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Investigation of the Intracellular Pathways Required for 5HT-induced Mitogenesis and their Role in Pulmonary Hypertension.

Kirsty Mary Marshall BSc. (Hons)

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University of Glasgow

ABSTRACT

Pulmonary arterial hypertension (PAH) is a rare and progressive disease characterised by increased pulmonary vascular resistance and elevated pulmonary artery pressure, leading to right ventricular failure and eventually death. The monoamine 5-hydroxytryptamine (5HT) has been implicated in the processes of pulmonary vasoconstriction and pulmonary artery remodelling that contribute to the development of the PAH. However, the signalling mechanisms utilised by 5HT that contribute to pulmonary vascular remodelling are still unclear and appear to be cell-type specific, with much of the work having been carried out in pulmonary artery smooth muscle cells (PASMCs). Fibroblasts also contribute significantly to the pulmonary vascular remodelling that occurs during PAH, however little is known of the role 5HT plays in this cell type. Using Chinese Hamster Lung Fibroblast (CCL-39) cells as a model system to investigate the mitogenic effects of 5HT, this study has characterised potential 5HT-mediated signalling pathways in fibroblasts that may contribute to pulmonary vascular remodelling.

5HT was found to induce a rapid and transient activation of extracellular regulated mitogen-activated protein kinase (ERK), a process central to the mitogenic effects of 5HT in CCL-39 cells. Furthermore, the 5HT transporter (5HTT), $5HT_{1B}$ and $5HT_{2A}$ receptors were all required for optimal ERK-dependent proliferation. Pharmacological inhibition of the Rho/ROCK (Rho-associated kinase) pathway significantly inhibited 5HT-stimulated ERK activation, cyclin D1 accumulation and proliferation. Inhibition of ROCK had no effect on the translocation of active ERK to the nucleus, but did however selectively inhibit 5HT-induced activation of a cytoplasmic pool of ERK. Additionally, ROCK inhibition had no effect on the ability of 5HT to activate mitogen-activated protein kinase kinase (MEK), suggesting ROCK is required for maintaining functional interactions between MEK and ERK. Sensitivity to ROCK inhibition is restricted to $5HT_{1B}$ receptor activation of the ERK pathway. Moreover, the role of ROCK in maintaining cytoskeletal integrity is important in mediating 5HT-induced ERK activation, as disruption of the actin cytoskeleton markedly and specifically reduces 5HT- stimulated ERK activation.

Using a model of PAH, arising from overexpression of 5HTT ($5HTT^+$), the effects of ROCK inhibition *in vivo* were investigated. ROCK 1 and ROCK 2 transcripts were upregulated in response to chronic hypoxia, with the upregulation of ROCK 1 potentiated in $5HTT^+$ mice. Administration of the ROCK inhibitor Y27632 had significantly greater effects in $5HTT^+$ mice compared to WT, highlighting the functional importance of the increase in ROCK 1 transcript. Hypoxia-induced pulmonary vascular remodelling and elevated right ventricular pressure were attenuated more significantly by ROCK inhibition in $5HTT^+$ mice than in WT. Furthermore, ROCK inhibition only reduced hypoxia-derived right ventricular hypertrophy significantly in $5HTT^+$ animals and not WT.

In conclusion, this study highlights a role for ROCK in the pulmonary vascular changes that occur during PAH and proposes a new mechanism by which cross-talk between ROCK and 5HT signalling systems occurs.

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Finally, I would like to dedicate this thesis to my Dad. He is often in my thoughts and I'm sure he would have been extremely proud.

CONTENTS

LIST OF EXPERIMENTAL FIGURES

LIST OF EXPERIMENTAL TABLES

ABBREVIATIONS

AA	Arachadonic acid
AN	Anisomycin
ANOVA	One-way analysis of variance
Ang-1	Angiopoetin-1
AT1aR	Angiotensin type 1a receptor
ATP	Adenosine 5’ triphosphate
AVD	Apoptotic volume decrease
BCA	Bicinchoninic acid
BMP	Bone morphogenetic protein
BMPR-2	Bone morphogenetic protein type 2 receptor
BSA	Bovine serum albumin
$[Ca^{2+}]_i$	Intracellular calcium concentration
CaM	Calmodulin
CaMK	Calmodulin kinase
cAMP	Cyclic 3’, 5’ adenosine monophosphate
CCL-39	Chinese hamster lung fibroblasts
Cdk	Cyclin dependent kinase
cGMP	Guanosine 3’, 5’ monophosphate
CNS	Central nervous system
Con A	Conconavilin A
COPD	Chronic obstructive pulmonary disease
Co-Smad	Common-mediator mothers against decapentaplegic protein
COX-2	Cyclo-oxygenase-2
CREB	cAMP response element binding protein
CRMP2	Collapsin response mediator protein 2
CTGF	Connective tissue growth factor
Cyto D	Cytochalasin D
DMEM	Dulbecco’s Modified Eagle Medium
DNA	Deoxyribonucleic acid
dRVP	Diastolic right ventricular pressure
DTT	Dithiothreitol

DUSP	Dual specificity phosphatase
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetra-acetic acid
EGTA	Ethyleneglycol-bis(2-aminoethyl)-N,N,N',N-tetra acetic acid
ELAM-1	Endothelial leukocyte adhesion molecule 1
eNOS	Endothelial nitric oxide
EPO	Eythropoietin
ERK	Extracellular signal-regulated protein kinase
ERM	Ezrin/radaxin/moesin
ET-1	Endothelin-1
ET_A	Endothelin A receptor
ET_B	Endothelin B receptor
ETC	Mitochondrial electron transport chain
FACS	Fluorescence-activated cell sorter.
FBS	Foetal bovine serum
GAP	GTPase-activating protein
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GDI	GDP dissociation protein
GDP	Guanine 5' diphosphate
GEF	Guanine nucleotide exchange factor
GFAP	Glial fibillary acidic protein
GI	Gastrointestinal tract
GPCR	G-protein-coupled receptor
GRK	G-protein-coupled receptor kinase
GTP	Guanine 5' triphosphate
HEPES	4-2-hydroxyethyl-1-piperazineethanesulphonic acid
HIF	Hypoxia inducible factor
HR	Heart rate
HRE	Hypoxic response element
HRP	Horseradish peroxidase
HPV	Hypoxic pulmonary vasoconstriction
IBS	Irritable bowel syndrome
Id	Inhibitor of differentiation

ICAM-1	Intracellular adhesion molecule-1
IgG	Immunoglobulin G
IL-1α	Interleukin-1α
IL-1β	Interleukin-1β
IL-6	Interleukin-6
iNOS	Inducible nitric oxide synthase
IPAH	Idiopathic pulmonary arterial hypertension
JNK	c-Jun N-terminal kinase
KDa	Kilo Daltons
KSR1	Kinase suppressor of Ras-1
K_v channels	Voltage-gated potassium channels
Lat B	Latrunculin B
LIMK	LIM Kinase
LPA	Lysophosphatidic acid
MAO	Monoamine oxidase
MAPK	Mitogen activated protein kinase
MAP2K	Mitogen activated protein kinase kinase
MAP3K	Mitogen activated protein kinase kinase kinase
MDC	Monodansylcadaverin
mDia	Mammalian diapharous protein
MEK	Mitogen activated protein kinase kinase
MLC	Myosin light chain
MLCK	Myosin light chain kinase
mRVP	Mean right ventricular pressure
mTOR	Mammalian target of rapamycin
MYPT1	Myosin phosphatase 1
NAC	N-acetyl-L-cysteine
NHE1	Na^+/H^+ exchange protein
NFκB	Nuclear factor kappa B
NO	Nitric oxide
NOS	Nitric oxide synthase
NP-40	Nonident P-40
PAEC	Pulmonary artery endothelial cell

PAF	Pulmonary artery fibroblast
PAH	Pulmonary arterial hypertension
PAP	Pulmonary artery pressure
PAR2	Protease-activated receptor 2
PASMC	Pulmonary artery smooth muscle cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDE	Phosphodiesterase
PDGF	Platelet derived growth factor
PDGFR	Platelet derived growth factor receptor
pERK	Phosphorylated extracellular regulated kinase
PH	Plextrin homology domain
PI3K	Phosphatidylinositol-3-kinase
PKA	Protein kinase A
PKB	Protein kinase B
PKC	Protein kinase C
PKN	Protein kinase N
PLC	Phospholipase C
PMA	Phorbyl 12-meristate-13-acetate
PMSF	Phenyl methyl sulphonyl fluoride
PP2A	Protein phosphatase 2A
PVR	Pulmonary vascular resistance
RB	Rho binding
RIPA	Radioimmunoprecipitation buffer
RKIP	Raf kinase inhibitor protein
RNA	Ribonucleic acid
ROC	Receptor operated caclium channel
ROCK	Rho-associated kinase
ROCK-ER	Rho-associated kinase – estrogen receptor fusion construct
ROS	Reactive oxygen species
RSmad	Receptor-activated mothers against decapentaplegic protein
RTK	Receptor tyrosine kinase
RV/LV+S	Ratio of right ventricle to left ventricle plus septum

RVH	Right ventricular hypertrophy
RVP	Right ventricular pressure
S6K1	p70 ribosomal S6 kinase
S1P	Spingosine-1-phosphate
SAP	Systemic arterial pressure
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
Smad	Mother against decapentaplegic protein
SMC	Smooth muscle cell
SOCC	Store operated calcium channel
SRF	Serum response factor
sRVP	Systolic right ventricular pressure
SSRI	Selective serotonin uptake inhibitor
TBS	TRIS-buffered saline
TEMED	N,N,N',N'-tetramethylethylenesiamine
TG	Transglutaminase
TGF-β	Transforming growth factor - β
TGF-βR	Transforming growth factor - β receptor
TMD	Transmembrane domain
TNFα	Tumour necrosis factor α
TPH	Tryptophan hydroxylase
TRIS	Hydroxymethyl-aminomethane
TRPC	Transient receptor potential channel
TXA_2	Thromboxane A_2
VEGF	Vascular endothelial derived growth factor
VEGFR	Vascular endothelial derived growth factor receptor
VIP	Vasoactive interstitial peptide
VSMC	Vascular smooth muscle cells
WT	Wildtype
5HT	5-hydroxytryptamine
5HTT	5-hydroxytryptamine transporter
$5HTT^+$	Mice overexpressing 5-hydroxytryptamine transporter

Chapter 1
INTRODUCTION

1.1 PULMONARY ARTERIAL HYPERTENSION

Pulmonary arterial hypertension can be characterised by sustained elevation of pulmonary vascular resistance (PVR) and pulmonary artery pressure (PAP), leading to impaired right-heart function and eventually failure. The condition is defined clinically by a mean pulmonary arterial pressure exceeding 25 mmHg at rest or 30 mmHg during exercise (Barst et al., 2004). Common symptoms include fatigue, exertional dyspnea, edema and syncope (Rich et al., 1987). As symptoms experienced by most patients are non-specific, diagnosis is frequently delayed.

Pulmonary hypertension is classified into 5 main categories: pulmonary arterial hypertension (PAH), pulmonary hypertension with left heart disease, pulmonary hypertension associated with lung diseases and/or hypoxaemia, pulmonary hypertension due to chronic thrombotic and/or embolic disease, and miscellaneous (Simonneau et al., 2004) (Table 1.1). Around 10% of patients that present with PAH without any identifiable cause have a family history of the disease, and are referred to as having familial PAH (Loyd et al., 1984), with remaining patients classified as having idiopathic PAH (IPAH). Familal PAH is an autosomal dominant disorder with incomplete penetrance and genetic anticipation, that was initially mapped to a locus designated PPH1 on chromosome 2q31-32 (Morse et al., 1997, Nichols et al., 1997). PPH1 was subsequently fine mapped to a 3cM region on chromosome 2q33 (Deng et al., 2000a), that has been associated with mutations in the BMPR2 gene encoding bone morphogenetic protein (BMP) type 2 receptor (BMPR-2) (Lane et al., 2000, Deng et al., 2000a). Germline mutations in BMPR-2 have been identified in 60% of patients with familial PAH and also in around 10% to 30% of those with the idiopathic form of the condition (Lane et al., 2000, Deng et al., 2000b, Thomson et al., 2000). Other genetic factors, such as polymorphisms in the 5-hydroxytryptamine (5HT) transporter gene, have also been associated with the development of the condition (Eddahibi et al., 2001). Moreover, PAH may occur secondary to other conditions, including collagen vascular disease, HIV infection or lung diseases such as chronic obstructive pulmonary disease (COPD) (Simonneau et al., 2004).

The idiopathic form of the disease occurs more frequently in women than men (>2:1) and is usually fatal within 3 years if untreated (Newman et al., 2004). Current therapies, such as prostacyclin and endothelin antagonists markedly improve physical function and survival, with the 5 year mortality rate around 50% (Newman et al., 2004).

Revised World Health Organisation Clinical Classification of Pulmonary Hypertension (Venice 2003)

Pulmonary arterial hypertension

Primary pulmonary hypertension
Idiopathic
Familial
Related to:
Collagen vascular disease
Congenital systemic to pulmonary shunts
Portal hypertension
HIV infection
Drugs/Toxins
Anorexigens
Other
Associated with significant venous or capillary involvement
Pulmonary veno-occlusive disease
Pulmonary capillary haemangiomatosis
Persistent pulmonary hypertension of the newborn

Pulmonary venous hypertension

Left-sided atrial or ventricular heart disease
Left-sided valvular heart disease

Pulmonary hypertension associated with lung diseases and/or hypoxaemia

Chronic obstructive pulmonary disease
Interstitial lung disease
Sleep-disordered breathing
Alveolar hypoventilation disorders
Chronic exposure to high altitude
Developmental abnormalities

Pulmonary hypertension due to chronic thrombotic and/or embolic disease

Thromboembolic obstruction of proximal pulmonary arteries
Thromboembolic obstruction of distal pulmonary arteries
Nonthrombotic pulmonary embolism (tumour, parasites, foreign material)

Miscellaneous

Sarcoidosis, histiocytosis X, lymphangiomatosis, compression of pulmonary vessels (adenopathy, tumour, fibrosing mediostinitis)

Table 1.1 Revised Clinical Classification of Pulmonary Hypertension.
The aim of classification was to identify different categories of pulmonary hypertension that shared similarities in pathophysiological mechanisms and clinical symptoms. Clinical classification is important in standardising diagnosis and treatment.

1.2 THE PULMONARY CIRCULATION

The pulmonary circulation is a closed circuit of vessels carrying blood between the heart and lungs. In healthy adults the system is a low-pressure, low-resistance circuit, with a mean PAP between 9 - 19 mmHg at sea level (Chemla et al., 2002). In addition to this, the pulmonary vessels are thin-walled and highly distensible, able to accommodate large increases in blood flow, such as that occurring during exercise, with minimal increases in PAP (Chemla et al., 2002, Vonk-Noordegraaf et al., 2005).

