptor. Therefore orexin A-mediated phosphorylation of ERK1/2 was detectable even when the receptor was not (Figure 4.3). Similar results were obtained for the cluster C1, the cluster C2 and the L152D mutant of the receptor. The higher the amount of expressed receptor the higher the increase in ERK1/2 activity (Figure 4.3 and data not shown). In contrast, stimulation of the I148E and the I148E-L152D mutant only caused a modest increase of ERK1/2 activity above the basal level no matter the amount of receptor expressed (Figure 4.2 and data not shown).

Since stimulation of the i2 loop mutants I148E-L152D and I148E failed to elicit any detectable movement of β-arrestin 2 to the plasma membrane (Figure 4.1), the inability of the i2 loop mutants to activate ERK1/2 could also be due to the fact that these mutants did not activate β-arrestins. Therefore to corroborate that activation of ERK1/2 by the orexin 1 receptor depends on G protein and not on β-arrestin, MEF wild type cells, MEF β-arrestin 1 and 2 knock out cells and MEF $G_{q/11}$ knock out (EF 88) cells were transiently transfected with wild type orexin 1 receptor using the AMAXA nucleofection system. Following agonist stimulation (0.5 µM orexin A, 5 min) the cells were lysed and the protein extracted. The amount of activated ERK1/2 was determined by immunoblotting using a phospho-specific ERK1/2 antibody. As depicted in Figure 4.4 A, MEF wild type cells expressing the orexin 1 receptor displayed a significant increase in phosphorylated ERK1/2 in response to orexin A. Similar levels of ERK1/2 phosphorylation were observed in β-arrestin 1 and 2 knock out cells. In contrast stimulation of the orexin 1 receptor in a $G_{q/11}$ null background (EF88 cells) did not cause any detectable phosphorylation of ERK1/2 above basal. Therefore EF88 cells were incubated with 1 µM PMA to demonstrate that ERK1/2 can be further activated in these cells (Figure 4.4 B). It is noteworthy that the basal level of ERK1/2 phosphorylation was elevated in these cells compared to the wild type cells. The reasons behind this observation are not clear. Taken together these findings indicate that G protein but not β-arrestin coupling of the receptor seems to be essential for ERK1/2 activation by the agonist-stimulated orexin 1 receptor. However the time course of activation looks as if it is influenced by the ability of the receptor to bind to β-arrestins with high affinity.

4.4 Cell proliferation in response to activation of the wild type orexin 1 receptor and the cluster C1 mutant

β-arrestins are reported to facilitate GPCR-stimulated ERK1/2 activation by functioning as scaffolds. As a functional consequence of the cytosolic retention of phospho-ERK1/2, ERK1/2-dependent cell proliferation is hindered since ERK1/2 re-localisation to the nucleus appears to be an important step for cell cycle re-entry (Tohgo *et al.,* 2002; Tohgo *et al.,* 2003; Brunet *et al.,* 1999).

To determine whether the stability of the receptor-β-arrestin interaction and the sustained phosphorylation of ERK1/2 mediated by the wild type orexin 1 receptor had any consequence on orexin A-induced cell proliferation, HEK293T cells were transiently transfected with VSV-G-orexin 1 receptor wild type or cluster C1 mutant cDNAs. The cells were plated in 96 well plates 24 h post-transfection and incubated for another 24 h in the absence of foetal bovine serum and orexin A, the presence of 10 % foetal bovine serum or the presence of 10 % foetal bovine serum and 0.5 μM orexin A. The preliminary results shown in Figure 4.5 indicate that the wild type form of the receptor failed to elicit any detectable proliferative response to orexin A, whereas the cluster C1 mutant displayed a small, but significant increase in cell proliferation above that produced by FBS. It is therefore possible that activation of the wild type orexin 1 receptor results in retention of activated ERK1/2 in the cytosol whereas a small fraction of phospho-ERK1/2 seems to be able to translocate to the nucleus in response to activation of the cluster C1 mutant. However this is only a suggestion at this point and still needs to be proven experimentally.

4.5 Effects of inhibitors of endocytosis on orexin 1 receptor-mediated ERK1/2 phosphorylation

The fact that many GPCRs undergo ligand-induced endocytosis via a clathrin/dynamin-mediated process has led to the assumption that MAPK activation depends on receptor endocytosis (Lefkowitz 1998). However it has emerged in the meantime that the requirement of receptor endocytosis for MAPK activation is not universal since several receptors have been shown to activate MAPK independently of internalisation (DeGraff *et al.,* 1999; Le *et al.,* 1999; Whistler *et al.,* 1999). To examine whether receptor endocytosis was required for activation of ERK1/2 by the orexin 1 receptor the effects of three different inhibitors of orexin 1 receptor sequestration, hypertonic sucrose, concanavalin A and dominant negative dynamin, were determined. HEK293T cells were transiently transfected with the orexin 1 receptor and the cells pre-treated with 0.45 M sucrose for 30 min before addition of orexin A (0.5 μM) for 5 min. Figure 4.6 shows that hypertonic sucrose had no effect on the levels of ERK1/2 activation by the orexin 1 receptor. In contrast incubation of the cells with 0.25 mg/ml concanavalin A for 30 min prior to agonist challenge (0.5 μM orexin A, 5 min) inhibited orexin 1 receptor-mediated ERK1/2 activation by 76 % ± 8 (Figure 4.6). Co-expression of N272 dynamin in CHO cells stably expressing the HA-orexin 1 receptor also blunted the ERK1/2 response to orexin A (0.5 μM for 5 min) (Figure 4.7). Similar to concanavalin A, dominant negative dynamin reduced ERK1/2 phosphorylation by 71 % ± 5. Taken together, these data suggest that an intact endocytic pathway is required for receptor-mediated activation of ERK1/2.

4.6 Involvement of Src in ERK MAPK activation by the orexin 1 receptor

The mechanism by which GPCRs activate the MAPK pathway seems to depend on the receptor and cell type. Some of these activation pathways involve tyrosine kinases (Dikic and Blaukat, 1999). To investigate the role of Src in signalling of the orexin 1 receptor, the VSV-G tagged orexin 1 receptor was transiently transfected into SYF cells using the Amaxa nucleofection system. Treatment of the cells with 0.5 μM orexin A for 5 min led to increased activity of ERK1/2 (Figure 4.8). Therefore the orexin 1 receptor seems to activate ERK1/2 by a mechanism that does not involve Src family tyrosine kinases.

4.7 Activation of the JNK MAPK pathway

Another group of MAPKs are the JNKs, which are also referred to as SAPKs since they can be activated by environmental stress such as UV light and hyperosmotic shock and also by cell surface receptors inlcuding tyrosine kinase receptors, cytokine receptors and GPCRs. GPCRs can activate JNK not only through G proteins but also through β-arrestin 2 (Marinissen and Gutkind, 2001)

To determine whether stimulation of the orexin 1 receptor leads to activation of JNKs and if so by what pathway, the cDNAs encoding the wild type form, the cluster C1 and the three different i2 loop mutants were transiently transfected into HEK293T cells. Following addition of 0.5 μM orexin A for 0-60 min and 0.3 M sorbitol for 30 min as positive control, the cells were lysed and the activity of JNK measured in an *in vitro* kinase assay using a

GST fusion protein containing c-Jun as substrate which is phosphorylated by JNK on Ser63 and Ser73. Figure 4.8 shows that stimulation of the wild type orexin 1 receptor with agonist for 30 min caused maximal activation of JNK, which was maintained for another 15 min after which the level of kinase activity started to decline. None of the mutants tested displayed any significant activation of JNK above basal during the time course of the experiment (Figure 4.9 and data not shown).

To determine whether the results described above are caused by problems in achieving high enough receptor expression, HEK293T cells were transiently transfected with different amounts of the various cDNAs and the cells then incubated with 0.5 μM orexin A for 30 min. Figure 4.10 (and data not shown) illustrates that stimulation of any form of the receptor resulted in activation of JNK. Moreover the amount of active JNK appeared to be dependent on the receptor expression levels. Unfortunately, the lysis buffer used was not strong enough to extract the receptor and therefore it was impossible to determine the amount of receptor present in the individual samples. Therefore the results depicted above are inconclusive and might be explained by the fact that receptor expression levels were indeed too low.

4.8 Activation of the p38 MAPK pathway

p38 protein kinases are the third group of MAPKs and like the JNKs they too belong to the class of SAPKs. Although they are also activated by environmental stress they are regulated by different MAPKKKs and p38 can be turned on independently of JNK (Derijard *et al.*, 1995; Raingeaud *et al.*, 1996). Just as for the ERK MAPK pathway GPCRs can induce p38 kinase activity. Depending on the GPCR this activation can be mediated by the βγ-subunit complex or the α-subunit of heterotrimeric G proteins (Yamauchi *et al.*, 1997). As for the ERK1/2 and JNK pathway there are reports implicating β-arrestin 2 in playing a crucial role in the activation of this kinase pathway by GPCRs (Sun *et al.*, 2002). The same cell lysates used to measure ERK1/2 activation (section 4.3) were probed with a phospho-specific p38 antibody to examine p38 stimulation. No form of the receptor tested showed any detectable activation of p38 under the same conditions where ERK1/2 were clearly activated (Figure 4.11 A and data not shown). To rule out any problems with the experimental set up the cells were stimulated with 250 ng/ml anisomycin for 5 min as positive control. Phospho p38 could be detected under these conditions proving the experimental procedure to be working (Figure 4.11 B).

Figure 4.1: Internalisation of the i2 loop mutants with β-arrestin 2

HEK293T cells were transiently co-transfected with plasmid DNA encoding VSV-G-orexin 1 receptor I1488E, -L152D, or –I148E-L152D and β-arrestin 2-GFP. 24 hr later the cells were stimulated with vehicle (panel 1, 3 and 5) or 0.5 μM orexin A (panel 2, 4 and 6) for 30 min in the presence of anti-VSV-G antibody and visualised under the confocal microscope following permeabilisation and addition of the Alexa 594 secondary antibody. The distribution of the orexin 1 receptor (i), β-arrestin 2 (ii) and a composite of these images (iii) are shown. Each image depicts a representative confocal microscopic image from one of three separate experiments.

Figure 4.1 a: Elevation of intracellular [Ca^{2+}] levels by the i2 loop mutants

N-terminally VSV-tagged forms of the i2 loop mutants of the orexin-1 receptor were transfected along with $G\alpha_{11}$ into $G\alpha_q/G\alpha_{11}$ knock-out EF88 cells. The effect of 0.5 μM orexin A on intracellular [Ca^{2+}] was then recorded in individual cells. Data are pooled from 6 cells expressing each construct.