Pulmonary blood vessels are arranged to facilitate efficient gas exchange, carrying blood into the pulmonary microvasculature of the alveoli, where uptake of oxygen and the unloading of excess carbon dioxide occurs. The pulmonary arteries run alongside the airways, branching with them and producing a large surface area within the alveolar region by means of a capillary network sheet flow (Mandegar et al., 2004, Hislop and Pierce, 2000). Blood flow in the pulmonary arteries must be sufficient and at a linear velocity with the counter current for gas exchange to occur (Hislop and Pierce, 2000). Larger vessels in the proximal region of the pulmonary arterial network are elastic in structure with several layers of smooth muscle separated by collagen and elastic laminae (Hislop and Pierce, 2000). As the arterial tree advances distally and the vessels decrease in diameter, there is a gradual decrease in the level of muscularisation, with arteries only partially muscular or completely non-muscular (Hislop and Pierce, 2000, Meyrick and Reid, 1983, MacLean et al., 2000).

Unlike the systemic circulation, where hypoxic conditions result in vasodilation, in the pulmonary circulation vasoconstriction occurs (Aaronson et al., 2006). Hypoxic pulmonary vasoconstriction (HPV) is thought to be an adaptive mechanism, unique to the vessels of the lungs. HPV is greatest in resistance pulmonary arteries (200-300 μm diameter). In contrast, large pulmonary arteries behave like systemic arteries, relaxing in response to hypoxic conditions. HPV appears to be an intrinsic property of pulmonary artery smooth muscle cells (PASMCs) (Murray et al., 1990, Yuan et al., 1990) and is important in redirecting blood flow from poorly ventilated areas of the lung to better ventilated areas, maximising oxygenation of pulmonary venous blood

(Mandegar et al., 2004, Moudgil et al., 2005). The mechanisms of HPV are not fully understood but appear to involve the coordinated inhibition of voltage-gated K^+ (Kv) channels and activation of voltage-gated L-type calcium channels in resistance PASMCs (Weir et al., 2005). Furthermore, mitochondrial generation of reactive oxygen species (ROS) have been implicated in the mechanism of HPV. However, controversy exists as to whether an increase or decrease in ROS production occurs in response to hypoxia (Weissmann et al., 2006).

1.3 PATHOBIOLOGY OF PAH

Pulmonary hypertension is a disease of the small pulmonary arteries, resulting from the combined effects of pulmonary vasoconstriction, vascular remodelling and thrombosis (Gurbanov and Shiliang, 2006, Mandegar et al., 2004). These processes contribute to the increases in PVR and PAP witnessed in patients with PAH, putting excessive burden on the right ventricle due to the increased workload required to compensate for elevated downstream pressure, and eventually results in right-sided heart failure (Mandegar et al., 2004, Humbert et al., 2004). The pathogenesis of PAH is complicated and multifactorial.

1.3.1 PULMONARY VASOCONSTRICTION IN PAH

Pulmonary vasoconstriction is a main contributing factor to PVR and thus elevated PAP. Hypoxia plays a major role in vasoconstriction during PAH. For instance, in patients with conditions such as COPD and high altitude pulmonary edema, sustained alveolar hypoxia results in vasoconstriction of pulmonary vessels and vascular remodelling leading to the development of PAH (Mandegar et al., 2004). Excessive pulmonary vasoconstriction has also been related to abnormal K^+ channel functions and/or expression as well as endothelial dysfunction and calcium sensitisation (Moudgil et al., 2005).

ROLE OF K^+ CHANNELS IN PULMONARY VASOCONSTRICTION

K^+ channels play an important role in the regulation of pulmonary vascular tone (Nelson et al., 1990). Inhibition of K^+ channels depolarises PASMCs, resulting in the opening of voltage-gated Ca^{2+} channels, increasing cytoplasmic Ca^{2+} levels (Nelson et al., 1990, Yuan, 1995), which may trigger pulmonary artery vasoconstriction. Indeed, blockade of K_v channels using 4-aminopyridine has been found to cause pulmonary vasoconstriction (Hasunuma et al., 1991). Hypoxia also inhibits K^+ currents and depolarises PASMC membranes (Post et al., 1992, Yuan et al., 1993), suggesting a role for K^+ channels in mediating HPV . Certain K^+ channels are sensitive to O_2 levels as they possess cysteine and methionine groups which are subject to reduction or oxidation by redox mediators, such as reactive oxygen species (Moudgil et al., 2005). Some K_v channels, such as the $K_v1.5$ channel, respond to redox mediators by altering their gating and open-state probability (Archer et al., 2004). Oxidants, including H_2O_2, increase potassium currents in PASMCs, whereas reducing agents inhibit them (Reeve et al., 1995). The redox theory of HPV suggests that under normoxic conditions, there is basal production of reactive oxygen species, such as H_2O_2 by the electron transport chain of PASMC mitochondria maintaining the K_v current (Archer et al., 1986, Reeve et al., 1995). However, this mechanism is upset during hypoxic conditions, resulting in the decreased production of H_2O_2 inhibiting K_v channels and resulting in activation of voltage gated L-type Ca^{2+} channels, which subsequently increases intracellular Ca^{2+} levels and induces HPV (Moudgil et al., 2005, Moudgil et al., 2006) (Figure 1.1). Several O_2-sensitive K_v channels may be involved in mediating HPV, including $K_v1.5$ (Archer et al., 1998, Archer et al., 2001), $K_v2.1$ (Archer et al., 1998, Patel et al., 1997) and possibly $K_v1.2$ (Hulme et al., 1999) and $K_v3.1b$ (Osipenko et al., 2000). For instance, in mice deficient in $K_v1.5$, HPV is markedly attenuated (Archer et al., 2001). Indeed, $K_v1.5$ channels have been found to be downregulated in PASMCs from patients with PAH (Yuan et al., 1998), as have both $K_v1.5$ and $K_v2.1$ channels in rats with chronic hypoxia-induced PAH (Michelakis et al., 2002b).

ROLE OF INTRACELLULAR Ca^{2+} HOMEOSTASIS IN PULMONARY VASOCONSTRICTION

Intracellular Ca^{2+} plays an essential role in pulmonary vasoconstriction, with both extracellular calcium influx and release of intracellular calcium from the sarcoplasmic reticulum involved in mediating HPV (Salvaterra and Goldman, 1993, Gelband and Gelband, 1997). Smooth muscle contraction is initiated by an elevation in intracellular free calcium concentration ($[Ca^{2+}]_i$). Calmodulin (CaM), an intracellular Ca^{2+}-binding protein, binds to Ca^{2+} as $[Ca^{2+}]_i$ rises. The resulting CaM/Ca^{2+} complex then binds to and activates myosin light chain kinase (MLCK) leading to phosphorylation of the myosin light chain (MLC) of the contractile apparatus. This process stimulates the activation of the myosin ATPase, hydrolysing ATP to generate energy for the cycling of myosin cross-bridges with actin filaments. The formation of these cross bridges brings about smooth muscle contraction, resulting in vasoconstriction (Somlyo and Somlyo, 1994) (Figure 1.2).

$[Ca^{2+}]_i$ is elevated in PASMCs exposed to hypoxia (Salvaterra and Goldman, 1993, Wang et al., 2005, Bakhramov et al., 1998, Cornfield et al., 1993). This hypoxia-induced increase in $[Ca^{2+}]_i$ results in PASMC contraction (Murray et al., 1990, Leach et al., 1994, Jin et al., 1992). Furthermore, hypoxia triggers increases in $[Ca^{2+}]_i$ and contraction in small intrapulmonary arteries that is sustained during the hypoxic period and reversed on return to normoxia (Robertson et al., 2000b). Several studies have reported the predominant source of Ca^{2+} contributing to elevated $[Ca^{2+}]_i$ during hypoxic conditions is extracellular and enters PASMCs *via* the voltage-gated L-type calcium channel (Bakhramov et al., 1998, Cornfield et al., 1993). Inhibition of these channels has been shown to attenuate hypoxic responses in pulmonary arteries (Jin et al., 1992, Leach et al., 1994) and HPV in isolated lungs and intact animals (Redding et al., 1984, Stanbrook et al., 1984, Simonneau et al., 1981, McMurtry et al., 1976).

In addition to this, the release of calcium from intracellular stores has also been proposed to mediate HPV. Gelband and Gelband (1997) reported that in rat pulmonary arteries, the initial event in HPV is the release of Ca^{2+} from intracellular stores, resulting in elevated $[Ca^{2+}]_i$ and increased pulmonary artery

tone. Furthermore, in canine pulmonary arteries, release of Ca^{2+} from the sarcoplasmic reticulum was found to contribute significantly to HPV (Jabr et al., 1997). Depletion of Ca^{2+} stores have also been shown to abolish hypoxic responses in PASMCs (Dipp et al., 2001). Similarly, release of intracellular calcium, from ryanodine-sensitive stores contributes significantly to HPV in perfused lungs (Morio and McMurtry, 2002).

Entry of Ca^{2+} *via* store-operated Ca^{2+} channels (SOCCs) may also be important in mediating HPV. As mentioned previously, in PASMC under acute hypoxic conditions, release of Ca^{2+} from intracellular stores has been observed resulting in increased $[Ca^{2+}]_i$. In some instances this has been reported to enhance capacitative Ca^{2+} entry *via* SOCCs (Kang et al., 2003, Ng et al., 2005) and may therefore contribute to HPV. Furthermore, in another study where intracellular Ca^{2+} stores were depleted and voltage-gated L-type Ca^{2+} channels blocked, hypoxia still resulted in an increase in $[Ca^{2+}]_i$. This was attributed to the ability of acute hypoxia to enhance Ca^{2+} entry *via* SOCCs (Wang et al., 2005). Chronic hypoxia has also been found to upregulate members of the canonical transient receptor potential channel (TRPC) gene family (TPRC1 and TPRC6) in pulmonary arteries, resulting in increased expression of SOCCs and receptor-operated Ca^{2+} channels (ROCs) in PASMCs. The enhanced activity of these channels under hypoxic conditions contributes to elevated $[Ca^{2+}]_i$ and increased pulmonary vascular tone (Lin et al., 2004) (Figure 1.3). Further highlighting the role of intracellular Ca^{2+} release in mediating HPV, cyclic ADP ribose, which acts on ryanodine receptors to stimulate intracellular Ca^{2+} release, is elevated by hypoxia in small pulmonary arteries. Moreover, contraction to hypoxia in these arteries was completely suppressed by using a cyclic ADP ribose antagonist (Wilson et al., 2001).

Smooth muscle cell contraction is dependent not only on $[Ca^{2+}]_i$ but also on the Ca^{2+} sensitivity of the contractile apparatus (Morgan, 1987). Under conditions of agonist-mediated contraction, Ca^{2+} sensitisation can occur. For example, in permeabilised SMCs, agonists can enhance the force of contraction when intracellular Ca^{2+} remains constant (Kitazawa et al., 1989). One mechanism of Ca^{2+} sensitisation suggests altered relations between myosin regulatory light chain phosphorylation and intracellular Ca^{2+}, involving the MLCK or phosphatase cascades (de Lanerolle and Paul, 1991). Another possible

mechanism of sensitisation involves alterations in Ca^{2+} affinity of regulatory proteins such as caldesmon and calponin. These proteins are associated with thin filaments and inhibit actin-myosin interaction, a process attenuated by Ca^{2+} (Winder et al., 1998). Ca^{2+} sensitisation also occurs in pulmonary arteries under hypoxic condition. During sustained HPV, force development continues to increase while $[Ca^{2+}]_i$ attains a constant level (Robertson et al., 1995, Robertson et al., 2003). In addition to this, Rho-associated kinase (ROCK) inhibition has been shown to suppress sustained HPV in pulmonary arteries and perfused lung (Robertson et al., 2000a). Furthermore, ROCK has been proposed to contribute to sustained HPV by mediating Ca^{2+} sensitisation in pulmonary arteries (Nagaoka et al., 2004).

ROLE OF REACTIVE OXYGEN SPECIES IN PULMONARY VASOCONSTRICTION

HPV is an important physiological response of the lung to alveolar hypoxia, required to redistribute pulmonary blood flow from areas of low oxygen to high oxygen availability. However, the underlying oxygen sensing and signal transduction mechanisms of HPV remain unclear. Several studies have reported that inhibition of the mitochondrial electron transport chain (ETC) specifically inhibits HPV (Michelakis et al., 2002a, Weissmann et al., 2003, Waypa et al., 2001). These findings suggest a role for mitochondria as oxygen sensors for HPV. Two conflicting hypotheses have developed concerning a role for mitochondria in HPV. (1) The redox hypothesis of HPV suggests that a decrease in mitochondrial ROS occurs during hypoxia, shifting the cellular redox balance towards a more reduced state. As mentioned previously, this can result in the inhibition and closure of K_v channels, a process mediated by the redox pairs GSH/GSSG and NADH/NAD (Moudgil et al., 2005, Michelakis et al., 2002c). (2) In direct contrast to this, a hypoxia-induced increase in mitochondrial ROS production has also been reported. This increase in ROS is thought to mediate HPV by triggering intracellular calcium release (Waypa et al., 2001, Waypa and Schumacker, 2005).

In support of the former hypothesis, rotenone and antimycin A (inhibitors of the proximal region of the ETC), were shown to mimick the effects of HPV in

isolated pulmonary arteries and PASMCs, decreasing ROS production and subsequently resulting in the inhibition of potassium channels (Michelakis et al., 2002a). Furthermore, under normoxic conditions, pharmacological inhibition of the ETC proximal to complexes II and III (sites of mitochondrial ROS release) resulted in a reduction in ROS production and an increased pulmonary artery pressure (Michelakis et al., 2004). Taken together these findings suggest a decrease in mitochondrial-produced ROS mediates HPV.

On the other hand, others have provided evidence that *increased* ROS release from ETC complex III occurs under hypoxic conditions in isolated rat lung, and that this increase in ROS mediates HPV (Waypa et al., 2001). Furthermore, inhibition of the proximal region of the ETC was found to attenuate hypoxia-induced constriction in PASMCs by decreasing intracellular Ca^{2+} levels (Waypa et al., 2002). Conversely, hypoxia has been reported to trigger increases in $[Ca^{2+}]_i$ by augmenting ROS signalling from mitochondria (Waypa et al., 2006). Therefore, these findings suggest an increase in mitochondrial-produced ROS mediates HPV by facilitating increases in $[Ca^{2+}]_i$.

NAD(P)H-oxidases, enzymes that function to generate superoxide, have also been proposed as possible oxygen sensors of HPV. Again, two conflicting concepts exist as to the contribution of NAD(P)H-oxidase-derived superoxide in HPV, with one reporting an upregulation and another reporting a downregulation of superoxide. Reports that NADPH-oxidase is activated in response to hypoxia in PASMCs (Marshall et al., 1996), and that the generation of superoxide and subsequent formation of H_2O_2 are required for HPV in perfused rabbit lung (Weissmann et al., 1998), suggest that NADPH-mediated increases in superoxide are required to mediate HPV. Increased superoxide generation and an increased expression of NADPH-oxidase has also been observed in pulmonary arteries from pulmonary hypertensive fetal lambs (Brennan et al., 2003). The hypothesis that NADPH-mediated superoxide production contributes to HPV is further confirmed by the ability of NADPH-oxidase inhibitors to attenuate HPV in intact lungs (Weissmann et al., 2000). But, it has also been suggested an NADPH-oxidase-mediated decrease in superoxide occurs during HPV (Wolin et al., 1999). Several studies have reported decreases in superoxide and H_2O_2 generation under hypoxic conditions. This has been suggested to attenuate the cellular levels of cyclic

guanosine 3', 5' monophosphate (cGMP) due to reduced stimulation of soluble guanylyl cyclase and thus results in vasoconstriction (Burke-Wolin and Wolin, 1989, Burke-Wolin and Wolin, 1990, Cherry et al., 1990, Wolin et al., 1999).
In summary, while ROS play a key role in mediating pulmonary vascular tone in hypoxia, there is no consensus as to whether ROS are increased or decreased under hypoxic conditions.

ROLE OF ENDOTHELIAL DYSFUNCTION IN PULMONARY VASOCONSTRICTION

Endothelial dysfunction results in an imbalance in the production of vasodilatory and vasoconstrictive agents. In PAH an insufficient level of vasodilators such as nitric oxide (NO) and prostacyclin, in addition to an increase in the production of vasoconstrictors such as endothelin-1 (ET-1), favours the vasoconstriction of pulmonary arteries. For instance, prostacyclin synthesis has been found to be decreased in endothelial cells from patients with PAH due to a reduction in the expression of prostacyclin synthase, the enzyme responsible for its synthesis (Christman et al., 1992, Tuder et al., 1999). Endothelium-derived vasodilators are further decreased in PAH by the reduction of endothelial NO synthesis (eNOS) expression, reported in endothelial cells of patients (Giaid and Saleh, 1995). In addition to this, vasoactive interstitial peptide (VIP), a potent pulmonary vasodilator, has also been found to be reduced in serum from PAH patients (Petkov et al., 2003). To further contribute to the vasoconstrictive environment, increased levels of ET-1, which induces vasoconstriction, have been reported in both animal models and patients with PAH (Giaid et al., 1993). It can therefore be seen that endothelial dysfunction results in an imbalance of endothelium-derived mediators, favouring vasoconstriction and contributing to the pathobiology of PAH.

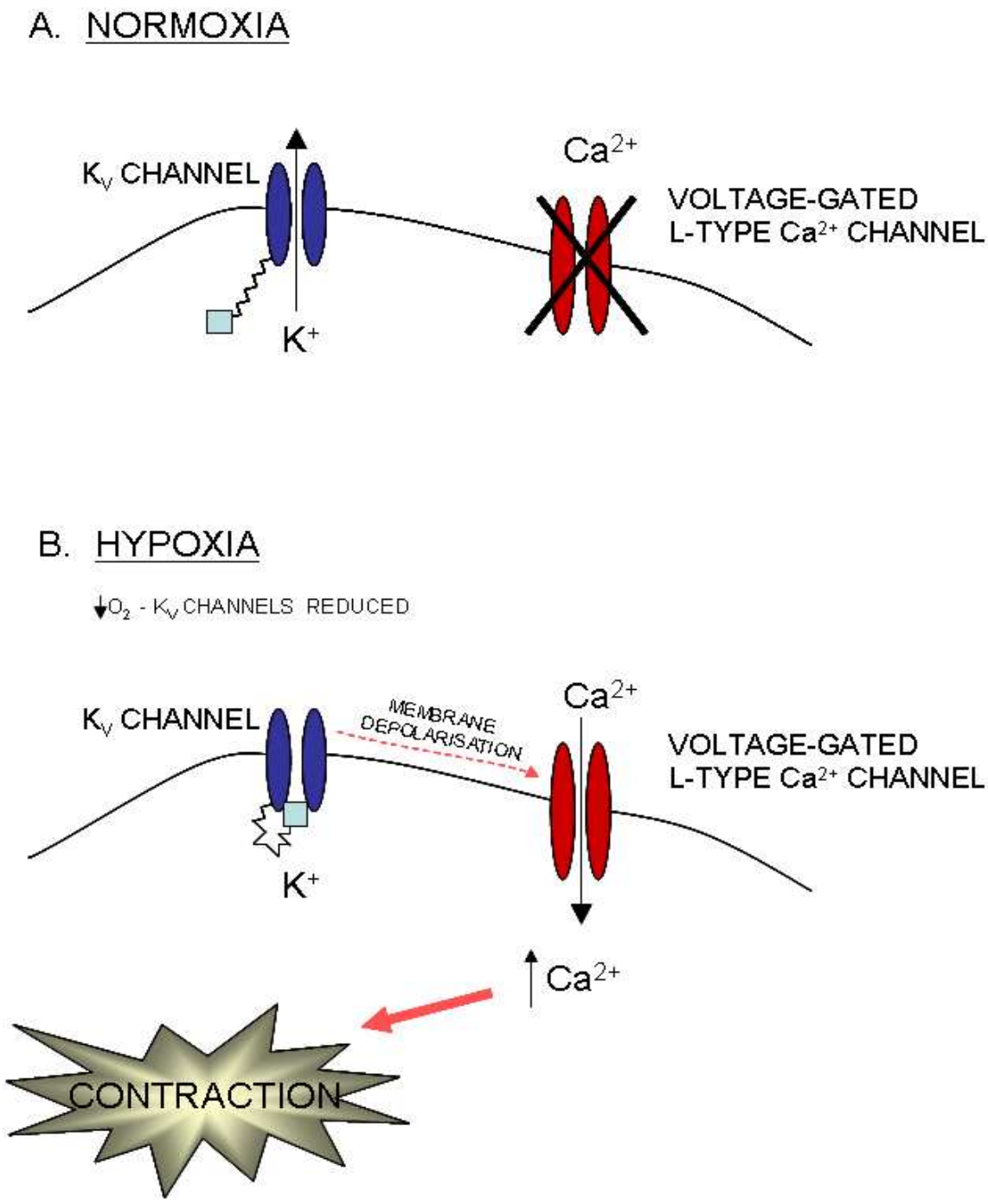

Figure 1.1 The role of potassium channel activity in mediating hypoxic pulmonary vasoconstriction.
Normoxia is associated with open, oxidised K_v channels. Under hypoxic conditions, K_v channels become reduced, resulting in their closure and membrane depolarisation. This facilitates the opening of voltage-gated L-type Ca^{2+} channels. Ca^{2+} influx *via* these channels elevates intracellular Ca^{2+} concentrations, resulting in vasoconstriction. Oxidising compounds mimic the effects of normoxia, while reducing agents mimic the effects of hypoxia.

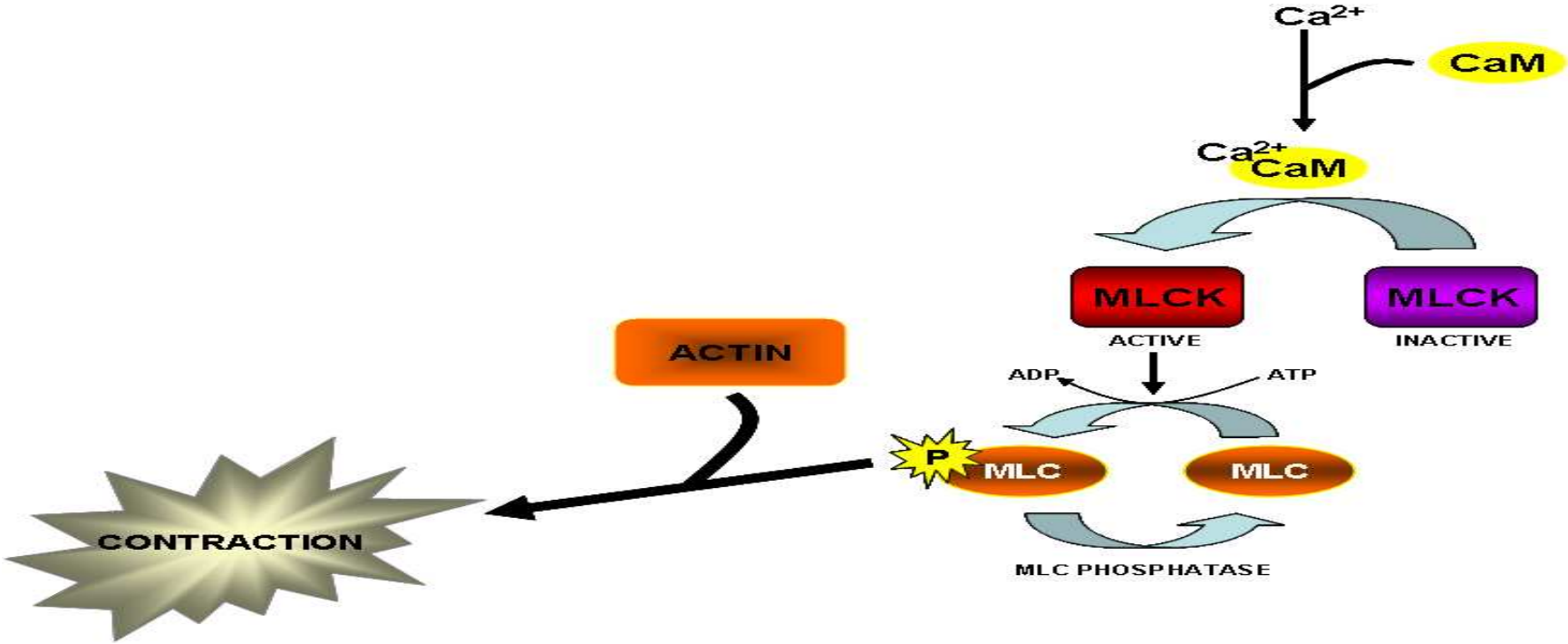

Figure 1.2 Signal transduction mechanisms mediating contraction of vascular smooth muscle.
The level of free intracellular calcium (Ca^{2+}) is a major determinant of smooth muscle contraction. As free intracellular calcium (Ca^{2+}) rises, it binds and forms a complex with calmodulin (CaM). The Ca^{2+}/CaM complex then activates myosin light chain kinase (MLCK), which subsequently phosphorylates the myosin light chain (MLC). This stimulates myosin ATPase activity and promotes crossbridge cycling with actin filaments. The formation of these crossbridges underlies smooth muscle cell contraction and results in vasoconstriction.

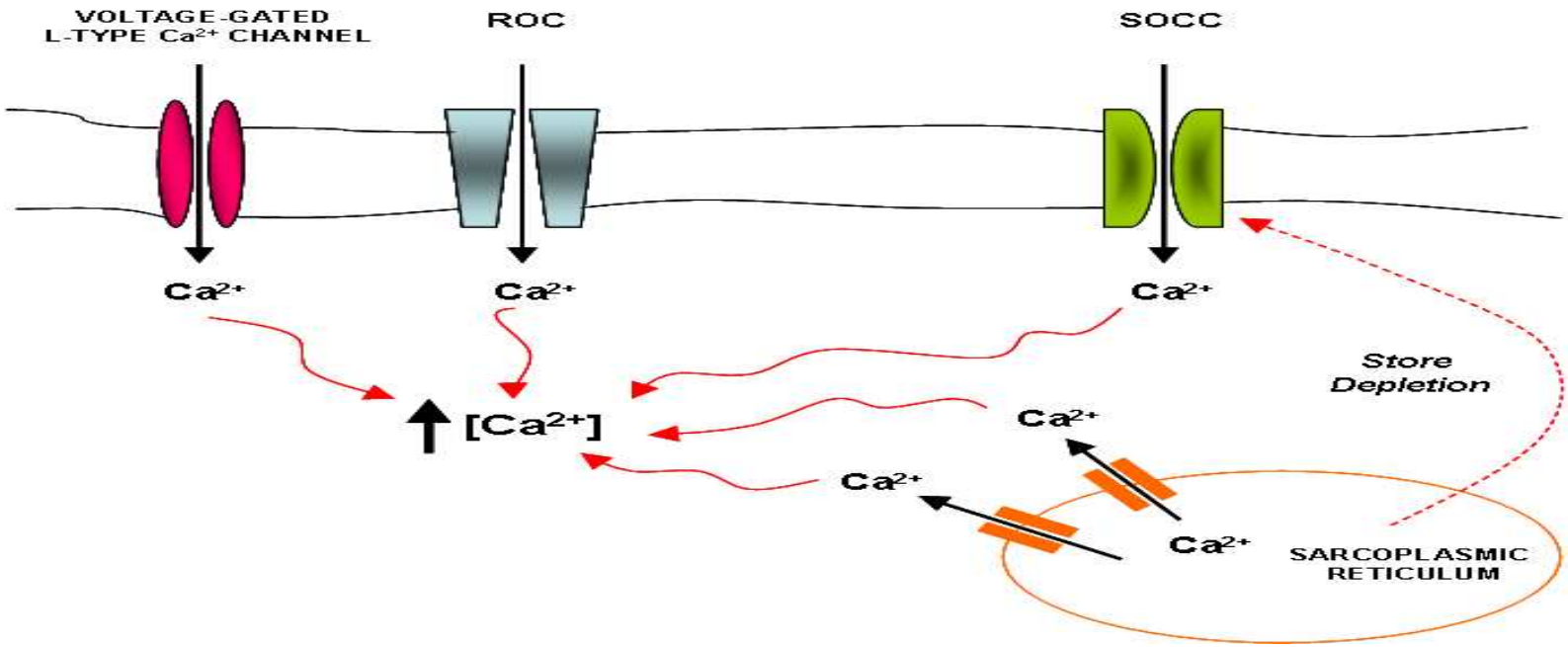

Figure 1.3 Diagram of the proposed mechanisms contributing to increased intracellular Ca^{2+} concentrations and hypoxic pulmonary vasoconstriction (HPV).
Hypoxia results in decreased activity of K_v channels and membrane depolarisation, thereby initiating the opening of L-type Ca^{2+} channels and allowing Ca^{2+} influx. Intracellular Ca^{2+} is also elevated by the hypoxic activation of receptor-operated Ca^{2+} channels (ROC) and store-operated Ca^{2+} channels (SOCC). In addition, hypoxia induces calcium release from intracellular stores in the sarcoplasmic reticulum, further elevating intracellular Ca^{2+}. The depletion of these stores is also thought to contribute to the activation of SOCC.