Figure 4.1

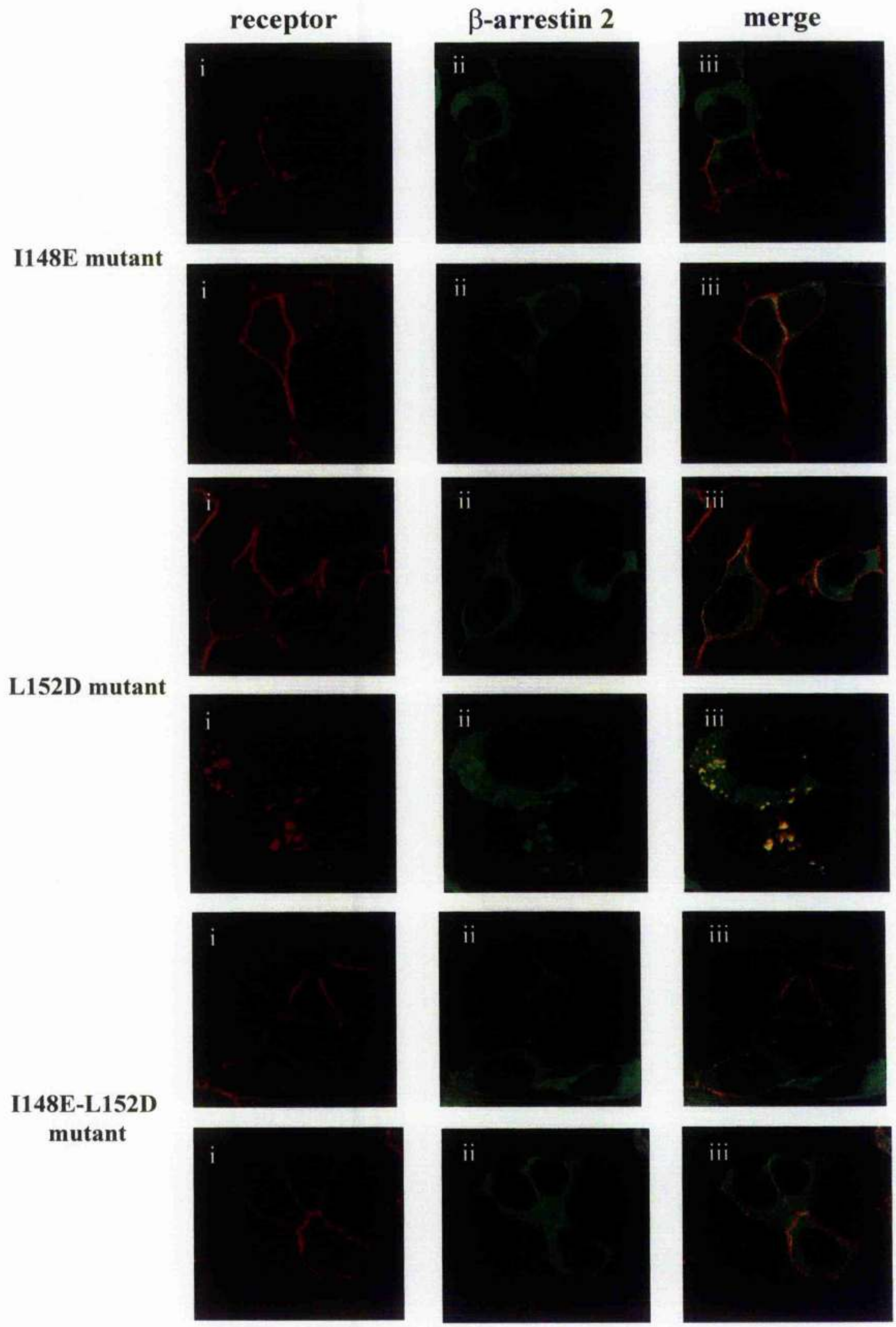

receptor
β-arrestin 2
merge
i
ii
iii
I148E mutant
i
ii
iii
i
ii
iii
L152D mutant
i
ii
iii
i
ii
iii
I148E-L152D mutant
i
ii
iii

Figure 4.1 a

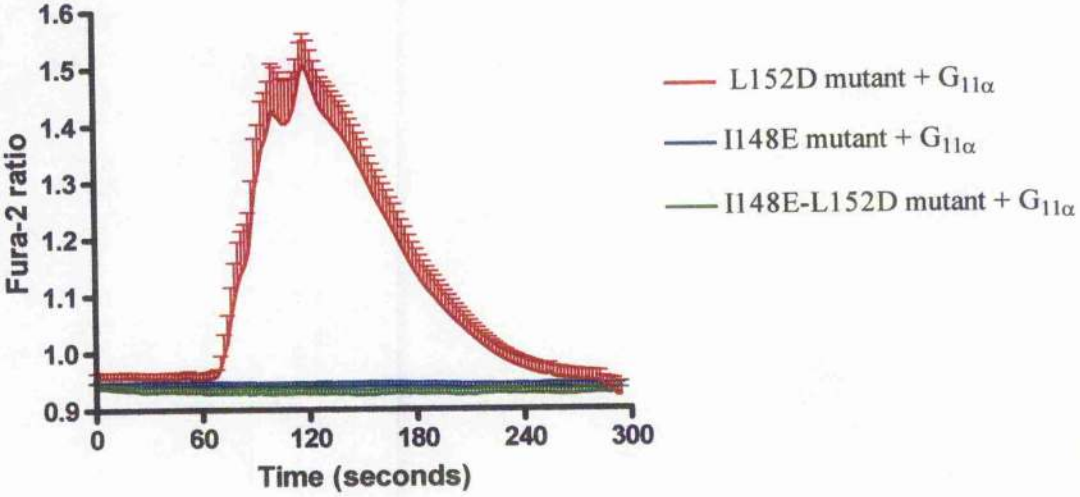

1.6
1.5
1.4
1.3
1.2
1.1
1.0
0.9
Fura-2 ratio
0
60
120
180
240
300
Time (seconds)
L152D mutant + $G_{11\alpha}$
I148E mutant + $G_{11\alpha}$
I148E-L152D mutant + $G_{11\alpha}$

Figure 4.2: Time course of orexin A-induced ERK1/2 activity

A. HEK293T cells transiently expressing the wild type orexin 1 receptor, the C1 mutant, or the I148E mutant N-terminally tagged with VSV-G for 24 h were serum starved for 2 hr and then stimulated with 0.5 µM orexin A for the indicated times. ERK1/2 activity was then detected using phospho-specific anti-ERK1/2 antibodies (P-ERK1/2). Expression levels of ERK1/2 were monitored using antibodies directed against total population of ERK1/2 (ERK1/2). This experiment was repeated twice with a similar result each time.

B. ERK1/2 phosphorylation is expressed as % of the maximum P-ERK1/2 signal in each experiment and represents the mean ± S.E.M. from four separate experiments, $p < 0.05$.

Figure 4.2

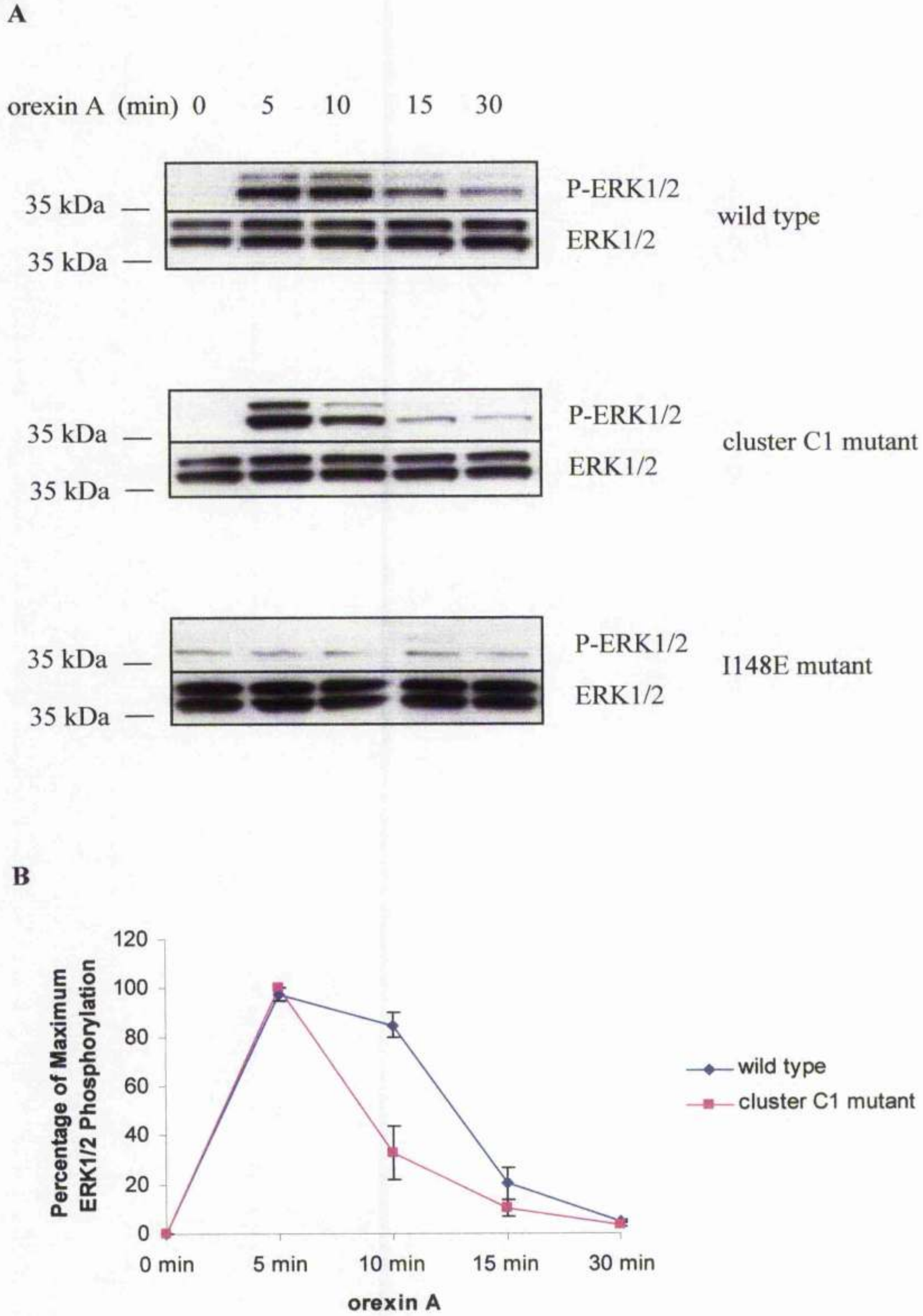

A
orexin A (min) 0 5 10 15 30
35 kDa
35 kDa
P-ERK1/2
ERK1/2
wild type
35 kDa
35 kDa
P-ERK1/2
ERK1/2
cluster C1 mutant
35 kDa
35 kDa
P-ERK1/2
ERK1/2
I148E mutant
B
Percentage of Maximum ERK1/2 Phosphorylation
120
100
80
60
40
20
0
0 min
5 min
10 min
15 min
30 min
orexin A
wild type
cluster C1 mutant

Figure 4.3: Activation of ERK1/2 by the different receptor mutants

HEK293T cells were transfected with the indicated amounts of plasmids for VSV-G-orexin 1 receptor wild type, -C1 cluster mutant, -I148E. The cells were serum starved for 2 hr and stimulated with 0.5 μM orexin A for 5 min. Activation of ERK1/2 was evaluated by immunoblot analyses using anti-phospho specific ERK1/2 antibodies (P-ERK1/2). To show equal loading, membranes were stripped and reprobed with antibody against total ERK1/2 (ERK1/2). Similar results were obtained in two further experiments.