1.3.2 PULMONARY VASCULAR REMODELLING

Vascular remodelling is a term often used to describe structural and functional alterations to blood vessels, allowing them to function more effectively. It is an adaptive process which occurs in response to long-term changes in haemodynamic conditions that occur during development (Mulvany et al., 1996). However, the process can contribute to the pathobiology of various vascular diseases, including PAH.

Under normal conditions the diameter of the pulmonary artery wall is maintained by a balance between proliferation and apoptosis of pulmonary artery fibroblasts (PAFs), PASMCs and pulmonary artery endothelial cells (PAECs) (Gurbanov and Shiliang, 2006, Mandegar et al., 2004). In PAH this balance is disrupted in the favour of proliferation, resulting in an increase in the thickness of the artery wall and a narrowing of the vessel lumen which eventually results in its complete occlusion. These structural changes result in the loss of vascular compliance and are responsible for the increase in PVR observed in PAH patients (Mandegar et al., 2004, Gurbanov and Shiliang, 2006). Pulmonary vascular remodelling can be initiated by a variety of stimuli, including anorectic drugs, collagen vascular disease and chronic hypoxia (Mandegar et al., 2004). This process involves all layers of the vessel wall and is complicated by the cellular heterogeneity that exists within the pulmonary artery wall (Jeffery and Morrell, 2002) (Figure 1.4).

CONTRIBUTION OF PULMONARY ARTERY SMOOTH MUSCLE CELLS (PASMCs) TO VASCULAR REMODELLING

Common to the pathogenesis of all forms of human PAH is an increase in the thickness of the medial layer of muscular pulmonary arteries and the muscularisation of distal, previously non-muscular pulmonary arteries (Humbert et al., 2004, Stenmark and Mecham, 1997, MacLean et al., 2000). The increased thickness of the medial compartment is due to hypertrophy and proliferation of SMCs and the increased synthesis and deposition of matrix proteins including elastin and collagen (Stenmark and Mecham, 1997). The

muscularisation of previously non-muscular arteries may be due to the differentiation and hypertrophy of intermediate cells and pericytes present in the vessel wall, which acquire a smooth muscle like appearance (Meyrick and Perkett, 1989, Durmowicz and Stenmark, 1999) . During PAH, changes in the phenotype of PASMCs occur, which contribute to the process of vascular remodelling. For instance, PASMCs taken from patients with PAH have been shown to have increased proliferative capabilities (Eddahibi et al., 2001). Furthermore, it has also been suggested that smooth muscle cells (SMCs) may contribute to intimal thickening and neointima formation by migrating from the media to the subendothelial layer, where they change phenotype from contactile to synthetic and secrete excessive extracellular matrix proteins (Olschewski et al., 2001). In addition to this, the pulmonary artery is composed of phenotypically diverse SMC populations (Frid et al., 1994). These subpopulations exhibit different proliferative responses to hypoxia-induced PAH, a property that be may important in the process of medial remodelling (Wohrley et al., 1995)(Figure 1.4).

CONTRIBUTION OF PULMONARY ARTERY FIBROBLASTS (PAFs) TO VASCULAR REMODELLING

Fibroblasts also play an important role in vascular remodelling. Located in the adventitial compartment, fibroblasts proliferate, hypertrophy and increase production of extracellular matrix proteins (Stenmark et al., 2002, Stenmark and Mecham, 1997). The most dramatic structural changes occur in the adventitia of small pulmonary arteries as a result of these processes (Stenmark et al., 1987, Murphy et al., 1981, Durmowicz et al., 1994). The pulmonary vasculature appears to contain multiple, functionally distinct subpopulations of fibroblasts. These subpopulations have marked differences in morphology and proliferative capabilities and this may play a crucial role in regulating vascular remodelling (Das et al., 2002). Unlike, SMCs, hypoxia induces proliferation in pulmonary artery fibroblasts in the absence of exogenous growth factors (Falanga and Kirsner, 1993, Storch and Talley, 1988). Furthermore, fibroblasts have been observed to proliferate earlier and have a greater response to hypoxia than endothelial or SMCs (Belknap et al., 1997). In addition to an increase in the

number of fibroblasts in the adventitial layer of hypoxic pulmonary arteries, a large increase in matrix protein deposition has been observed, with increases in collagen, elastin and tropoelastin reported (Durmowicz et al., 1994). Fibroblasts have also been shown undergo changes in cytoskeletal and contractile protein expression, significantly altering their function. This process results in the expression of α-smooth muscle actin within the cells, suggesting conversion to myofibroblasts, which may contribute to the development of PAH (Short et al., 2004). The hypoxic activation of fibroblasts may also have some bearing on the increase in SMC proliferation observed in PAH (Rose et al., 2002). Moreover, fibroblasts have been proposed to migrate to the medial and intimal layers of pulmonary vessels during PAH contributing to neointimal formation (Humbert et al., 2004) (Figure 1.4).

CONTRIBUTION OF PULMONARY ARTERY ENOTHELIAL CELLS (PAECs) TO VASCULAR REMODELLING

In chronic hypoxic PAH, increases in intimal thickness occur due to hypertrophy and hyperplasia in both the endothelial and sub-endothelial layers (Stenmark and Mecham, 1997). Plexiform lesions occur in a large percentage of patients with PAH (Tuder et al., 1994). These lesions originate in small precapillary vessels usually at blood vessel bifurications (Stevens, 2005). Increases in endothelial cell proliferation contribute to the formation of such lesions (Tuder et al., 1994, Voelkel and Tuder, 1995) and in some instances plexiform lesions occur as a result of monoclonal endothelial cell proliferation (Lee et al., 1998a). Plexiform lesions grow into the vessel resulting in lumen occlusion (Stevens, 2005). Enothelial cells within these lesions have a pro-proliferative, anti-apoptotic phenotype and no longer grow in a monolayer, with the resulting intravascular growth resembling a tumour (Tuder et al., 2001). Other abnormalities have been observed in the endothelial cells of these lesions compared to normal pulmonary endothelial cells (Loscalzo, 1992). For example, a decreased production of vasodilators (Tuder et al., 1999, Giaid and Saleh, 1995) and increased production of vasoconstrictors have been reported (Giaid et al., 1993). Furthermore, these endothelial cells also express pro-angiogenic molecules such as vascular endothelial derived growth factor

(VEGF) and VEGF receptor (VEGFR), which may contribute to cell proliferation and vascular remodelling (Tuder and Voelkel, 2001). Changes in surface coagulant properties and proinflammatory cytokine production have also been observed in endothelial cells from PAH patients. For instance, under hypoxic conditions, thrombomodulin production is suppressed and procoagulant activity increased (Ogawa et al., 1990). Furthermore, hypoxia also increases interleukin-1α (IL-1α) production by endothelial cells and upregulates endothelial-leukocyte adhesion molecule-1 (ELAM-1) and intracellular adhesion molecule-1 (ICAM-1) on the cell surface (Shreeniwas et al., 1992). This suggests hypoxia induces changes in the endothelial layer promoting coagulation and increasing the interaction of endothelial cell with circulating inflammatory cells. In support of this hypothesis, increased adherence of leukocytes and platelets has been observed in *in vivo* models of PAH (Hung et al., 1986). Increased inflammatory infiltrates have also been observed in a variety of plexiform lesions (Balabanian et al., 2002). Recently, it has also been proposed that PAECs may contribute to vascular remodelling under chronic hypoxic conditions by transdifferentiating into smooth muscle-like cells (Zhu et al., 2006). This may be an alternative explanation for the muscularisation of previously non-muscular vessels in the process of PAH (Figure 1.4).

ROLE OF APOPTOSIS IN VASCULAR REMODELLING

Apoptosis, or programmed cell death, is a fundamental biological function involved in many physiological and pathological processes. It can be defined as a well ordered form of cell death comprised of a regulated sequence of events, resulting in removal of cell material without the release of harmful substances to surrounding tissue (Renehan et al., 2001). In PAH the balance between proliferation and apoptosis is disturbed. In PASMCs, increased proliferation and decreased apoptosis contribute to the thickening of the vessel wall and vascular remodelling (Stenmark and Mecham, 1997, Mandegar et al., 2004). Several lines of investigation implicate decreases in apoptosis in the development and maintenance of PAH. For instance, PASMCs from patients with PAH have been shown to be resistant to apoptotic inducers such as BMP - 2, 5 and 7 (Zhang et al., 2003). Furthermore, a reduction in K_v channel

expression and function in PASMCs attenuates programmed cell death by decelerating apoptotic volume decrease (AVD) and inhibiting cytoplasmic caspases (Zhang et al., 2003). The anti-apoptotic protein Bcl-2 has also been reported to be increased in the lungs of PAH patients. Further highlighting the role of apoptosis in pulmonary vascular remodelling, the induction of apoptosis in models of PAH has been found to result in the regression of hypertrophied PASMCs (Cowan et al., 1999, Rabinovitch, 1998)(Figure 1.4).

1.3.3 VASCULAR THROMBOSIS

Thrombosis often occurs in the pulmonary arterioles of many PAH patients and may result from injury to the endothelium, abnormal fibrinolysis, enhanced procoagulant activity and platelet abnormalities (Humbert et al., 2004, Veyssier-Belot and Cacoub, 1999, Herve et al., 2001). Indeed, decreased fibrinolytic activity has been observed in the plasma of patients with PAH (Welsh et al., 1996, Frank et al., 1997). Furthermore, the endothelium plays an important role in regulating coagulation. The decrease in production of prostacyclin and NO by endothelial cells of PAH patients is likely to contribute to thrombosis as both of these mediators inhibit platelet aggregation (Moncada and Vane, 1979, Moncada et al., 1991). Moreover, the production of thrombomodulin, a co-factor produced by endothelial cells that binds and inactivates thrombin to attenuate coagulation, is significantly decreased in individuals with PAH (Welsh et al., 1996). The imbalance in the level of pro- and anti-coagulant factors in the favour of coagulation contributes to thrombus formation in PAH patients (Figure 1.4).

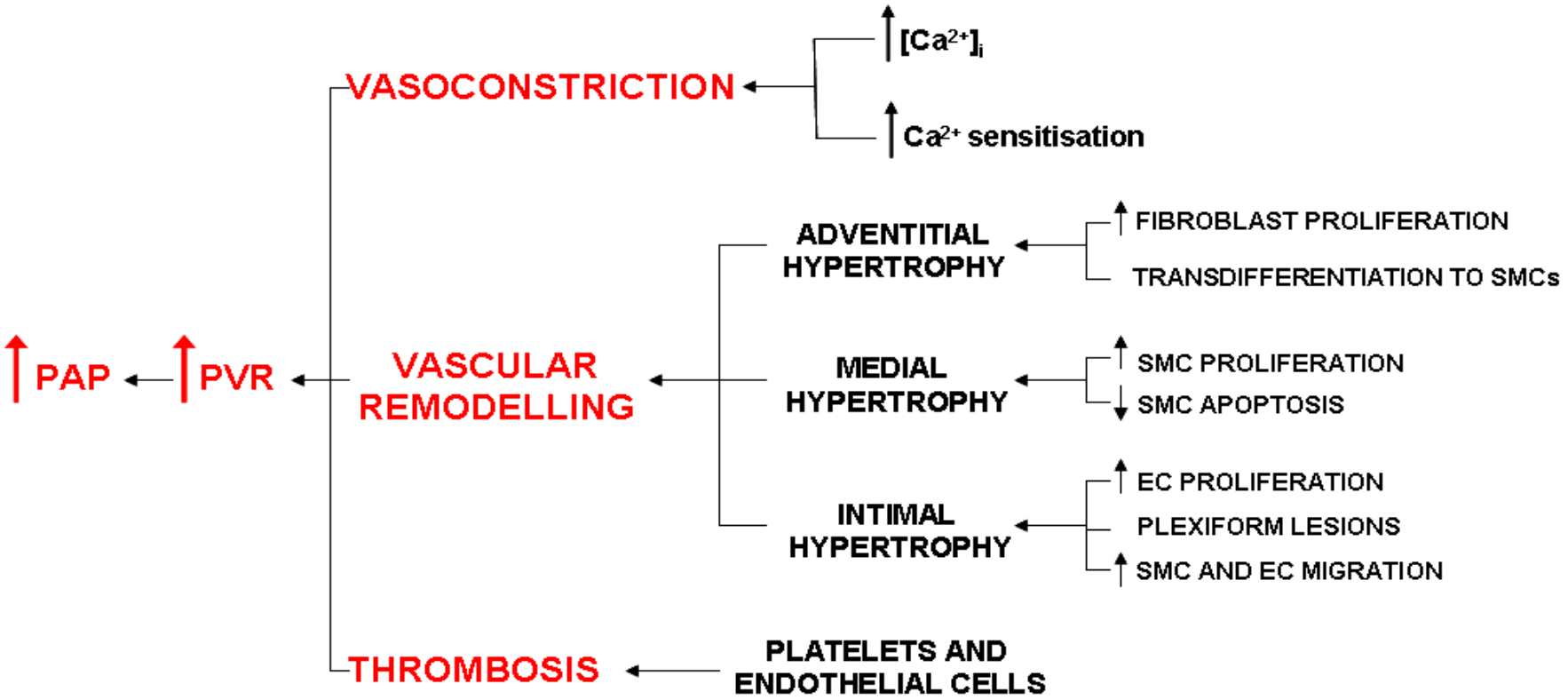

Figure 1.4 Schematic diagram of how individual pathophysiological components contribute to increased pulmonary vascular resistance and pulmonary artery pressure during pulmonary hypertension.
Changes in cellular function within the wall of pulmonary arteries contribute to pulmonary vascular remodelling, vasoconstriction and thrombosis, leading to increased pulmonary vascular resistance (PVR) and pulmonary artery pressure (PAP), characteristic of pulmonary hypertension. Figure adapted from Mandegar *et al*., (2004).

1.3.4 MEDIATORS OF PAH

Given the multifactorial pathobiology of PAH, various mediators have been implicated in the development and progression of the disease. Several factors mentioned previously, which affect vascular tone, also promote vascular remodelling e.g. ET-1 and Kv channels (Mandegar et al., 2004). Genetic factors, such as mutations in the BMPR-2 gene also contribute to the development of the condition, however in most cases PAH is not attributed to inherited genetic mutations (Thomson et al., 2000). Therefore, other external stimuli play a major role in contributing to the development of PAH. Some of the key mediators involved in the development and maintenance of PAH are described below.

ROLE OF THE TRANSFORMING GROWTH FACTOR-β (TGF-β) SUPERFAMILY AND SIGNALLING PATHWAYS IN VASCULAR REMODELLING

The TGF-β superfamily of structurally related cytokines includes TGF-β, BMPs and activins. The TGF-β superfamily members induce a multitude of effects such as cell differentiation, proliferation, migration and apoptosis in a variety of cell types (Roberts and Sporn, 1993). As mentioned previously, mutations in the BMPR-2 gene have been associated with the pathogenesis of PAH, as have somatic mutations in the TGF-β type 1 receptor (TGF-βR1) and abnormalities in TGF-β signalling (Yeager et al., 2001, Richter et al., 2004). Briefly, signal transduction of TGF-β superfamily receptor ligands requires the ligand-induced formation of heteromeric complexes of type 1 and type 2 transmembrane serine/threonine kinase receptors (Derynck and Zhang, 2003). Different receptor combinations allow for differential ligand binding properties and diverse signalling responses to the same ligand (Derynck and Zhang, 2003). Ligand binding induces the phosphorylation of the GS segment in the type 1 receptor by the type 2 receptor kinases. The active type 1 receptor then phosphorylates the receptor-activated mothers against decapentaplegic (R-Smad) proteins. Activated R-Smads can then dimerise with a common mediator-Smad (Co-

Smad) to form a signalling complex capable of translocating to the nucleus, resulting in the transcription of Smad-responsive genes, some of which encode anti-proliferative and pro-apoptotic proteins (Derynck and Zhang, 2003) (Figure 1.5). The BMPR-2 receptor has been documented to interact with three different type 1 receptors (ALK-2, ALK-3 and ALK-6) and signals *via* the R-Smads, Smad 1,5 and 8 (Derynck and Zhang, 2003, Moustakas et al., 2001) (Figure 1.5).

The exact mechanisms as to how BMPR-2 and TGF-β mutations and alterations in signalling pathways contribute to vascular remodelling in PAH are not fully understood, but it is thought they have a negative effect on pro-apoptotic Smad complexes. In normal human PASMCs, treatment with BMPs results in increased apoptosis and a reduction in levels of the anti-apoptotic protein Bcl-2, thereby triggering the release of cytochrome C from mitochondria and activating caspases 3, 8 and 9 (Lagna et al., 2006, Zhang et al., 2003). However, in cells taken from patients with PAH, BMP-induced apoptosis is significantly attenuated (Zhang et al., 2003, Yang et al., 2005, Lagna et al., 2006). These cells displayed mutations in the kinase domain or carboxy-teminus of BMPR-2 and were also found to be deficient in Smad signalling (Lagna et al., 2006, Yang et al., 2005, Zhang et al., 2003). Loss of function mutants in BMPR-2 have also been reported to contribute to increased endothelial cell survival and decreased apoptosis (Teichert-Kuliszewska et al., 2006). In addition to this, in the monocrotaline-rat model of PAH, TGF-β receptor (TGF-βR) 1 and TGF-βR2, as well as Smad 3 and 4, were all found to be reduced in the lung and PASMCs, resulting in decreased TGF-β-induced signalling and apoptosis (Zakrzewicz et al., 2007). Furthermore, in mice developed to incorporate an inducible dominant-negative mutant of the TGF-βR2 receptor, chronic hypoxia-induced pulmonary hypertension was significantly attenuated (Chen et al., 2006).

TGF-β activity may also influence other factors implicated in vascular remodelling. For instance, TGF-β induces ET-1 production in human pulmonary arteries (Markewitz et al., 2001), as well as stimulating connective tissue growth factor (CTGF) production in pulmonary fibroblasts (Kucich et al., 2001). These findings suggest a role for the TGF-β superfamily of receptors and downstream signalling cascades in the development of pulmonary hypertension, and also

highlight the requirement for a balance between apoptosis and proliferation in the maintenance of a healthy vascular system.

POTASSIUM CHANNELS AND VASCULAR REMODELLING

As mentioned previously, K_v channels play an important role in mediating vasoconstriction during PAH. The downregulation of $K_v1.5$ and $K_v2.1$ channels that occurs during PAH also play a significant role in pulmonary vascular remodelling. Chronic decreases in K^+ channel expression induces PASMC proliferation and hypertrophy (Mandegar et al., 2004). This is thought to occur as loss of K_v current depolarises the cell membrane resulting in the intracellular accumulation of calcium, with the Ca^{2+}/Calmodulin complex subsequently formed responsible for activating several steps of the cell cycle and thus favours cellular proliferation (Hardingham et al., 1997). In addition to the downregulation of K_v channels contributing to proliferation, it also appears to have an effect on apoptotic processes. Previously, activation of K_v channels has been reported to play an important role in both early and late volume decrease associated with the induction of apoptosis (Platoshyn et al., 2002). Therefore, the downregulation of these channels in PAH has an inhibitory effect on apoptosis. Furthermore, the resulting intracellular accumulation of K^+ ions due to the loss of K_v channels has been shown to inhibit caspases and thus decrease apoptosis (Thornberry and Lazebnik, 1998, Bortner and Cidlowski, 1999, Bortner et al., 1997). In addition to this, survivin, a known inhibitor of apoptosis has been reported to be expressed in PAH, resulting in K_v channel downregulation in PASMCs (McMurtry et al., 2005). Overexpression of survivin in normal PASMCs promotes proliferation and decreases K_v current. Conversly, treatment with a dominant negative survivin mutant was found to significantly increase K_v current in PASMC from PAH patients and attenuate the effects of experimental PAH *in vivo* (McMurtry et al., 2005).

In summary, it can be seen that K_v channels play an important role in maintaining the balance between apoptosis and proliferation in pulmonary smooth muscle cells, and that the downregulation of these channels in PAH tips the balance in favour of proliferation and thus contributes to vascular remodelling.

ENDOTHELIUM-DERIVED FACTORS AND VASCULAR REMODELLING

As mentioned previously, endothelial-derived factors play an important role in regulating vascular tone. However, changes in the synthesis and expression of these mediators during PAH also contribute to vascular remodelling. In addition to its potent vasodilatory effects NO also has anti-proliferative capabilities. Therefore its reduction in PAH may be a factor in the remodelling process. Indeed, decreases in endothelial NOS (eNOS) expression were reported in the vascular endothelium of patients with PAH, with expression levels correlating inversely to the severity of the histological changes observed (Giaid and Saleh, 1995). Furthermore, in a hypoxic model of PAH, prolonged inhaled NO therapy resulted in a marked attenuation in the level of muscularised arteries and right ventricular hypertrophy (RVH) (Horstman et al., 1998). Inhaled NO has also been used in the treatment of patients, markedly reducing pulmonary artery pressure and PVR (Channick et al., 1996).

NO mediates it effects by binding to and activating guanylyl cyclase, thus elevating intracellular levels of cGMP (Stasch et al., 2002). Phosphodiesterase (PDE) enzymes are important in regulating the cellular levels of cGMP and cyclic 3', 5' adenosine monophosphate (cAMP), by controlling their rates of degradation (Bender and Beavo, 2006). Several studies have reported the upregulation of PDEs during PAH, including elevated levels of PDE1, 3 and 5 (Schermuly et al., 2007, Murray et al., 2002, Murray et al., 2007, Maclean et al., 1997). Selective inhibition of these PDEs has been found to have antiproliferative effects (Murray et al., 2007, Wharton et al., 2005) and reduce pulmonary vascular remodelling (Schermuly et al., 2004, Schermuly et al., 2007, Sebkhi et al., 2003, Garg et al., 2006).

In addition to its vasoconstrictive effects, ET-1 acts as a growth factor (Yanagisawa, 1994) and has been found to play a crucial role in vascular remodelling during PAH. ET-1 predominantly binds to two receptors, endothelin-A (ET_A) and endothelin-B (ET_B) receptors. Responses to ET-1 appear to be mainly *via* the ET_A receptor subtype (Arai et al., 1990, Barnes and Liu, 1995). ET_A receptors are found on smooth muscle cells only and, when activated, induce vasoconstriction and cellular proliferation. ET_B receptors on smooth muscle cells, when activated, cause vasoconstriction, whereas those on

endothelial cells produce vasodilation (*via* production of NO) and clearance of circulating ET-1 (Galie et al., 2004). Hypoxia has been shown to attenuate ET_B-induced vasoconstriction in rat pulmonary arteries. This study suggested that the hypoxia-induced production of ROS increases ET-1 release from endothelial cells and results in the downregulation of ET_B receptors on SMCs (Wang et al., 2006).

The upregulation of ET-1 occurs in the lungs of patients with various etiologies of PAH (Giaid et al., 1993). Confirming the role of ET-1 in the development of PAH, treatment with ET antagonists have been found to attenuate the development of pulmonary vascular remodelling in a variety of models of the condition (Kim et al., 2000, Oparil et al., 1995, Eddahibi et al., 1995, Okada et al., 1995). Furthermore, bosentan, a competitive antagonist at both ET_A and ET_B receptors, is benefical in the treatment of human PAH (Rubin et al., 2002). Therefore, endothelial-derived mediators play an important role in regulating vascular tone and cell proliferation within the pulmonary vasculature, with dysregulation of these mediators significantly contributing to the development of PAH.

THE POTENTIAL ROLE OF EICOSANOIDS IN PULMONARY VASCULAR REMODELLING

Eicosanoids such as prostacyclin and thromboxane A_2 (TXA_2) have been implicated in the pathophysiology of PAH. As mentioned previously, TXA_2 levels are elevated in endothelial cells from patients with PAH. This contributes to vasoconstriction during PAH and also has mitogenic effects, in addition to contributing to platelet aggregation. Patients with PAH have elevated levels of urinary 11-dehydro-TXB_2, a major urinary metabolite of TXA_2, suggesting a role for TXA_2 in the pathogenesis of PAH (Christman et al., 1992). In an *in vivo* model of the condition, inhibition of TXA_2 significantly reduced arterial media thickness, as well as RVH, delaying the onset of PAH. These beneficial effects were suggested to be mediated mainly by inhibiting platelet aggregation (Nagata et al., 1997).

Prostacyclin synthesis is also altered in PAH. In addition to its vasodlilatory properties, prostacyclin inhibits smooth muscle cell proliferation and platelet

aggregation (Fetalvero et al., 2007). As mentioned earlier, endothelial cells from patients with PAH have reduced expression of prostacyclin synthase (Tuder et al., 1999) and decreased levels of prostacyclin metabolites have been observed in the urine of such patients (Christman et al., 1992). Prostacylin therapy is a mainstay in the treatment of PAH, with many prostacylin analogues such as epoprostenol, treprostinil and beraprost improving the pulmonary function of patients (Lee and Rubin, 2005). Another such analogue, iloprost, has been reported to have beneficial effects on pulmonary vascular remodelling *in vivo*; reducing RVH, medial wall thickness and the number of muscularised pulmonary arteries in monocrotaline-induced PAH (Schermuly et al., 2005b). In summary, the balance between the actions of TXA_2 and prostacyclin are important in maintaining a healthy pulmonary vasculature. Alterations in levels of these mediators during PAH contribute to the progression of the disease.

ANGIOGENIC AND GROWTH FACTORS AND VASCULAR REMODELLING

Abnormalities in the expression of various angiogenic factors and growth factors have been suggested to play a role in PAH. One such factor, namely VEGF, has been implicated in various etiologies of PAH. VEGF is a vascular endothelial cell-specific mitogen with pro-angiogenic properties and is secreted by various cell types. In systemic vessels, increases in VEGF bioavailability at sites of endothelial injury accelerate repair and attenuate neointimal formation (Asahara et al., 1995). In addition to this, VEGF overexpression within the vascular wall restores endothelium dependent relaxation and protects against vasoconstriction and platelet aggregation (Thomas, 1996). VEGF is abundant in the adult lung (Monacci et al., 1993) and can be regulated by hypoxia (Monacci et al., 1993, Liu et al., 1995). Indeed, increase in VEGF, VEGFR1 and VEGFR2 have been observed in the lungs of hypoxic rats (Tuder et al., 1995, Christou et al., 1998). Similary, the upregulation of VEGF has also been observed in platelets from patients with PAH (Eddahibi et al., 2000b), in addition to increased levels of VEGF and VEGFRs in the lungs of such patients (Hirose et al., 2000). In particular VEGF and VEGFR expression appears to be upregulated in plexform lesions (Tuder and Voelkel, 2001), with VEGF levels elevated in SMCs inside and adjacent to these areas (Hirose et al., 2000).

Furthermore, VEGFRs were also observed to be expressed on endothelial cells in and around these lesions (Hirose et al., 2000). In addition to its beneficial effects in the systemic circulation, overexpression of VEGF also appears to play a protective role in the pulmonary circulation, as highlighted by various in vivo studies. For instance, VEGFR2 inhibition in chronically hypoxic rats results in severe PAH (Taraseviciene-Stewart et al., 2001). Conversly, $VEGF_A$ overexpression markedly attenuates the development of hypoxic PAH in rats. Comparable effects were also observed in rats where gene transfer of $VEGF_A$ to the pulmonary microvasculature had been undertaken. In these animals the effects of monocrotaline-induced PAH were significantly reduced even in PAH that was already established (Campbell et al., 2001). $VEGF_A$ overexpression in chronically hypoxic rats also has a protective effect (Partovian et al., 2000). Similarly, overexpression of VEGFB has also been reported to have beneficial effects (Louzier et al., 2003). It has been suggested the advantageous effects of VEGF overexpression may be due to its ability to protect endothelial function and upregulate mediators such as eNOS (Kroll and Waltenberger, 1998, Hood et al., 1998, Partovian et al., 2000). Furthermore, it has also been proposed that endogenous levels of VEGF do not counteract PAH, but that it is the upregulation of VEGF that assists in combating the condition. This is supported by data showing that in mice, where $VEGF_B$ has been knocked out, haemodynamic changes in response to hypoxia remain unaffected (Louzier et al., 2003). Moreover, in patients with PAH receiving prostacyclin therapy, levels of VEGF are elevated compared to control (Eddahibi et al., 2000b).

Angiopoetin-1 (Ang-1), a smooth muscle-secreted ligand that plays a pivitol role in vasculogenesis and is an important mediator of both physiological and pathological angiogenesis (Hayes et al., 1999), has also been implicated in the development of PAH. Ang-1 signal transduction occurs *via* the endothelial-specific receptor Tie2 (Davis et al., 1996). Ang-1 is absent in normal adult lung tissue, but appears to be constitutively expressed in lungs from PAH patients (Du et al., 2003). A potential role for Ang-1 in PAH is also highlighted by development of the condition in rodents genetically engineered to constitutively express Ang-1 in the lung (Sullivan et al., 2003). Furthermore, Ang-1 has also been reported to attenuate the expression of BMPR1a, which is required for BMPR2 signalling in PAECs (Du et al., 2003). Given the role of BMPR2 in

PAH, inhibition of its signalling by Ang-1 may contribute to vascular remodelling. It has also been suggested that Ang-1 may contribute to pulmonary artery remodelling by stimulating the proliferation of PASMCs. Human PAECs treated with Ang-1 have been shown to produce 5HT, which then acts on SMCs resulting in their proliferation (Sullivan et al., 2003). However, in contrast to a notion that Ang-1 contributes to the development of PAH, a protective role has also been reported. In this study, Ang-1 gene therapy reduced mortality, systolic right ventricular pressure (sRVP) and RVH in monocrotaline-induced PAH (Zhao et al., 2003). Although contradictory, these studies imply a role for Ang-1 in the pulmonary hypertensive process. More recently, other studies have confirmed the pathogenic effects of Ang-1 in PAH. For instance in some rodent models of PAH, gene transfer of a Tie2 receptor antagonist markedly reduced the development of PAH (Kido et al., 2005). Furthermore, another study showed increased levels of Tie2 receptor expression and phosphorylation in PAECs from PAH patients (Dewachter et al., 2006). This also suggests the Ang-1/Tie 2 pathway is potentiated in PAH patients, resulting in the increased production of endothelial cell-derived growth factors such as ET-1 and 5HT which may then contribute to SMC proliferation.