Figure 4.3

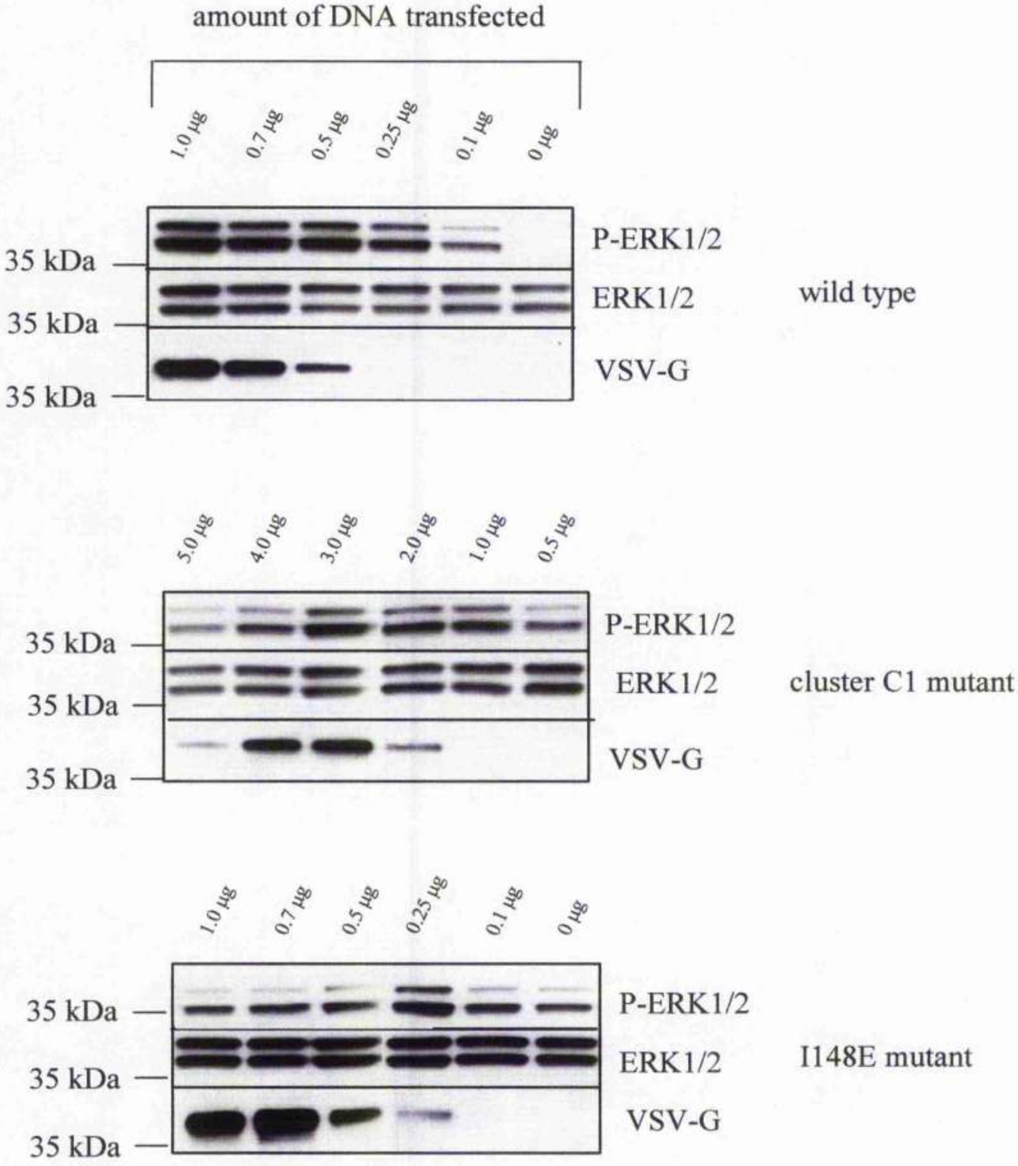
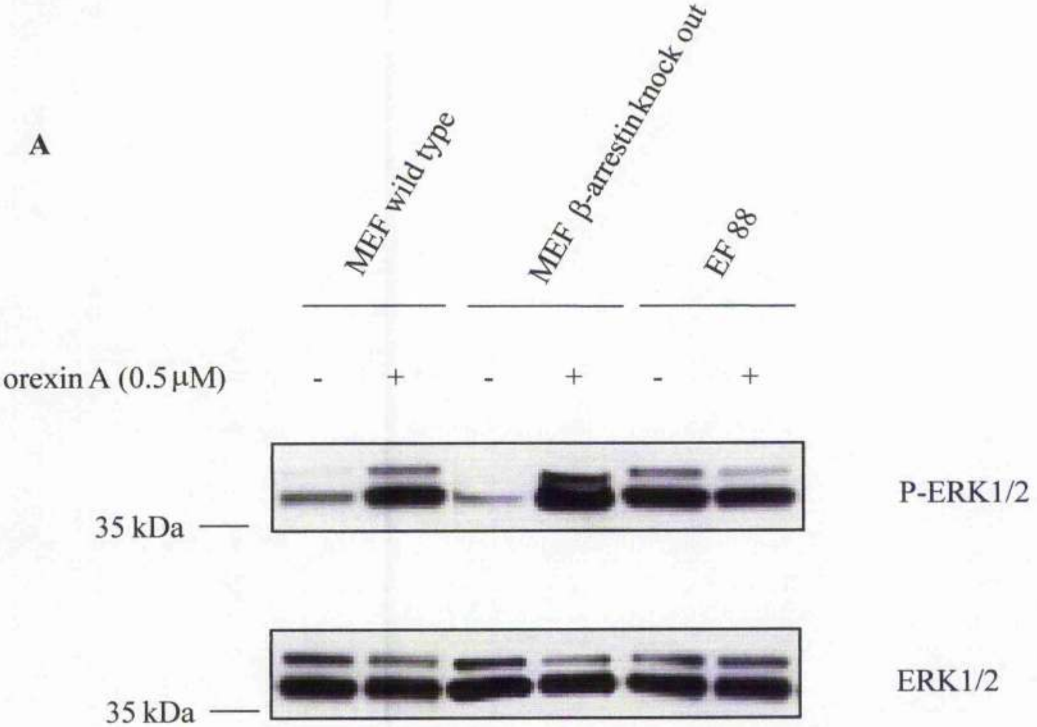

amount of DNA transfected
1.0 µg
0.7 µg
0.5 µg
0.25 µg
0.1 µg
0 µg
35 kDa
35 kDa
35 kDa
P-ERK1/2
ERK1/2
VSV-G
wild type
5.0 µg
4.0 µg
3.0 µg
2.0 µg
1.0 µg
0.5 µg
35 kDa
35 kDa
35 kDa
P-ERK1/2
ERK1/2
VSV-G
cluster C1 mutant
1.0 µg
0.7 µg
0.5 µg
0.25 µg
0.1 µg
0 µg
35 kDa
35 kDa
35 kDa
P-ERK1/2
ERK1/2
VSV-G
I148E mutant

Figure 4.4: Activation of ERK1/2 by the orexin 1 receptor is G protein-dependent

A, MEF wild type cells, MEF β-arrestin 1 and 2 knock out cells and MEF $G_{q/11}$ knock out cells (EF88) were transiently transfected with VSV-G-orexin 1 receptor. Following serum starvation for 2 h the cells were stimulated with 0.5 μM orexin A for 5 minutes and whole cell lysates prepared. *B*, EF88 cells transiently transfected with VSV-G-orexin 1 receptor were serum starved for 2 h and stimulated for 5 min with 1 μM PMA. Activation of ERK1/2 was measured by immunoblot using a polyclonal rabbit antibody specific to the phosphorylated form of ERK1/2 (P-ERK1/2). To show equal loading, membranes were stripped and reprobed with antibody against total ERK1/2 (ERK1/2). The data shown are representative of three independent experiments.

Figure 4.4


A
MEF wild type
MEF β-arrestin knock out
EF 88
orexin A (0.5 μM)
- + - + - +
P-ERK1/2
35 kDa
ERK1/2
35 kDa

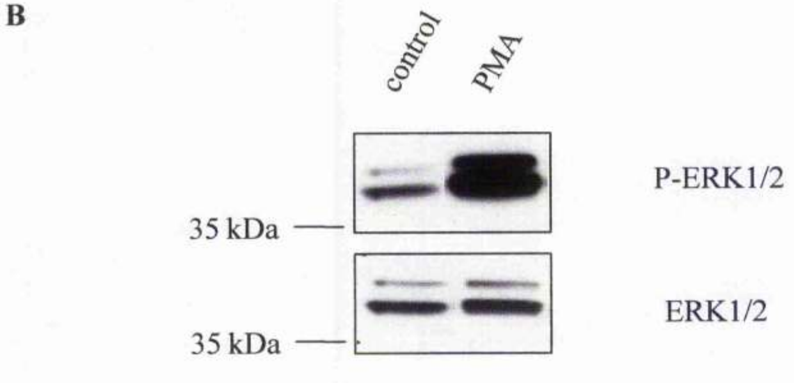

B
control
PMA
P-ERK1/2
35 kDa
ERK1/2
35 kDa

Figure 4.5: Effect of the stability of receptor-β-arrestin binding on orexin A-stimulated cell proliferation

HEK293T cells transiently expressing VSV-G-orexin 1 receptor wild type or C1 mutant were incubated for 24 h in media without FBS (NS), in media containing 10 % FBS (FBS) and media containing 10 % FBS and 0.5 μM orexin A (orexin A) prior to determination of cell proliferation using the WST-1 reagent (section 2.8.3). Results are expressed as -fold increase relative to cells stimulated with media in the absence of any additions. The experiments were performed in triplicates and independently repeated three times.