Platelet-derived growth factor (PDGF) may also contribute to PAH. In an animal model of the condition, increased levels of PDGF receptors (PDGFR) have been observed (Balasubramaniam et al., 2003). Furthermore, it has also been reported that PDGF antagonists have beneficial effects in rats with monocrotaline-induced PAH, in addition to the chronic hypoxic mouse model of the condition, reversing vascular remodelling in both instances (Schermuly et al., 2005a).

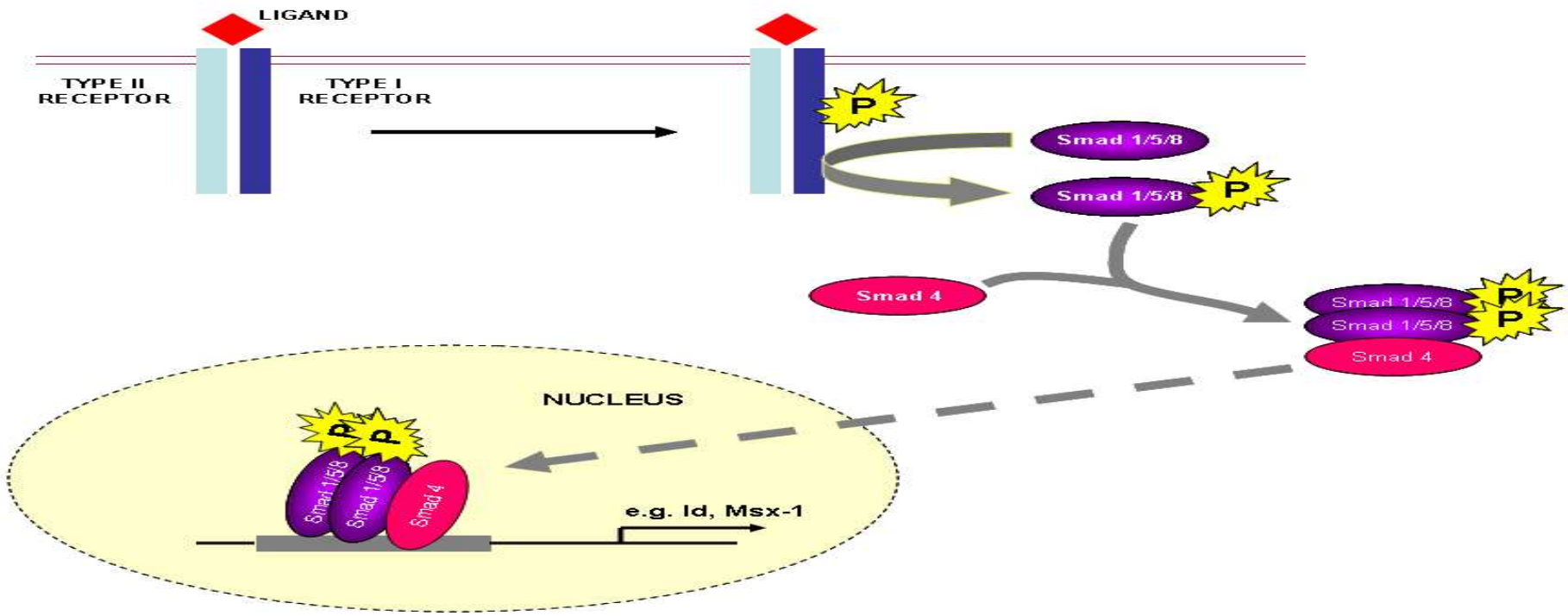

Figure 1.5 TGF-β receptor signal transduction pathway induced by BMP ligands.

BMP ligands (BMP-2,-4,-7) bind the receptor complex at the cell surface inducing phosphorylation of the type 1 receptor by the type 2 receptor. The active type 1 receptor then phosphorylates and activates Smad1/5/8, which subsequently dimerise and interact with the common mediator Smad, Smad 4. This complex then translocates to the nucleus resulting in the transcription of Smad-responsive genes such as Id (inhibitor of differentiation) and Msx-1 (Miyazono et al., 2005, Alvarez Martinez et al., 2002). Figure adapted from Derynck and Zhang (2003).

1.4 5-HYDROXYTRYPTAMINE (5HT)

In addition to the mediators of PAH mentioned previously, 5HT has been shown to play a pivitol role in the disease process, as will be described in the following section.

1.4.1 5HT STRUCTURE, SYNTHESIS AND METABOLISM

5HT, or “serotonin”, was chemically identified by Rapport *et al* (1948) as one of the major vasoconstricting substances in defibrinated blood, originating from platelets. It was subsequently found to also act as a major neurotransmitter, being involved in a variety of processes carried out by the central nervous system (CNS), as well as regulating several functions in the periphery (Hoyer et al., 2002). 5HT is a monoamine, its structure comprising an amino group connected to an “indole” group by an ethyl chain (Figure 1.6).

Production of 5HT occurs in a variety of cells including neurons, enterochromafin cells and endothelial cells. The biosynthesis pathway converts dietary tryptophan to 5-hyroxytryptophan by the action of the enzyme tryptophan hydroxylase (TPH). This process is the rate-limiting step in the generation of 5HT. Two isoforms of TPH exist; TPH2 which is expressed abundantly in the brain, and TPH1, which is responsible for the synthesis of 5HT in the periphery (Nakamura and Hasegawa, 2007). 5-hydroxytryptophan is then decarboxylated by a ubiquitous animo-acid decarboxylase, resulting in the formation of 5HT (Figure 1.7). Upon synthesis, 5HT is taken up and stored by platelets as they pass through the intestinal circulation, as well as being stored in neurons and chromaffin cells.

Degradation and inactivation of 5HT occurs through the action of monoamine oxidases (MAO), which catalyse its oxidative deamination. Monoamine oxidases are found in most tissues and exist in 2 forms encoded by different genes, MAO-A and MAO-B. It is MAO-A that preferentially catalyses the degradation of 5HT (Youdim and Bakhle, 2006). Deamination by MAO-A is then followed by oxidation to 5-hydroxyindoleacetic acid, which is excreted in the urine (Figure 1.7).

1.4.2 PHYSIOLOGICAL FUNCTIONS OF 5HT

The actions of 5HT are numerous and complex, varying between species and this is reflected by the large array of 5HT receptors that have been identified. The physiological effects of 5HT are mediated by 14 different receptor subtypes. These receptors are divided into 7 distinct classes ($5HT_1$ – $5HT_7$), mainly on the basis of their structural and functional characteristics (Hoyer et al., 2002). 5HT is also a substrate for the 5HT transporter (5HTT), which actively uptakes 5HT into cells (Torres et al., 2003).

The gastrointestinal tract is one of the main sites of action of 5HT, where the monoamine stimulates gastrointestinal motility, fluid secretion and is involved in eliciting nausea and vomiting (Gershon and Tack, 2007). In addition to this, 5HT is also involved in the process of platelet aggregation, the dysregulation of which can contribute to thrombus formation and vascular disease (McNicol and Israels, 2003, Torr et al., 1990, Noble and Drake-Holland, 1990). 5HT also plays a role in nociception, stimulating nociceptive sensory nerve endings in a variety of tissues (Goadsby, 2000, Graven-Nielsen and Mense, 2001, Jeong et al., 2004). Drugs targeting 5HT receptors are widely used in the treatment of migrane (Villalon et al., 2003). Furthermore, a variety of processes in the CNS are regulated by 5HT; these include hallucinations and behavioural changes, sleep wakefulness, mood, feeding behaviour and control of sensory transmission (Barnes and Sharp, 1999). Clinical conditions associated with disturbed 5HT function include depression, anxiety, schizophrenia, carcinoid syndrome, and, as mentioned previously, migraine. (Bleich et al., 1988),

1.4.3 GPCR REGULATION

With the exception of the $5HT_3$ receptor, which is a ligand-gated ion channel, the 5HT receptors are members of the G-protein-coupled receptor (GPCR) suprefamily (Hoyer et al., 2002). GPCRs regulate many physiological processes and include receptors for a range of chemically diverse hormones, neurotransmitters and chemokines (Pierce et al., 2002). Comprised of an extracellular N-terminus, 7 transmembrane spanning helices and an intracellular C-terminus, most GPCRs signal by activating heterotrimeric G-proteins (Pierce

et al., 2002) (Figure 1.8). G-proteins consist of α, β and γ subunits, and are generally referred to by their α-subunit. To date 16 Gα subunits have been identified and grouped into 4 families, G_s, G_q, G_i and $G_{12/13}$ (Milligan and Kostenis, 2006). In addition to this, 5 β and 12 γ subunits have been reported (Milligan and Kostenis, 2006). The pathways stimulated by GPCRs are dependent on the type of G-protein they associate with and receptors typically couple with one or more type of G-protein (Hamm, 1998). The association of G-protein with receptor is generally *via* the second or third intracellular loop of the GPCR (Hawes et al., 1994, Lameh et al., 1992). The binding of agonist to its receptor results in a conformational change, facilitating the activation of the G-protein by promoting the exchange of GDP for GTP at the guanine nucleotide binding site of Gα-subunit (Hamm, 1998, Pierce et al., 2002). This process results in the dissociation of the Gα subunit from the Gβγ subunits, which remain bound together. The activated Gα subunit and βγ dimer are then free to act as mediators of receptor-stimulated signalling pathways (Hamm, 1998) (Figure 1.9). Gi_α subunits mediate intracellular signalling by inhibiting adenylyl cyclase, where as Gs_α subunits stimulate adenylyl cyclase (Pierce et al., 2002). In addition to this, Gq_α, activates phospholipase Cβ, while $G_{12/13}$ have been shown to be involved in small G protein activation and cytoskeletal remodelling (Pierce et al., 2002, Ulloa-Aguirre et al., 1999). Furthermore, Gβγ subunits can activate a variety of proteins, including PLCβ, (Clapham and Neer, 1997).

1.4.4 5HT RECEPTOR CLASSES

5HT mediates its physiological effects *via* activation of its multiple receptor subytpes. The $5HT_1$ receptor class consists of 5 receptor subtypes: $5HT_{1A}$, $5HT_{1B}$, $5HT_{1D}$, $5ht_{1E}$ and $5ht_{1F}$ receptors. In humans these receptors share 40-63% sequence identity and mediate their effects by coupling preferentially to Gi_α, thus inhibiting adenylyl cyclase and cAMP production (Hoyer et al., 2002). $5ht_{1E}$ and $5ht_{1F}$ receptors are designated with lower case to indicate that a physiological role for these endogenous receptor has not yet been demonstrated (Hoyer et al., 2002). However, physiological functions for $5HT_{1A}$, $5HT_{1B}$ and $5HT_{1D}$ receptors have been well characterised. $5HT_{1A}$ receptors are largely distributed throughout the CNS and in the raphe nuclei act as

autoreceptors to attenuate serotonergic neuron firing (Hoyer et al., 2002). This receptor subtype is also involved in modulating many behavioural effects such as anxiety and depression (Hoyer et al., 2002). Currently, the $5HT_{1A}$ receptor agonist buspirone is used clinically in the treatment of these conditions (Den Boer et al., 2000).

$5HT_{1B}$ receptors are expressed in the CNS where they act as terminal autoreceptors (Hoyer et al., 2002) and may also be involved in controlling the release of other neurotransmitters such as acetylcholine and noradrenaline (Pauwels, 1997). Also located on cerebral arteries and other vascular tissues, they play a role in mediating contraction (Hoyer et al., 2002). The $5HT_{1D}$ receptor possesses 63% structural homology with the $5HT_{1B}$ receptor, although its expression is low compared with the $5HT_{1B}$ (Hoyer et al., 2002). $5HT_{1D}$ receptors have been found in the human heart where they modulate 5HT release (Hoyer et al., 2002). In addition to this, the receptor may also play a role in neurogenic inflammation and nociception. Indeed, $5HT_{1D}$ receptor antagonists have been shown to suppress these processes in guinea pig models, suggesting it may be a therapeutic target in the treatment of migraine (Cutrer et al., 1999). Many currently available drugs used in the treatment of migraine act as antagonists at both $5HT_{1B}$ and $5HT_{1D}$ receptors (Hoyer et al., 2002).

The $5HT_2$ class of receptor includes the $5HT_{2A}$, $5HT_{2B}$ and $5HT_{2C}$ receptors which exhibit 46-50% overall sequence homology. These receptors couple preferentially to Gq, activating phospholipase C (PLC) to increase the formation of inositol 1,4,5 trisphosphate and elevate cytosolic Ca^{2+} (Hoyer et al., 2002). $5HT_{2A}$ receptors are widely expressed in both the CNS and periphery, where they are involved in mediating vascular SMC contraction in bronchial, uterine and urinary tissues (Hoyer et al., 2002). $5HT_{2A}$ receptors are also involved in mediating platelet aggregation, thrombosis and increased capillary permeability following exposure to 5HT (Nagatomo et al., 2004). In the CNS, stimulation of this receptor mediates secretion of hormones such as renin and prolactin (Van de Kar et al., 2001). Furthermore, activation of this receptor class also mediates many behavioural functions and has been implicated in conditions such as schizophrenia (de Angelis, 2002). The $5HT_{2B}$ receptor has been located in the gastric fundus where it mediates fundic SMC contraction (Hoyer

et al., 2002). They are also expressed in the heart, brain and lung (Hoyer et al., 2002). The $5HT_{2B}$ receptor has been associated with promoting cell cycle progression in fibroblasts (Nebigil et al., 2000b) and cell survival in cardiomyocytes (Nebigil et al., 2003). It may also contribute to cardiac hypertophy (Nebigil and Maroteaux, 2003). Furthermore, the $5HT_{2B}$ receptor is required for normal heart formation during embryogenesis (Nebigil et al., 2000a). Expression of the $5HT_{2C}$ receptor is widespread throughout the CNS and also found in the choroid plexus (Hoyer et al., 2002). $5HT_{2C}$ receptors have been found to mediate various behavioural effects and are involved in conditions such as anxiety, depression and panic disorders (Lacivita and Leopoldo, 2006). This receptor subtype also plays a role in regulating dopaminergic function and inhibits dopaminergic transmission (Di Matteo et al., 2002). Additionally, $5HT_{2C}$ receptors have been found to be involved in mediating seizures and convulsion and as such are being considered in the treatment of epilepsy (Isaac, 2005). Furthermore, appetite and glucose homeostasis as also controlled, in part by the $5HT_{2C}$ receptor (Lacivita and Leopoldo, 2006). Notably, many antipsychotic drugs mediate their effects *via* antagonism of $5HT_{2A}$ and $5HT_{2C}$ receptors (Leysen, 2004).

Located on both central and peripheral neurones, the $5HT_3$ receptors are ligand-gated ion channels which trigger rapid depolarisation due to Na^+ and Ca^{2+} influx and K^+ efflux (Hoyer et al., 2002). $5HT_3$ receptors play a particularly important role in the gut, regulating gastric motility and intestinal secretion (Hoyer et al., 2002). These receptors are also involved in mediating chemotherapy- and radiotherapy-induced nausea and emesis (Gandara et al., 1998, Hoyer et al., 2002).

$5HT_4$, $5ht_6$ and $5HT_7$ receptors all couple preferentially to Gs, promoting cAMP formation (Hoyer et al., 2002). Located in the GI tract, heart and CNS, $5HT_4$ receptors mediate a variety of functions (Hoyer et al., 2002). In the gut, $5HT_4$ receptors are involved in regulating gastric motility (Degen et al., 2001) and as a result, $5HT_4$-selective agonists such as prucalopride are used in the treatment of some forms of irritable bowel syndrome (IBS) (De Schryver and Samsom, 2000, Spiller, 2004). In addition to this, $5HT_4$ receptors have been found in atria and ventricles of the heart, where they regulate heart rate, atrial contractile force and relaxation (Bach et al., 2001, Hegde and Eglen, 1996). Furthermore,

CNS effects of the $5HT_4$ receptor include modulation of neurotransmitter release, enhanced synaptic transmission and a potential role in memory (Hoyer et al., 2002). $5ht_6$ receptors are almost exclusively expressed in the CNS, where they have been shown to regulate glutamatergic and cholinergic neuronal activity (Woolley et al., 2004). This receptor may also be involved in the regulation of cognition and feeding (Woolley et al., 2004). $5HT_7$ receptors are expressed extensively in the vasculature and also in nonvascular smooth muscle, where it mediates relaxation (Thomas and Hagan, 2004). In the CNS, this receptor may also be involved in anxiety and cognitive disturbances (Thomas and Hagan, 2004). Possible roles in circadian rhythms, sleep, thermoregulation and learning and memory have also been reported (Hedlund and Sutcliffe, 2004, Jovanovska and Prosser, 2002, Thomas and Hagan, 2004). Little is known about the $5ht_5$ receptor class. Two subtypes ($5ht_{5A}$ and $5ht_{5b}$) have been identified in rodents, however only the $5ht_{5A}$ gene has been found to encode a functional protein in humans (Nelson, 2004). $5ht_5$ receptors are thought to couple to $G_{i/o}$, decreasing levels of cAMP (Carson et al., 1996, Francken et al., 1998). With widespread expression in the CNS, the $5ht_{5A}$ receptor has been proposed to act as an autoreceptor and may also be involved in the control of circadian rhythms (Thomas, 2006) (Figue 1.10).

1.4.5 5HT TRANSPORTER (5HTT)

The 5HT tansporter is a member of the NaCl-dependent transporter family that cotransport their substrate together with Na^+ and Cl^- ions (Torres et al., 2003). 5HTT is responsible for the clearance of 5HT from the synaptic cleft following release of the neurotransmitter, thereby terminating its action (Torres et al., 2003). Other cell types also express 5HTT, including platelets (Talvenheimo and Rudnick, 1980), which acquire extracellular 5HT for subsequent release in the process of platelet activation (Cirillo et al., 1999). Expression has also been observed in the intestinal tract (Wade et al., 1996), adrenal gland (Schroeter et al., 1997), blood vessels, heart (Ni and Watts, 2006) and lungs (Marcos et al., 2004). Furthermore, 5HTT is one of the primary targets for drugs used in the treatment of depression, including tricyclic antidepressants and selective serotonin reuptake inhibitors (SSRIs) (Zohar and Westenberg, 2000), as well as

being a target for drugs of abuse such as amphetamines, NMDA and cocaine (Ricaurte et al., 2000, Koob, 2000).

Structurally, 5HTT consists of 12 transmembrane domains (TMDs), connected by 6 extracellular and 5 cytoplasmic loops, with both the amino and carboxyl termini residing in the cytoplasm (Torres et al., 2003, Nelson, 1998) (Figure 1.11). In TMD1, an aspartic acid residue (D98) is key for substrate recognition, possibly through interactions between the carboxyl group and the positive change of the amine group of 5HT (Barker et al., 1999). In addition to this TMD3 and the third intracellular loop are also important in the function of 5HT binding and translocation. The TMD3 binds 5HT at a site located in the translocation path, while the third intracellular loop contains a reactive cysteine residue (Cys357), sensitive to conformational changes that result from ion and ligand binding (Androutsellis-Theotokis et al., 2001).

The driving force for 5HT uptake is the ion concentration gradient generated by the plasma membrane Na^+/K^+ ATPase. 5HTT cotransports 5HT with one Na^+ and one Cl^- ion, while the counter-transport of K^+ outwards is required in the translocation mechanism (Torres et al., 2003, Nelson, 1998). Binding of 5HT, Na^+ and Cl^- induces a conformational change in the transporter allowing exposure of the 5HT binding site to the opposite side of the membrane and thus transport of substrate and ions (Nelson, 1998, Torres et al., 2003). The reorientation of 5HTT then requires the binding and outward transport of intracellular K^+ ions (Nelson, 1998, Torres et al., 2003). In certain circumstances 5HTT may act in reverse, transporting 5HT out of the cell. This mechanism is especially important in the action of amphetamines, which induce massive release of monoamines (Seiden et al., 1993).

The activity of 5HTT can be regulated by both pre- and post-transcriptional modifications. 5HTT is encoded by a single gene on chromosome 17q11.2 (Ramamoorthy et al., 1993). Polymorphisms in this gene have been identified and found to alter the activity of 5HTT. These polymorphisms occur in the 5'-flanking promoter region and consist of multi-allelic 17-bp tandem repeat and an insertion/deletion of a 44-bp sequence (Lesch et al., 1996). This results in long (L) and short (S) alleles, with the S form shown to have reduced transcriptional efficiency, decreased 5HTT expression and 5HT uptake (Lesch et al., 1996).

Posttranscriptionally, protein kinase C (PKC), protein phosphatase 2A (PP2A) and p38 are all involved in the regulation of 5HTT activity. Multiple serine and threonine phosphorylation sites have been located on the cytoplasmic domains of 5HTT and phosphorylation appears to play a major role in regulating 5HTT trafficking and thus its ability to take up 5HT. Activation of PKC has been shown to phosphorylate 5HTT in a calcium-dependent manner, resulting in its internalisation and a decreased activity (Jayanthi et al., 2005, Ramamoorthy et al., 1998, Samuvel et al., 2005). Conversly, inhibition of protein phosphatase 2A (PP2A) also increases 5HTT phosphorylation and subsequent internalisation (Ramamoorthy et al., 1998), highlighting the important role phosphorylation plays in regulating 5HTT. Interestingly 5HT has been found to decrease 5HTT phosphorylation, thus inhibiting its internalisation (Ramamoorthy et al., 1998). This may be a negative feedback mechanism to prevent 5HTT downregulation when high levels of extracellular 5HT are present. The mitogen-activated protein kinase (MAPK) p38 also regulates the activity of 5HTT in manner distinct from that of PKC, by regulating the delivery of 5HTT to the plasma membrane (Samuvel et al., 2005). In this study inhibition of p38 decreased 5HT uptake by attenuating the levels of the transporter present at the membrane. Furthermore, receptor mediated activation of p38 has been shown to result in enhanced 5HTT activity, by a process that was independent of 5HTT trafficking (Zhu et al., 2005). This effect was dependent on activity of PP2A. Taken together, these finding suggest the importance of phosphorylation in the regulation of 5HTT activity, in addition to its regulation at a genetic level (Figure 1.12).

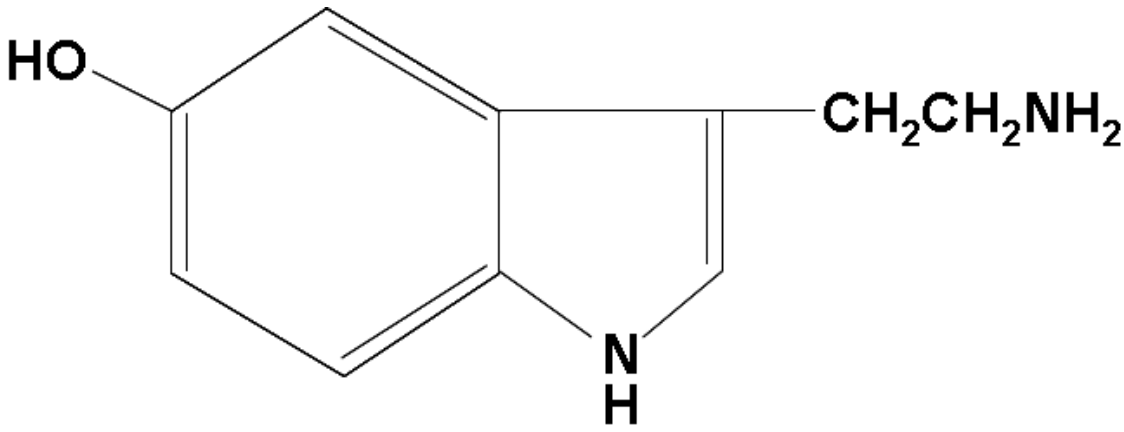

Figure 1.6 Diagram of the chemical structure of 5-hydroxytryptamine (5HT).

5HT is a biogenic amine. It structure comprises of an amino group, which is attached to an indole group by an ethyl chain.

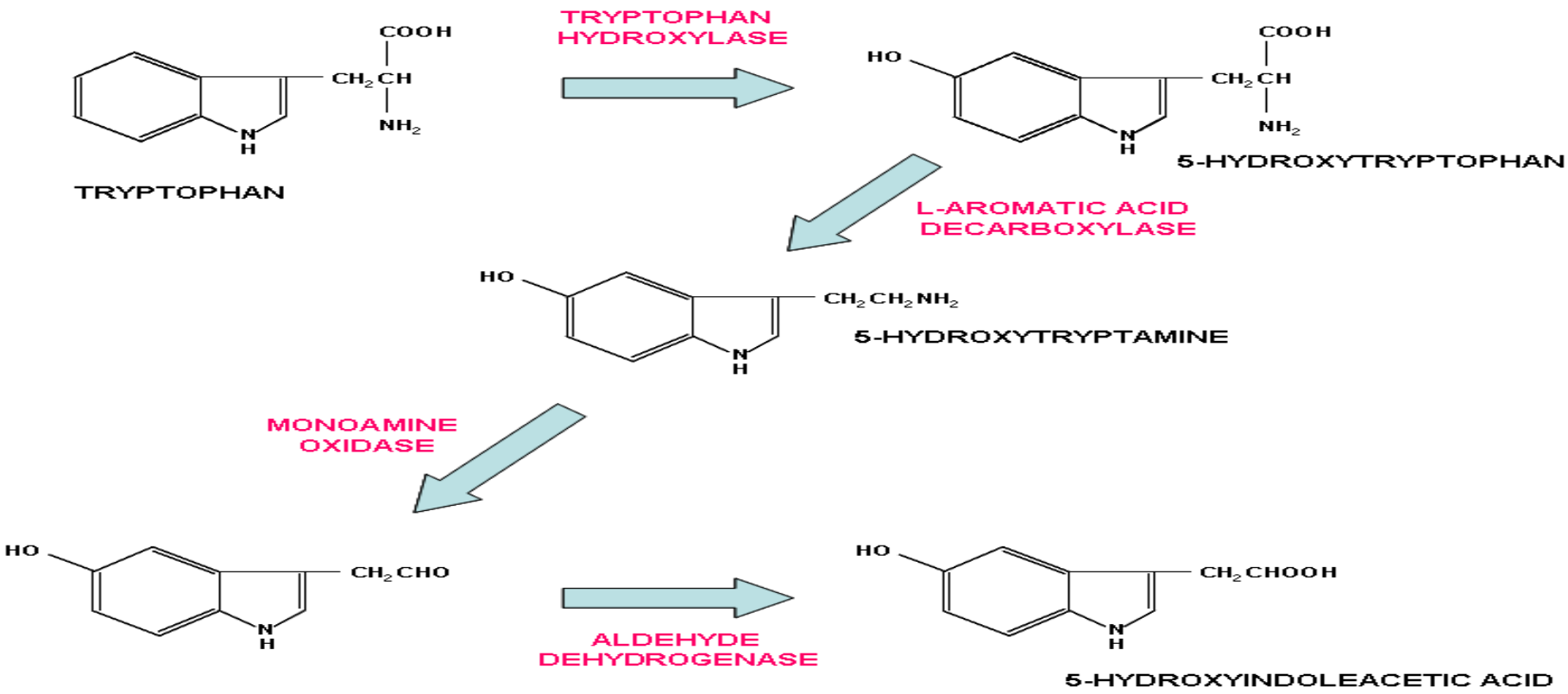

Figure 1.7 5HT biosynthesis and metabolism pathway.

5HT is synthesised from dietary tryptophan, which is converted to 5-hydroxytryptophan by the enzyme tryprophan hydroxylase, then to 5HT by a non-specific decarboxylase. Degradation occurs by the enzyme monoamine oxidase-A, resulting in the formation of 5-hydroxyindoleacetic acid, which is excreted in urine.