Figure 4.5

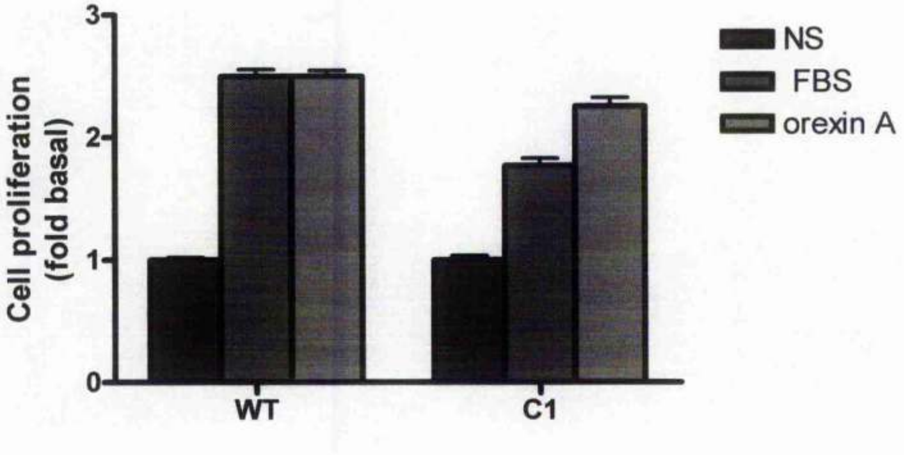


Figure 4.6: Effect of concanvalin A and sucrose on ERK1/2 activation by the orexin 1 receptor

A, HEK293T cells transiently expressing VSV-orexin 1 receptor were serum starved for 2 h and pre-treated with 0.45 M sucrose or 0.25 mg/ml concanavalin A for 30 min before addition of 0.5 μM orexin A for 5 min. Activation of ERK1/2 was determined using phospho-specific ERK1/2 antibodies (P-ERK1/2). Expression levels of ERK1/2 were monitored using antibodies directed against total population of ERK1/2 (ERK1/2). *B*, The levels of P-ERK1/2 were quantified and normalised to the P-ERK1/2 signal induced by orexin A. The graphs shown represent the mean ± S.E. of three independent experiments.

Figure 4.6

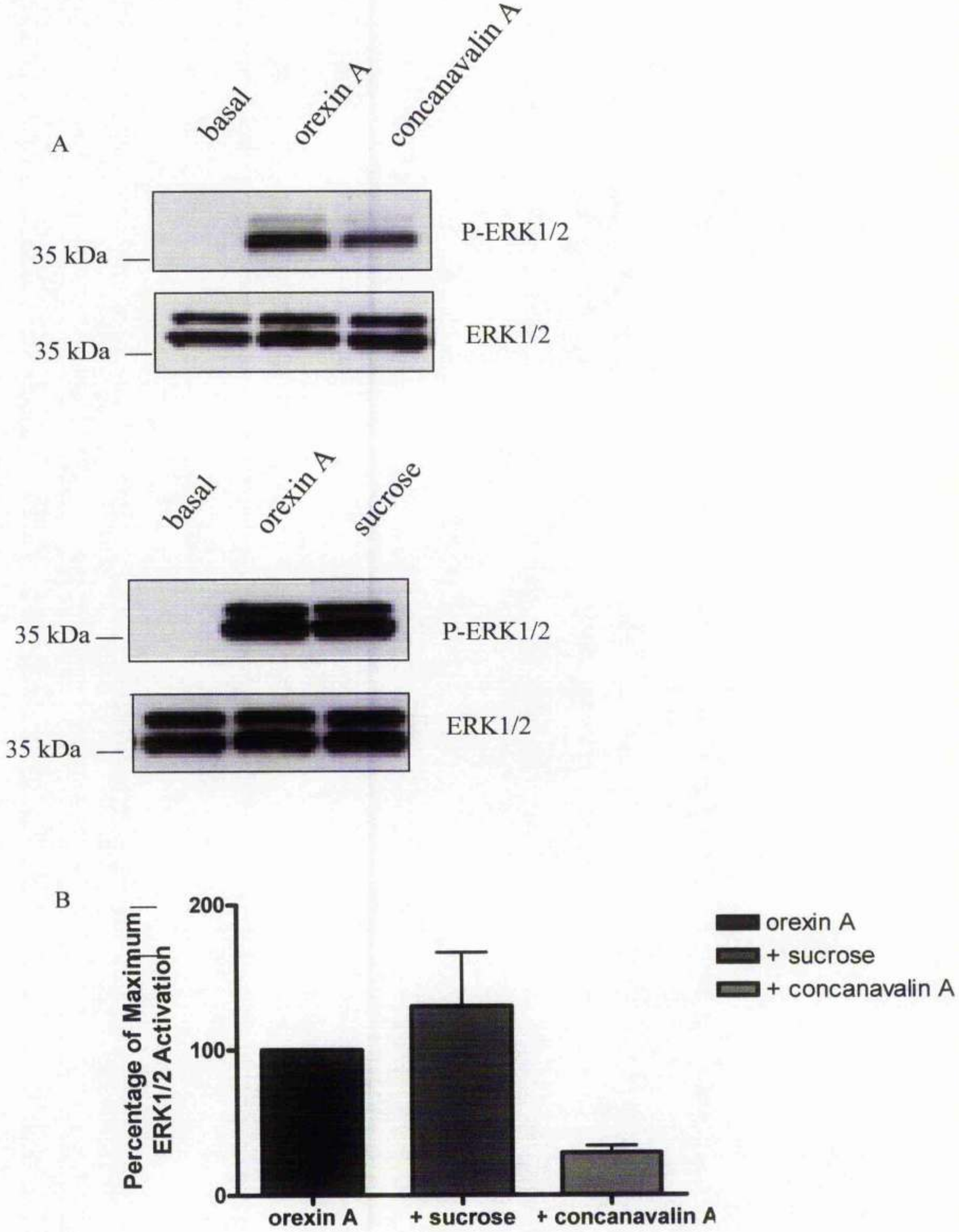
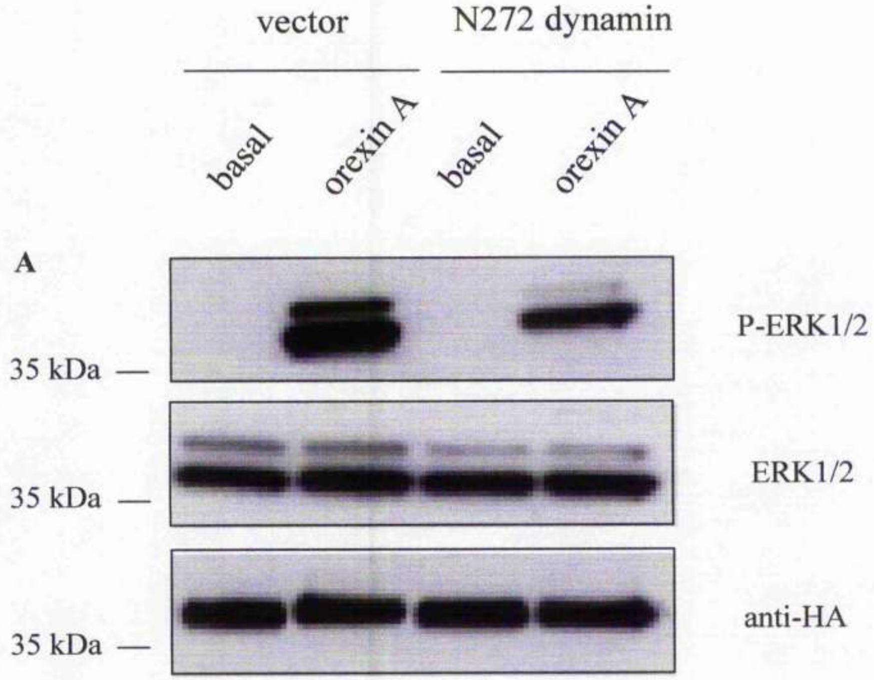

A
basal
orexin A
concanavalin A
P-ERK1/2
35 kDa
ERK1/2
35 kDa
basal
orexin A
sucrose
35 kDa
P-ERK1/2
ERK1/2
35 kDa
B
Percentage of Maximum ERK1/2 Activation
200
100
0
orexin A
+ sucrose
+ concanavalin A
orexin A
+ sucrose
+ concanavalin A

Figure 4.7: Involvement of dynamin in orexin 1 receptor mediated ERK1/2 activation

A, CHO cells stably expressing HA-orexin 1 receptor were transiently transfected with vector or N272 dynamin and grown for 24 h. Cells were serum starved for 2 h and then treated or not with 0.5 μM orexin A for 5 min. The activation of ERK1/2 was determined by immuno-blotting with a phospho-ERK1/2-specific antibody. To check for equal protein loading, membranes were stripped and reprobed with total ERK1/2 antibody. The expression level of the receptor was assessed by Western blot analysis of the same samples using anti-HA antibody. *B*, The effect of N272 dynamin on orexin A-induced ERK1/2 activation was determined by normalising the P-ERK1/2 signal to the response induced by orexin A in cells transfected with vector. Data shown are the mean ± S.E. of three independent experiments.

Figure 4.7


vector
N272 dynamin
basal
orexin A
basal
orexin A
A
P-ERK1/2
ERK1/2
anti-HA
35 kDa
35 kDa
35 kDa

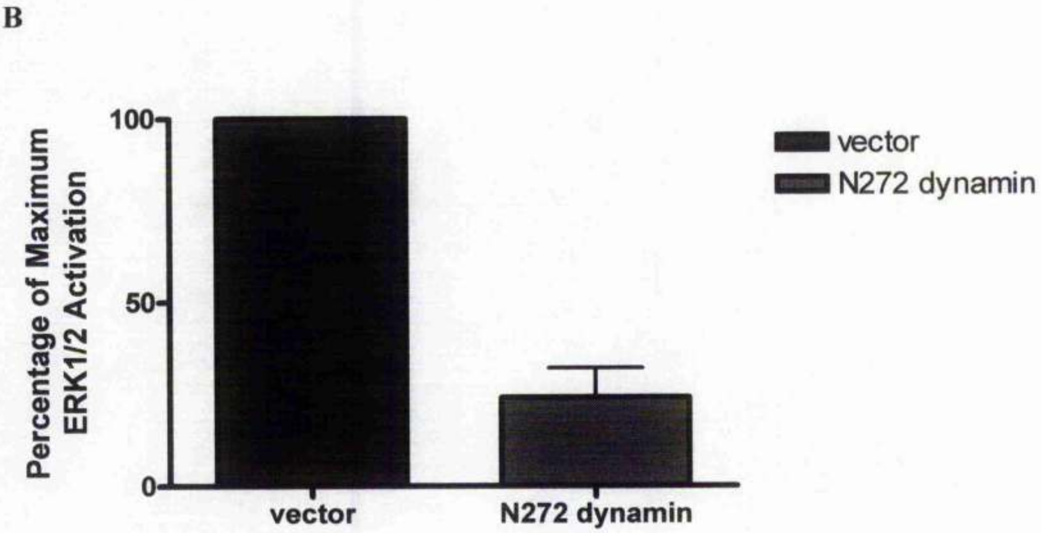

B
Percentage of Maximum ERK1/2 Activation
100
50
0
vector
N272 dynamin
vector
N272 dynamin

Figure 4.8: Src-family tyrosine kinases are not required for orexin A-mediated ERK1/2 activation

SYF cell were transiently transfected with VSV-G-orexin 1 receptor using the Amaxa nuclefection system. 24 h later the cells were deprived of serum for 2 h and treated with vehicle or 0.5 μM orexin A for 5 min. Whole cell lysates were prepared and the activation of ERK1/2 measured by immunodetection using a phospho-ERK1/2 antibody. To show that similar amounts of cell lysates were used in each lane, membranes were stripped and reprobed with a total ERK1/2 antibody. This experiment was repeated twice.

Figure 4.8

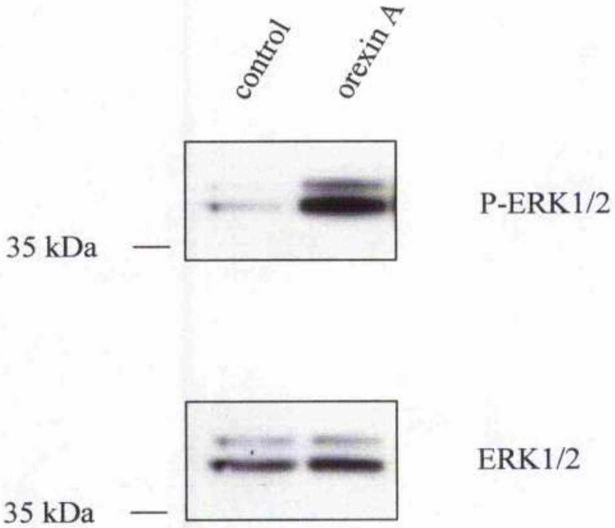

control
orexin A
35 kDa
P-ERK1/2
35 kDa
ERK1/2

Figure 4.9: Time course of JNK MAPK activation by the orexin 1 receptor

Insert, HEK293T cells transiently expressing the different forms of the orexin 1 receptor N-terminally tagged with VSV-G were serum starved for 2 h and stimulated with 0.5 μM orexin A or 0.3 M sorbitol for the indicated times. Reactions were stopped using lysis buffer and JNK MAPK activity determined by an *in vitro* kinase assay (section 3.5). *Diagram,* graphic representation of the data after normalising the phospho-c-jun levels to total c-jun. Similar results were obtained in three further experiments.

Figure 4.9

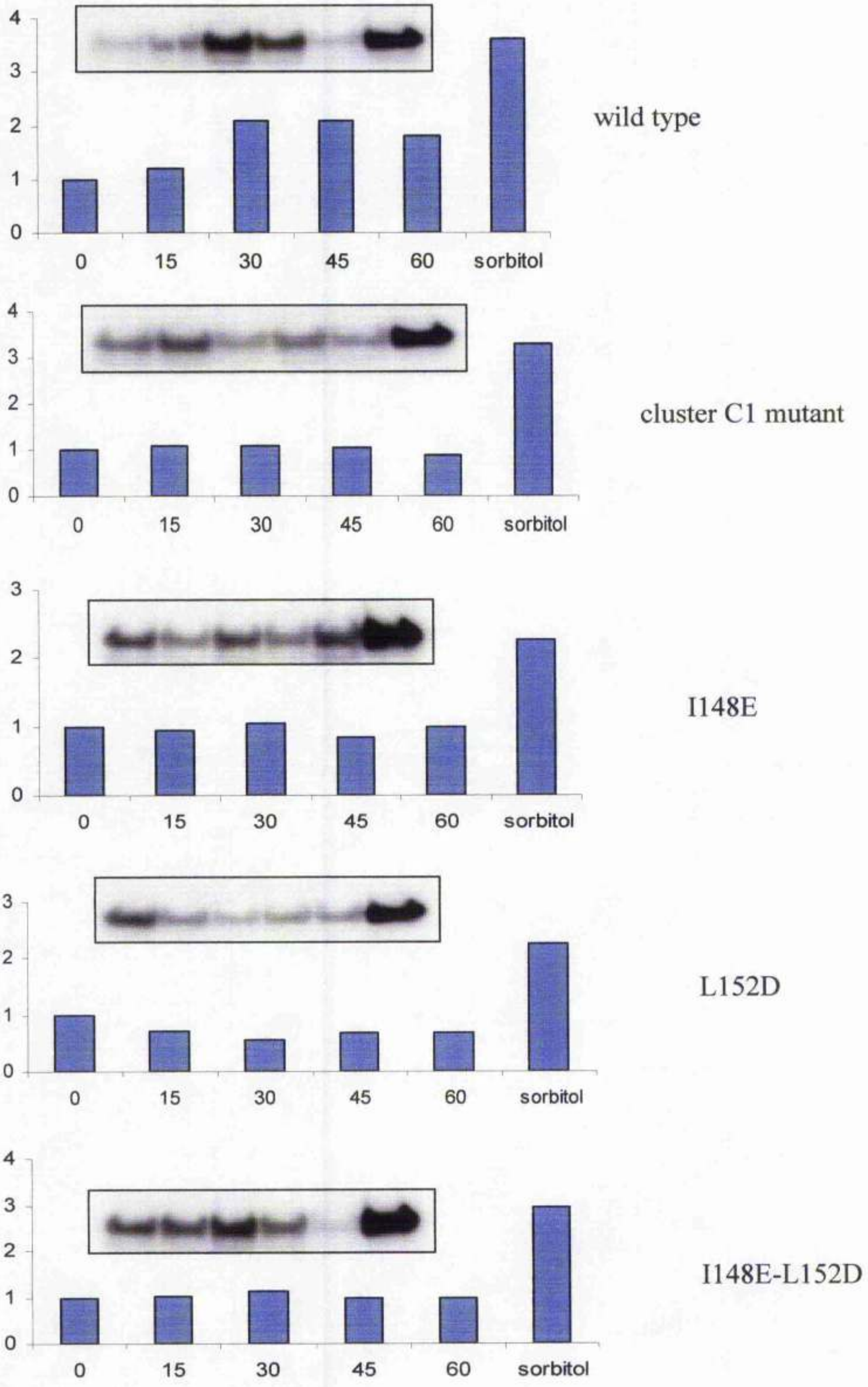
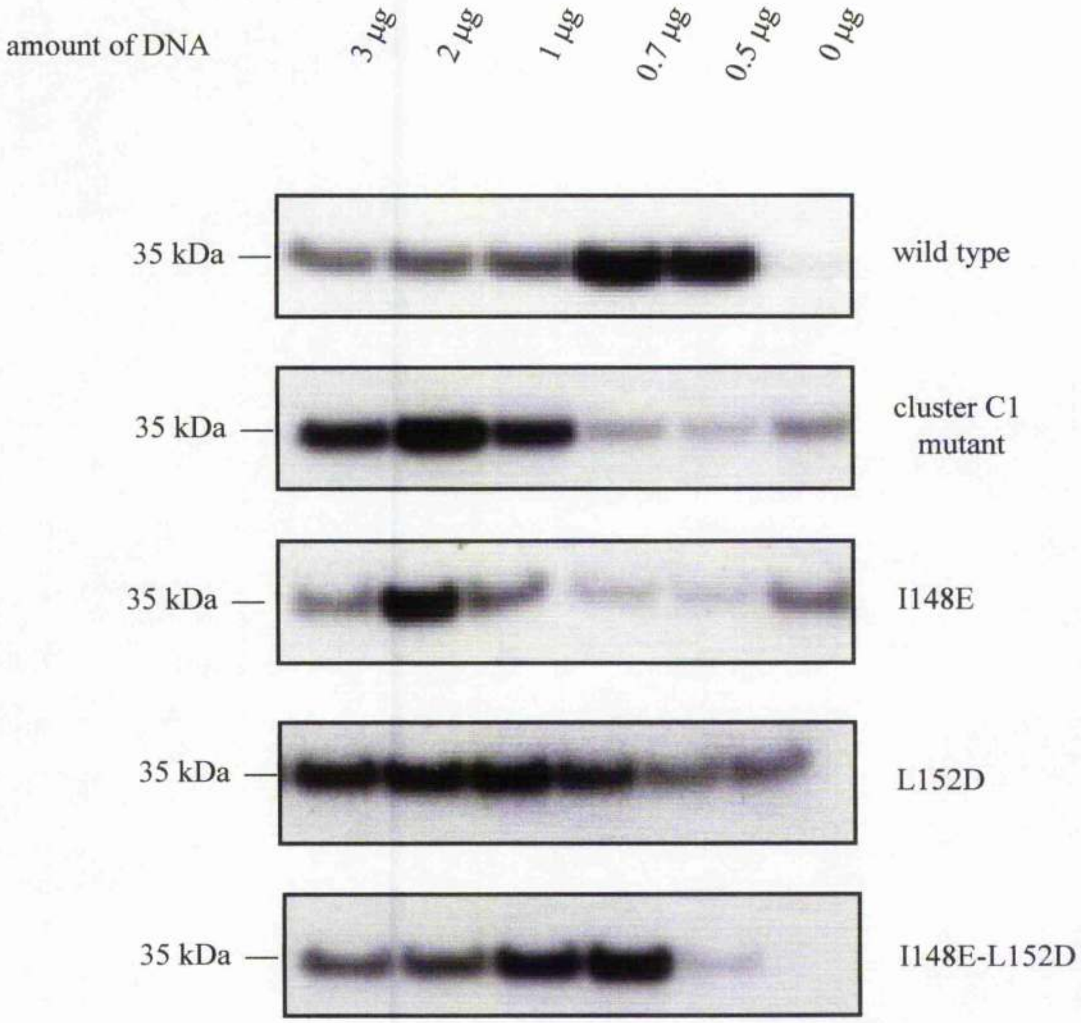

4
3
2
1
0
0
15
30
45
60
sorbitol
wild type
4
3
2
1
0
0
15
30
45
60
sorbitol
cluster C1 mutant
3
2
1
0
0
15
30
45
60
sorbitol
I148E
3
2
1
0
0
15
30
45
60
sorbitol
L152D
4
3
2
1
0
0
15
30
45
60
sorbitol
I148E-L152D

Figure 4.10: Activation of JNK MAPK by the orexin 1 receptor

HEK293T cells were transfected with the indicated amounts of the VSV-wild type orexin 1 receptor, -C1 mutant, -I148E mutant, -L152D mutant, or -I148E-L152D mutant and serum starved for 2 hr before addition of 0.5 μM orexin A for 30 min. As positive control the cells were treated with 0.3 M sorbitol. The reaction was terminated by addition of lysis buffer and JNK MAPK activity was measured by the phosphorylation of substrate GST-c-jun that was detected by autoradiography as described in section 2.5. Data are representative of three experiments.

Figure 4.10

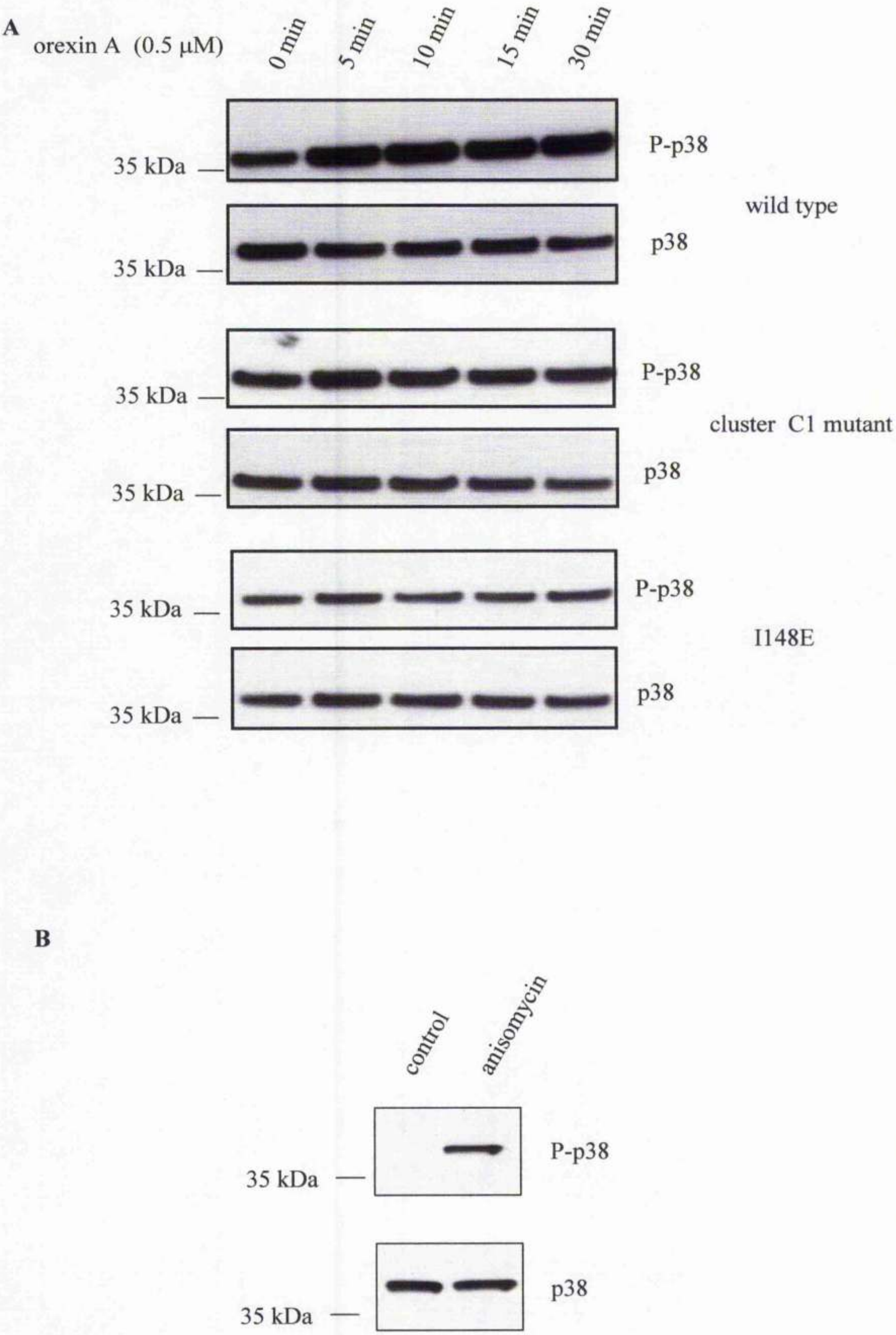


amount of DNA
3 µg
2 µg
1 µg
0.7 µg
0.5 µg
0 µg
35 kDa
wild type
35 kDa
cluster C1 mutant
35 kDa
I148E
35 kDa
L152D
35 kDa
I148E-L152D

Figure 4.11: Activation of p38 MAPK by the orexin 1 receptor over time

A, HEK293T cells were transiently transfected with expression vector encoding the different forms of the orexin 1 receptor tagged with VSV-G at the N-terminus. After serum starving the cells for 2 h 0.5 μM orexin A was added for the times indicated. *B*, HEK293T transiently expressing VSV-G-orexin 1 receptor wild type were serum starved for 2 h and incubated with 250 ng/ml anisomycin for 5 min. The cells were solubilised and extracts were subjected to immunoblotting by using anti-phospho-p38 MAPK antibodies (P-p38) to evaluate the activation of p38. After stripping and blocking, the same blots were reprobed with anti-p38 antibodies to check for total protein content (p38). Results are representative of three independent experiments.

Figure 4.11


A
orexin A (0.5 μM)
0 min
5 min
10 min
15 min
30 min
35 kDa
P-p38
35 kDa
p38
wild type
35 kDa
P-p38
cluster C1 mutant
35 kDa
p38
35 kDa
P-p38
I148E
35 kDa
p38
B
control
anisomycin
35 kDa
P-p38
35 kDa
p38

4.9 Discussion

The role of β-arrestins in agonist-mediated receptor desensitisation and internalisation, by uncoupling the receptor from heterotrimeric G proteins and thereby targetting it for endocytosis, has been well established. However evidence has been accumulating to indicate that β-arrestins also function as adaptors, localising signalling proteins to ligand activated GPCRs and initiating additional β-arrestin-dependent signalling events. Thus, β-arrestins appear not only to be involved in termination of GPCR activity, but also in the initiation of GPCR signalling. The finding that β-arrestins can interact directly with enzymatic effectors such as Src family tyrosine kinases (DeFea *et al.,* 2000 b; Miller *et al.,* 2000; Luttrell *at al.,* 1999) as well as components of the ERK1/2 (Luttrell *et al.,* 2001; Tohgo *et al.,* 2002) and JNK3 (McDonald *et al.,* 2000; Scott *et al.,* 2002; Miller *et al.,* 2001) MAPK modules suggests that β-arrestins may serve in a variety of signalling roles.

GPCRs are able to employ several distinct mechanisms to activate the ERK1/2 cascade (Pierce *et al.,* 2001). For example the erythropoietin receptor stimulates ERK1/2 via a G_i protein βγ-subunit-initiated pathway (Guillard *et al.,* 2003) and the G_s-coupled serotonin receptors 5-$HT_{4(b)}$ and 5-$HT_{7(a)}$ activate ERK1/2 in a PKA-dependent manner (Norum *et al.,* 2003). The neurokinin 1 receptor on the other hand activates ERK1/2 by β-arrestin-dependent mechanisms (DeFea *et al.,* 2000a). To delineate the pathway of ERK1/2 MAPK activation by the orexin 1 receptor, i2 loop mutants unable to activate G proteins were created and the ability of the wild type, the cluster C1 mutant and also of the i2 loop mutants to regulate ERK1/2 activation was compared. Stimulation of the i2 loop mutants I148E and I148E-L152D with agonist did not cause any detectable translocation of β-arrestin to the plasma membrane or internalisation of the receptor. The L152D mutant on the other hand behaved in a similar fashion to the wild type receptor. These findings together with the Ca^{2+} data show only the I148E and I148E-L152D mutants but not the L152D mutant to be impaired in G protein activation. The wild type orexin 1 receptor, the L152D mutant as well as the cluster C1 and C2 mutants were able to activate ERK1/2 in response to agonist challenge. However, stimulation of the wild type receptor, the L152D and the cluster C2 mutant resulted in prolonged phosphorylation of ERK1/2 compared to the cluster C1 mutant. Moreover preliminary results indicate that the cluster C1 mutant was weakly, but significantly mitogenic, whereas the wild type receptor failed to elicit a detectable mitogenic response. However, more experiments need to be carried out to support this observation. These results correspond well with recent findings showing that

G protein-independent and β-arrestin-dependent activation of ERK1/2 by the angiotensin II type 1A receptor does not induce nuclear translocation of ERKs and therefore no proliferation, and the activated ERKs phosphorylate only their cytoplasmic targets (Luttrell *et al.*, 2001; Seta *et al.*, 2002; Tohgo *et al.*, 2002). Interestingly β-arrestin 1 and GRK2 belong to the cytoplasmic targets of ERK1/2 (Lin *et al.*, 1999; Pitcher *et al.*, 1999; Elorza *et al.*, 2003). In both cases, phosphorylation exerts an inhibitory effect on protein function. So the β-arrestin-ERK complexes might provide a negative feedback mechanism to control GPCR singalling. It is also noteworthy that the extent of β-arrestin-bound ERK activation seems to be regulated by the stability of the receptor-β-arrestin complex which in turn is controlled by the presence of clusters of Ser/Thr residues within the receptor C-terminus (Tohgo *et al.*, 2003). However the situation might be yet more complicated due to the fact that β-arrestin 1 and 2 seem to have reciprocal effects on ERK1/2 activation by the angiotensin II type 1A receptor (Ahn *et al.*, 2004). In this context it might be interesting to see whether the same is true for the orexin 1 receptor as this receptor is clearly able to interact with both β-arrestin proteins (Evans *et al.*, 2001).

In contrast to the wild type orexin 1 receptor, the L152D, the cluster C1 and the cluster C2 mutant, the i2 loop mutants I148E and I148E-L152D were unable to cause any significant activation of ERK1/2. This could be due to a few factors. Firstly these mutants were shown to be unable to activate G proteins. Secondly these mutants did not internalise in response to agonist challenge and thirdly they did not cause translocation of β-arrestin from the cytosol to the plasma membrane. Inhibition of orexin 1 receptor endocytosis using hypertonic sucrose, concanavalin A or N272 dynamin indicates that the clathrin-mediated internalisation pathway is not required for receptor-stimulated activation of ERK1/2, since N272 dynamin and concanavalin A attenuated ERK1/2 phosphorylation, whereas hypertonic sucrose had no effect, indicating that internalisation *via* caveolae might play a role. These findings are somewhat surprising since the orexin 1 receptor was shown to internalise *via* clathrin coated-vesicles. A scenario is possible where receptor internalisation *per se* is not important for ERK1/2 activation, but an intact internalisation pathway is. This hypothesis is supported by reports suggesting that dynamin-regulated endocytosis of MAPKK, rather than activated receptors, is a critical step in the MAPK activation cascade (Kranenburg *et al.*, 1999). Similar findings were described for the thyrotropin-releasing hormone receptor with the difference that ERK1/2 phosphorylation required clathrin- and not caveolae-dependent endocytosis (Smith *et al.*, 2001). Receptor endocytosis has also been shown to be important for stimulation of the ERK MAPK cascade by the δ-opioid receptor (Ignatova *et al.*, 1999) and the m1 muscarinic acetylcholine receptor (Vogler *et al*, 1999). In case of the calcium sensing receptor ERK1/2 activation is dependent on dynamin- and β-arrestin-independent receptor internalisation (Holstein *et al.*, 2004), whereas ERK1/2 stimulation by the m3 muscarinic acetylcholine receptor is independent of receptor internalisation since concanavalin A and cytochalasin did not ablate signalling of the receptor to ERK1/2 (Budd *et al.*, 1999). It is also noteworthy that in the case of the gonadotropin-releasing hormone receptor, disruption of lipid rafts by removal of cholesterol leads to a loss of receptor-mediated ERK1/2 activation and constitutive localisation of the receptor to lipid rafts was shown to be necessary for signalling of the receptor to ERK1/2 (Navratil *et al.*, 2003). Maybe the orexin 1 receptor like the gonadotropin-releasing hormone receptor is similarly localised in such membrane microdomains. However more experiments are needed to study the possible involvement of caveolae in orexin 1 receptor-mediated ERK1/2 activation.

In agreement with recent findings for the β_2–adrenergic receptor (Huang *et al.*, 2004) stimulation of the orexin 1 receptor expressed in SYF cells resulted in activation of ERK1/2. Hence in MEF cells Src family kinases do not seem to be essential for orexin 1 receptor-mediated stimulation of ERK1/2.

To determine whether ERK1/2 activation by the orexin 1 receptor was mediated by G proteins, β-arrestins or both, the orexin 1 receptor stimulated activation of ERK1/2 in MEF wild type cells, β-arrestin 1 and 2 knock out cells and in $G_{q/11}$ knock out cells was investigated. Agonist challenge of the orexin 1 receptor resulted in phosphorylation of ERK1/2 in MEF wild type and β-arrestin 1 and 2 knock out cells, but not in $G_{q/11}$ knock out cells. This contrasts with studies for the angiotensin II type 1A receptor reporting that ERK1/2 is activated by β-arrestin- and G protein-dependent pathways (Wei *et al.*, 2003). Therefore in case of the orexin 1 receptor β-arrestins seem to play an important role in the modulation G protein-dependent activated ERK1/2 by prolonging activation.

In addition to acting as an adaptor protein in the ERK1/2 MAPK pathway β-arrestin 2 has also been shown to function as a scaffold to enhance signal transmission to the MAPK JNK3 therefore effectively bringing the MAPK activity under the control of the angiotensin II type 1A receptor (McDonald *et al.*, 2000). Moreover specific residues in the C-terminus of β-arrestin 2 are involved in the assembly of the multiprotein complex that contains JNK3 (Miller *et al.*, 2001). However, GPCRs can also stimulate JNK MAPK in a pathway that involves G proteins. In case of the cholecystokinin B receptor addition of gastrin leads to the activation of the JNK pathway by a mechanism involving certain protein kinase C isoforms and Src family kinases (Dehez *et al.*, 2002). Similar results were obtained for the gonadotropin-releasing hormone receptor (Levi *et al.*, 1998). Stimulation of the wild type orexin 1 receptor resulted in JNK activation, which was maximal after 30 min of agonist addition. JNK kinase was also activated in response to agonist challenge of the cluster C1 and the i2 loop mutants. Unfortunately, the time course of JNK activation could not be determined for these mutants. These results indicate JNK activation to be independent of G proteins and receptor endocytosis. However an involvement of β-arrestins cannot be ruled out. Although the i2 loop mutants did not cause translocation of β-arrestin 2 from the cytosol to the plasma membrane in response to orexin A, β-arrestins might still be activated in these cells. In support of this theory is the finding that G protein activation does not seem to be necessary for the binding of β-arrestin to the activated receptor since the orexin 1 receptor was shown to internalise in $G_{q/11}$ knock out cells. Therefore it could be possible that β-arrestins were not able to bind to the receptor at the plasma membrane because these mutants did not release G proteins once bound. Hence additional experiments are necessary to examine whether β-arrestins are involved in activation of JNK MAPK in response to the orexin 1 receptor. One possibility would be to determine the activation of JNK MAPKs by the orexin 1 receptor in β-arrestin 1 and 2 knock out cells.

p38 is another member of the family of MAPKs, that was shown to be activated in response to stimulation of GPCRs. For example addition of the GPCR agonist thrombin to vascular smooth muscle cells causes activation of p38 MAPK (Ghosh *et al.*, 2002). Furthermore stimulation of the CXCR4 chemokine receptor resulted in activation of p38 MAPK in a pathway dependent on β-arrestin 2 (Sun *et al.*, 2002). However none of the constructs of the orexin 1 receptor tested caused any significant increase in p38 MAPK activation hence excluding an involvement of the MAPK pathway in the signalling of the orexin 1 receptor.

In summary, the present chapter demonstrates that stimulation of the orexin 1 receptor results in ERK1/2 phosphorylation by a mechanism depending on G-protein activation and an intact endocytic pathway, but not necessarily receptor sequestration. Although β-arrestins did not seem to be directly involved in the stimulation of ERK1/2 by the receptor, they seem to play an important role in controlling the time course and spatial distribution of ERK1/2 activity. Furthermore agonist challenge of the orexin 1 receptor also caused activation of the JNK MAPK cascade whereas no effect on the p38 MAPK pathway could be observed.

Chapter 5

Final Discussion

Final discussion

GPCRs transduce extracellular signals that modulate a wide variety of biological processes including neurotransmission, taste, smell and hormonal control. Agonist activation of most GPCRs is quickly followed by receptor desensitisation. Rapid desensitisation is a result of receptor phosphorylation by GRKs or second messenger-dependent kinases. This phosphorylation event promotes the interaction with β-arrestins thereby uncoupling the receptor from its cognate G protein (Krupnick and Benovic, 1998). β-arrestins also target GPCRs for endocytosis *via* clathrin-coated pits by binding to components of the internalisation machinery such as clathrin and the clathrin adaptor AP2 (Goodman *et al.*, 1996; Laporte *et al.*, 2000). Two classes of GPCRs can be distinguished on the basis of their internalisation properties. Class A receptors, for example the β_2–adrenergic receptor, prefer binding to β-arrestin 2 over β-arrestin 1. The receptor and β-arrestin co-localise in clathrin-coated pits at or near the plasma membrane and rapidly dissociate upon internalisation. Class B receptors like the vasopressin V2 receptor on the other hand have no preference for β-arrestin 2 over β-arrestin 1. The receptor and β-arrestin form stable complexes that can be found in endosomes. The factor determining into which class a given GPCR fits seems to be clusters of hydroxy residues within the receptor C-terminus.

Studies conducted in recent years indicate that β-arrestins may not only be involved in the termination of GPCR signalling but also function as adaptor proteins coupling GPCRs to alternative, G protein-independent signalling pathways. The finding that β-arrestins can interact with different signalling proteins such as Src kinases (Luttrell *et al.*, 1999) and components of the ERK1/2 (DeFea *et al.*, 2000a) and JNK3 (McDonald *et al.*, 2000) MAPK cascades suggests that β-arrestins may be involved in a variety of signalling pathways.

The aim of the present thesis was to investigate the involvement of β-arrestins in the regulation of orexin 1 receptor internalisation and signalling. Orexins are of therapeutical interest as they play a pivotal role in the regulation of the sleep-wake cycle, energy metabolism and neuroendocrine function. They also seem to be involved in influencing arterial pressure and other cardiovascular factors. In addition these neuropeptides are implicated in neurodegeneration and nociceptive processing. However little is known about the cellular mechanisms underlying these effects.

Stimulation of the orexin 1 receptor with orexin A resulted in trafficking of β-arrestin 2 from the cytosol to the plasma membrane and co-internalisation of these two proteins *via* clathrin-coated pits into acidic endosomes. In addition internalisation of the orexin 1 receptor was shown to be G protein independent, as the receptor still internalised in $G_{q/11}$ mouse embryonic knock out cells. Furthermore addition of agonist caused rapid receptor phosphorylation in a process partly mediated by PKC and PKA.

Mutational analysis of the orexin 1 receptor C-terminus identified a cluster of hydroxyl residues consisting of three Thr and one Ser residue in the distal portion of the carboxyl tail, Thr418, Ser419, Thr421, and Thr422 (cluster C1), that was responsible for high affinity interaction between the receptor and β-arrestin 2. Disruption of this cluster by replacing any two of the four hydroxyl residues with Ala ablated co-internalisation of the receptor and β-arrestin 2 into acidic endosomes in response to orexin A. However in contrast to recent studies that describe these clusters of hydroxyl residues to serve as primary sites of receptor phosphorylation upon agonist challenge (Oakley *et al.*, 2001), there was no detectable difference in the phosphorylation levels of the wild type receptor and the cluster C1 mutant after addition of orexin A. Similar findings were reported for the human prostaglandin E_2 receptor, subtype EP4 (Neuschafer-Rube *et al.*, 2004).

Although mutation of the Ser/Thr residues in cluster C1 to Ala abolished co-internalisation of the receptor and β-arrestin 2, it had no effect on the endocytic pathway. Internalisation of the wild type receptor and the cluster C1 mutant was ablated in β-arrestin 1 and 2 mouse embryonic fibroblast knock out cells. Moreover pre-treatment of the cells with either hypertonic sucrose or concanavalin A blocked receptor endocytosis whereas filipin or nystatin had no effect. Also co-expression of dominant negative dynamin (N272 dynamin) with either form of the receptor inhibited receptor sequestration. However receptor internalisation seemed to be independent of Src family kinases. Taken together these results indicate that the wild type receptor as well as the cluster C1 mutant internalised *via* clathrin coated pits in a dynamin- and β-arrestin-dependent, but Src- and G protein-independent manner.

Stimulation of the orexin 1 receptor also lead to activation of the ERK MAPK cascade in a pathway depending on endocytosis since pre-treatment of the cells with concanavalin A or co-expression of N272 dynamin reduced orexin A-induced phosphorylation of ERK1/2. Similar observations were reported for the β_2–adrenergic receptor and the m1 muscarinic acetylcholine receptor (Pierce *et al.*, 2000; Vogler *et al.*, 1999). Surprisingly, hypertonic sucrose did not inhibit ERK1/2 activation. These results imply that a caveolae-mediated internalisation pathway is required for orexin A-stimulated ERK1/2 activation but not necessarily clathrin-mediated receptor endocytosis. Results obtained in MEF β-arrestin 1 and 2 knock out cells support this idea, since ligand stimulation of the orexin 1 receptor activated ERK1/2 while receptor internalisation was blocked in these cells. As described for the β_2–adrenergic receptor (Huang *et al.*, 2004) ERK1/2 stimulation by the orexin 1 receptor was independent of Src family kinases. However, in contrast to the orexin 1 receptor internalisation of the β_2–adrenergic receptor was impaired under these conditions. Since dynamin seemed to be required for receptor sequestration as well as orexin A-mediated ERK1/2 activation, an additional mechanism to activate dynamin apart from the one described for the β_2-adrenergic receptor depending on Tyr phosphorylation by c-Src (Ahn *et al.*, 1999) must, at least in MEF cells, exist. An alternative explanation could be that the orexin 1 receptor internalises *via* a different pathway independent of dynamin in SYF cells. Additional experiments ought to be carried out to test which scenario is true. Similar levels of ERK1/2 activation in response to orexin A could be observed for the cluster C1 mutant. However phosphorylation of ERK1/2 was significantly longer when the wild type orexin 1 receptor was stimulated compared to experiments performed with the cluster C1 mutation. In addition preliminary observations imply that activation of the cluster C1 mutant in the presence of FBS resulted in a small mitogenic response. No increase in cell proliferation could be detected for the wild type orexin 1 receptor under the same conditions. This could be due to the fact that orexin A stimulation results in the generation of a functionally distinct cytosolic pool of activated ERK1/2. Similar findings have been reported for the angiotensin II type 1A receptor showing that over-expression of β-arrestins facilitates the angiotensin-stimulated activation of ERK1/2 MAPK activity implicating β-arrestins as being a major component in ERK MAPK activation (Tohgo *et al.*, 2002). However binding of β-arrestins to ERK1/2 inhibits ERK-dependent transcription as ERK coupled to β-arrestins is retained within the cytosol (Tohgo *et al.*, 2002). One role of cytosolic retention of activated ERK1/2 could be to target ERK1/2 to cytoplasmic substrates involved in the regulation of GPCR signalling such as β-arrestin 1 and GRK2 (Lin *et al.*, 1999; Pitcher *et al.*, 1999; Elorza *et al.*, 2003). Though, additional experiments need to be carried out to test whether this is also the case for the orexin 1 receptor.

In contrast, ERK1/2 did not become activated in response to agonist challenge of mutant forms of the orexin 1 receptor unable to activate G proteins. This observation is supported by experiments carried out in $G_{q/11}$ and β-arrestin1/2 knock out cells showing, that ERK1/2 phosphorylation was dependent on G protein activation and independent of β-arrestins. This is inconsistent with findings described by Seta *et al.* (2002). They have recently reported that a mutant form of the angiotensin II type 1A receptor impaired in G protein coupling was still able to induce ERK1/2 activation.

In addition to activation of ERK1/2, stimulation of the orexin 1 receptor also led to phosphorylation of JNK MAPKs by an unknown mechanism. The p38 MAPK on the other hand did not seem to play a role in orexin 1 receptor signalling.

In summary, the orexin 1 receptor, a class B GPCR, co-internalised rapidly with β-arrestin 2 in response to agonist into acidic endosomes *via* clathrin-coated pits in a β-arrestin- and dynamin-dependent, but G protein- and Src-independent manner. Mutation of a cluster of Ser/Thr residues at the extreme C-terminus (cluster C1) of the receptor transformed the orexin 1 receptor to a class A GPCR unable to co-internalise with β-arrestins. Orexin 1 receptor stimulation led to activation of the ERK MAPK cascade and this activation was dependent on G protein activation and an intact endocytic pathway but not on Src, β-arrestin or receptor internalisation. Although without effect on the internalisation pathway, the C-terminally mutated form of the orexin-1 receptor was unable to sustain phosphorylation of ERK1/2 to the same extent as the wild type receptor. These studies indicate that a single cluster of hydroxy amino acids within the C-terminal seven amino acids of the orexin 1 receptor determines the sustainability of interaction with β-arrestin 2. They also imply that β-arrestins by serving as adaptor proteins play an important role in defining the kinetics and spatial distribution of orexin 1 receptor-mediated, G protein-dependent ERK MAPK activation.

The findings described in this thesis correlate well with the literature. The current model of GPCR desensitisation and internalisation involves receptor phosphorylation and β-arrestin binding in response to agonist. β-arrestin then targets the receptor to clathrin-coated pits for sequestration and pinching off of the vesicles from the plasma membrane is regulated by dynamin. C-terminal clusters of Ser/Thr residues in the neurotensin-1 receptor, the oxytocin receptor and the angiotensin II type 1A receptor were shown to promote the formation of high affinity receptor-β-arrestin complexes that remain associated during endocytosis. Mutation of these residues to Ala abolished co-internalisation of the receptor and β-arrestin (Oakley *et al.*, 2001). The observation, however, that these sites did not serve as primary agonist-stimulated phosphorylation site in the orexin 1 receptor is more unusual. Studies on the vasopressin V2 and the β_2-adrenergic receptor suggest that the stability of the receptor-β-arrestin interaction also determines the mechanism and consequences of ERK1/2 activation (Tohgo *et al.* 2003). Stimulation of the vasopressin V2 receptor resulted in cytoslic retention of phospho-ERK1/2 and failed to elicit a detectable mitogenic response. A chimeric receptor generated from the N-terminus of the vasopressin V2 receptor and the C-terminus of the β_2-adrenergic receptor caused greater nuclear translocation of activated ERK1/2 and a weak, but significant proliferative response. In contrast to the results obtained for the orexin 1 receptor they observed a more persistent ERK1/2 activation in case of the chimeric receptor compared to the wild type receptor. It is noteworthy that prolonged MAPK activation in pseudopodia during protease-activated receptor-2-induced chemotaxis seemed to be associated with a β-arrestin-dependent scaffold (Ge *et al.,* 2003). So far no reports exist implicating caveolae-mediated endocytosis in GPCR-dependent MAPK activation.

One problem encountered throughout the work of this thesis was the low receptor expression levels and the lack of appropriate stable cell lines. Viral transfection systems might be able to solve this problem.

Further research is required to define, in more depth, the sequestration and signalling of the orexin 1 receptor elicited by addition of orexin A. It would be interesting to find out whether there are any differences in the recycling and resensitisation rate between the wild type form of the orexin 1 receptor and the cluster C1 mutant. Also the time course of internalisation was determined by visualising stained receptors using the confocal microscope. This method might not be sensitive enough to detect small differences. In this regard the recent development of a radiolabelled antagonist, [H^3]-SB-674042, to the orexin 1 receptor should be of great benefit (Langmead *et al.,* 2004). Moreover the discovery of the tritiated antagonist allows pharmacological examination of the different forms of the orexin 1 receptor. It would also be of interest to investigate the additional sites in the receptor that are involved in the low affinity binding to β-arrestins and to examine the role of agonist-mediated phosphorylation in this context.

Additional experiments need to be carried out to determine the importance of the different endocytic pathways, clathrin *versus* caveolae, in the activation of the ERK1/2 cascade. Although in MEF cells an intact endocytic pathway seems to be sufficient for ERK1/2 phosphorylation it is not clear whether orexin 1 receptor sequestration is involved in signalling to the ERK1/2 MAPK cascade in other cell types. Also the experimental proof that β-arrestins serve as adaptor proteins in the orexin 1 receptor-mediated ERK1/2 MAPK cascade is still outstanding. Therefore co-immunoprecipitation experiments should be performed examining the effects of orexin A on binding of ERK1/2 to β-arrestins and on phosphorylation of bound ERK1/2 and to determine whether there are any differences between the wild type receptor and the cluster C1 mutant. Additionally the cellular distribution, cytosol *versus* nucleus, of phospho-ERK1/2 after stimulation of either from of the receptor ought to be investigated. Another way of examining whether high affinity binding of β-arrestins to the receptor has a functional consequence on ERK1/2 activation would be to measure ERK1/2-dependent transcription using an Elk-1-driven luciferase reporter system. Furthermore the cell proliferation experiments should be repeated after serum starvation and ideally/preferentially on cells stably expressing either from of the receptor. It is also noteworthy that recent reports indicate β-arrestin 1 and 2 to have reciprocal effects on ERK1/2 activation (Ahn *et al.,* 2004). In theory, the wild type receptor should bind both β-arrestin proteins with the same affinity as it belongs to class B, whereas the cluster C1 mutant, a class A GPCR, should prefer β-arrestin 2 over 1. Therefore it would be very interesting to see what the implications are for the two different forms of the receptor.

The internalisation and signalling behaviour of GPCRs seems to depend in many cases on the cellular environment in which they are expressed. Consequently the experiments carried out in MEF knock out cells should be repeated in HEK cells, ideally stably or inducibly expressing the receptor, using for example siRNA technology to knock out the individual proteins.

It was previously reported that β-arrestin 2 is necessary for β_2-adrenergic receptor and vasopressin V2 receptor ubiquitination thereby targetting the receptors for degradation (Shenoy *et al.,* 2001; Martin *et al.,* 2003). Binding of β-arrestin to the receptors itself requires ubiquitination of β-arrestin by E3 ubiquitin ligase, Mdm2. Stimulation of the β_2-adrenergic receptor, a class A GPCR, resulted in transient β-arrestin ubiquitination whereas activation of the vasopressin V2 receptor, a member of the class B, caused stable β-arrestin ubiquitination. Moreover the time course of β-arrestin ubiquitination and deubiquitination correlates with its receptor association and dissociation, respectively (Shenoy *et al.,* 2003). It might therefore be intriguing to examine the ubiquitination status of the orexin 1 receptor wild type, cluster C1 mutant and β-arrestin in response to receptor activation and to determine if this has any functional consequence on the degradation of either form of the receptor. β-arrestins were also shown to be regulated by sumoylation (unpublished observations). Small ubiquitin-related modifier (SUMO) is the best characterised member of a growing family of ubiquitin-related proteins. However in contrast to ubiquitination, sumoylation does not appear to mark proteins for degradation. The data available indicates a role of SUMO in the regulation of protein-protein interactions and /or subcellular localisation. Virtually nothing is known about the function of β-arrestin-sumoylation in the control of GPCR function. Hence, it might be exciting to study the effects of sumoylation on the signalling of the orexin 1 receptor. Furthermore, Hilairet and co-workers (2003) provided evidence for cross-talk between the orexin 1 receptor and the cannaboid receptor CB1 indicating heterodimerisation to play a role in the regulation of orexin 1 receptor function. Future work should be carried out to identify additional binding partners of the orexin 1 receptor and to determine their effects on orexin 1 receptor signalling.

In this thesis only preliminary data was obtained regarding the activation of the JNK MAPK cascade in response to orexin A. More studies need to be undertaken to identify the mechanisms underlying this signalling pathway and its implications. The β-arrestin 1 and 2 knock out cells might provide a useful tool to examine whether β-arrestins are involved in activation of JNK MAPK in response to the orexin 1 receptor.

Chapter 6

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