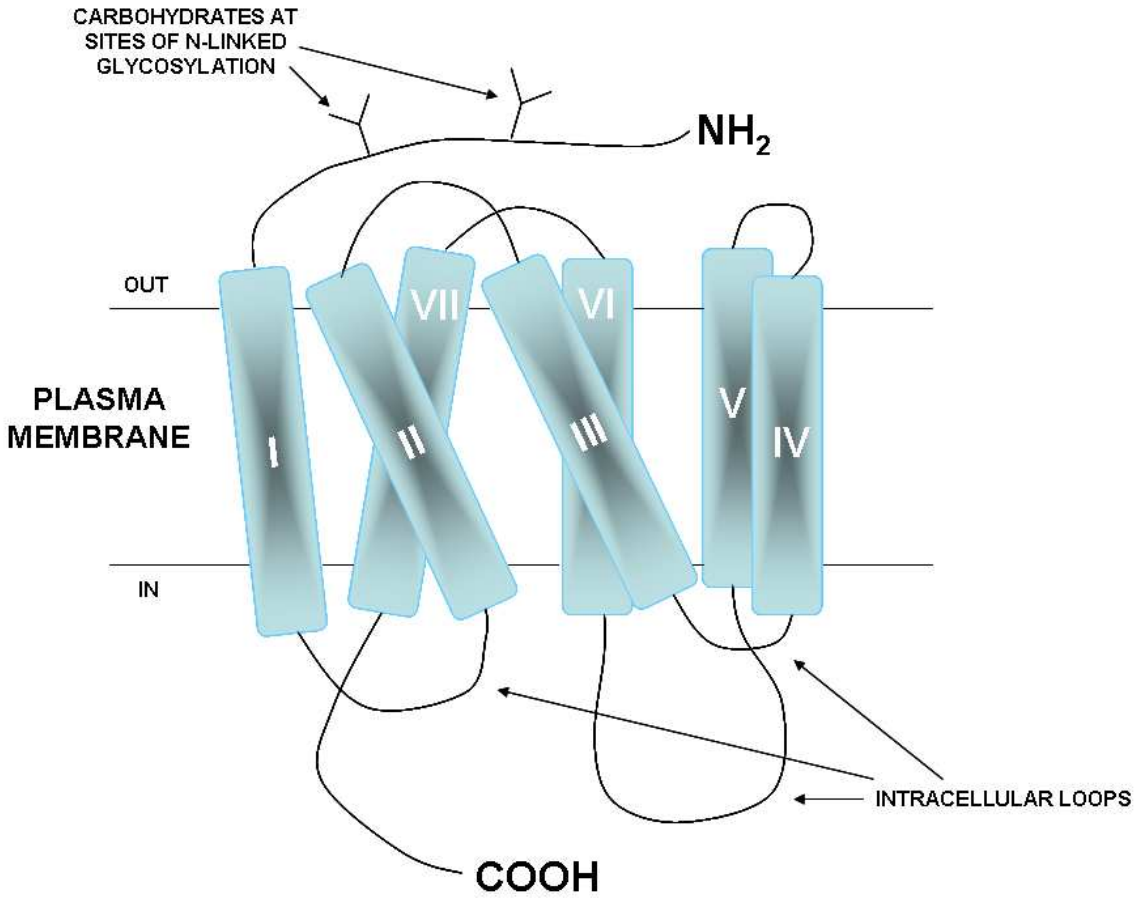

Figure 1.8 General Structure of a GPCR.

Diagram represents a typical GPCR, consisting of seven transmembrane-spanning α-helices, with an extracellular N-terminus and intracellular C-terminus. The central core comprises transmembrane domain II, III, V and VI, and is essential in ligand binding. Glycosylation sites towards the N-terminus as thought to be involved in receptor trafficking. Figure adapted from Ulloa-Aguirre *et al* (1999).

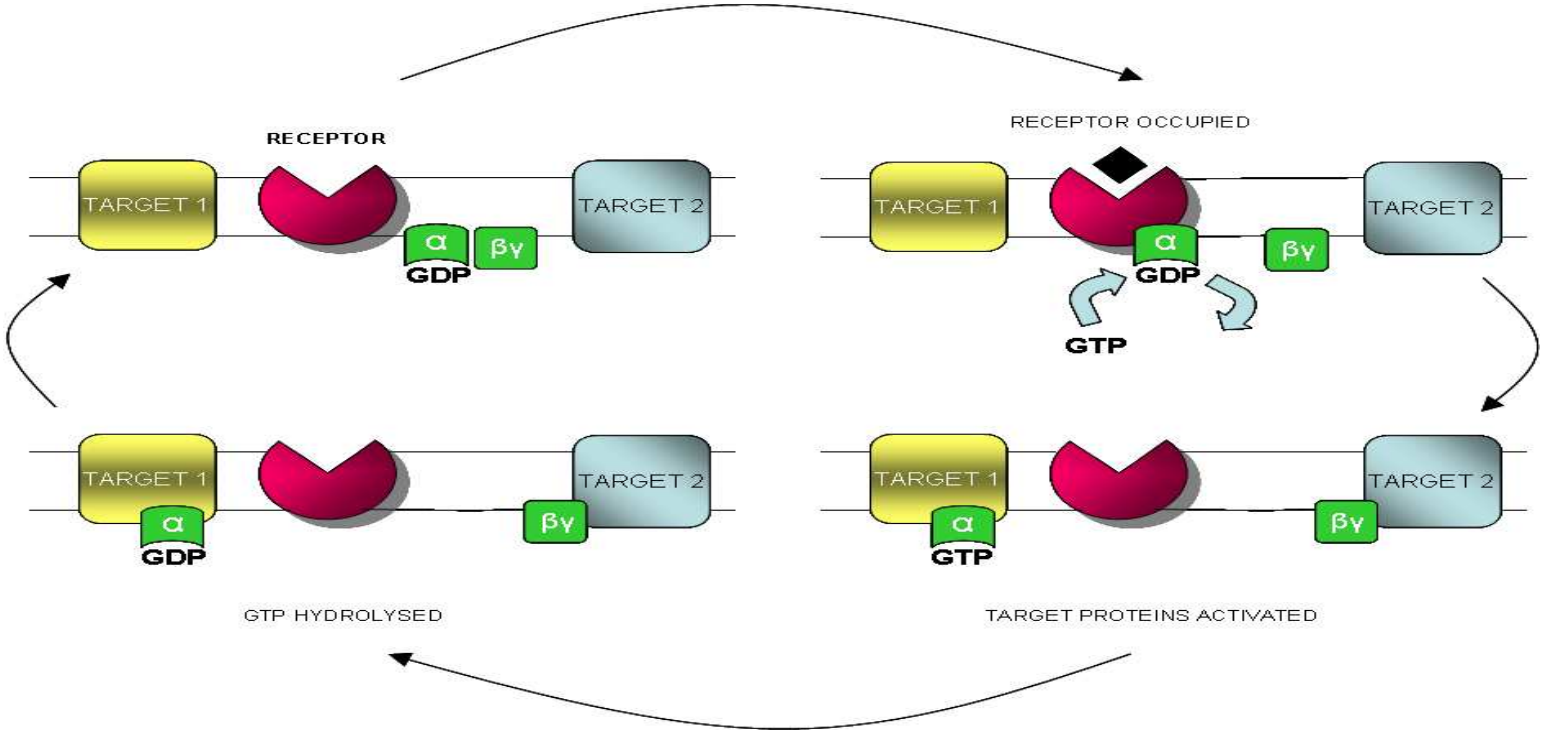

Figure 1.9 The function of the G-protein in mediating agonist induced signal transduction.

The binding of agonist to a GPCR results in coupling of the α-subunit of its associated G-protein to the receptor and subsequent exchange of GDP for GTP. Exchange of GDP for GTP results in the dissociation of α and βγ subunits, allowing them to interact with effector proteins and initiate cellular responses. The intrinsic GTPase activity of the α-subunit hydrolyses GTP to GDP, resulting in the reassociation of α and βγ subunits and terminates receptor signalling.

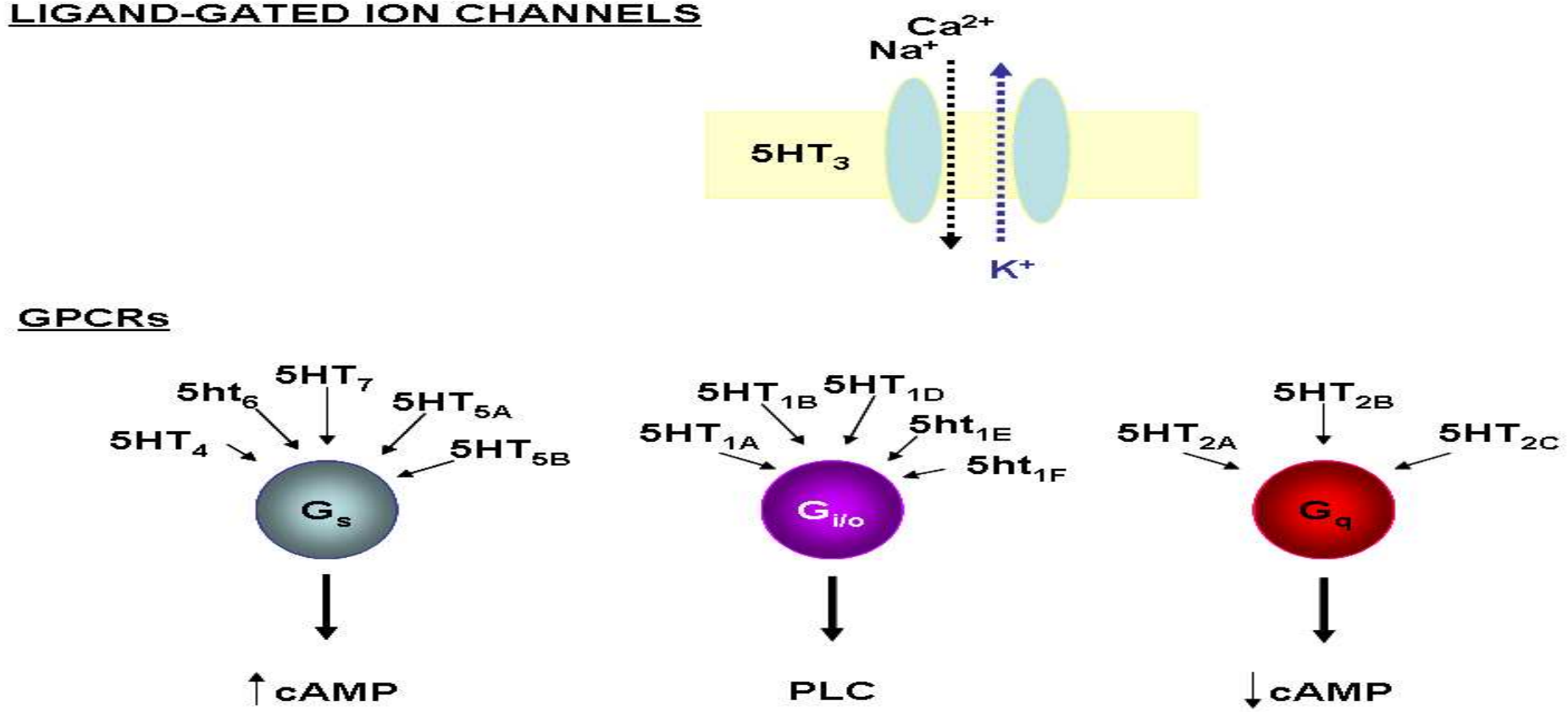

Figure 1.10 Diagram summarising mechanisms by which 5HT receptors mediate their response.

All 5HT receptors except the $5HT_3$ are G-protein-coupled receptors, with different receptors coupled to different G-proteins to elicit a reponse. The $5HT_3$ receptor is a ligand-gated non-selective cation channel.

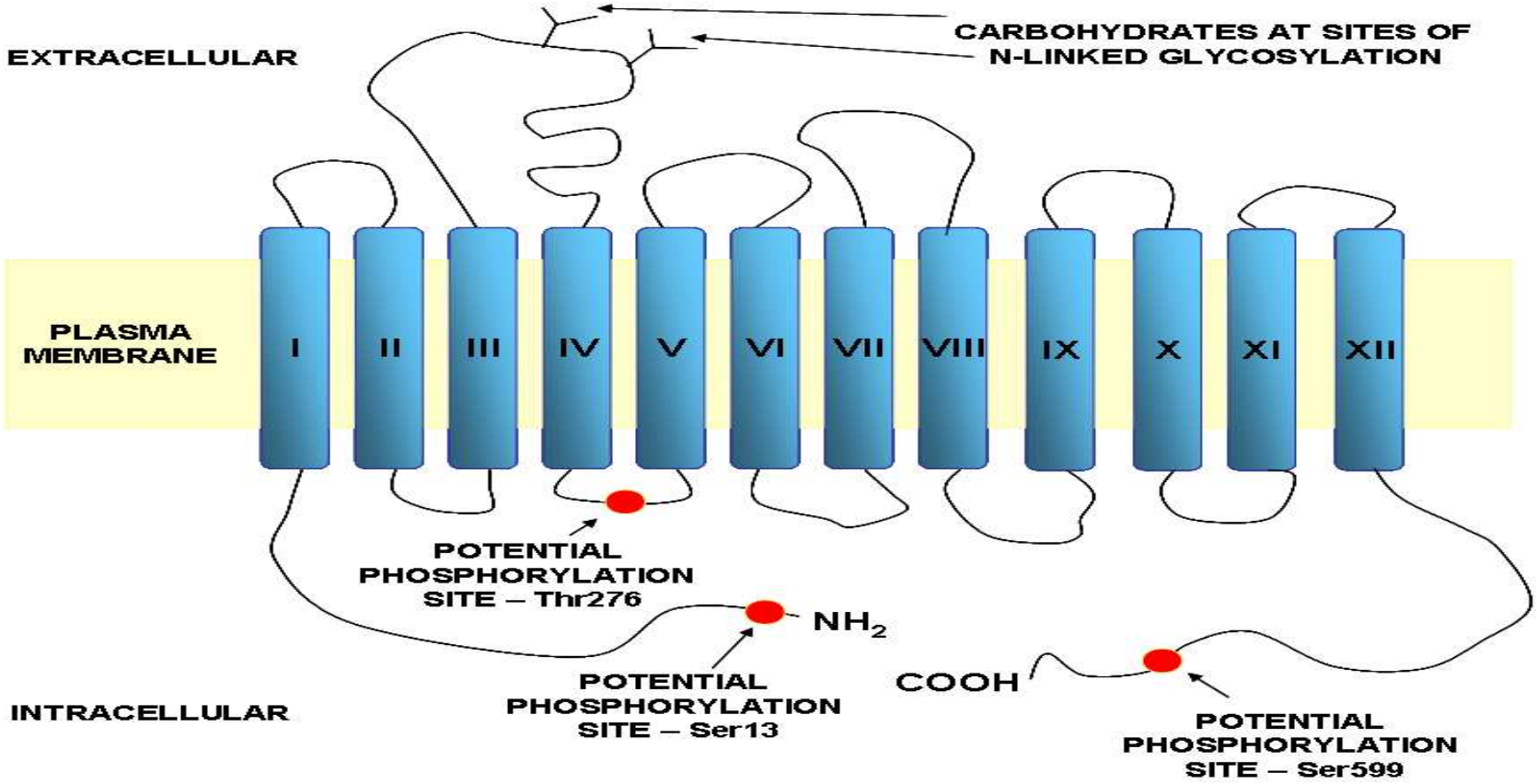

Figure 1.11 Diagram of proposed topology of the 5HT transporter (5HTT).

5HTT is comprised of 12 transmembrane domains connected by intracellular and extracellular loops with both the C- and N-terminus located in the cytoplasm. Figure adapted from Torres *et al* (2003).

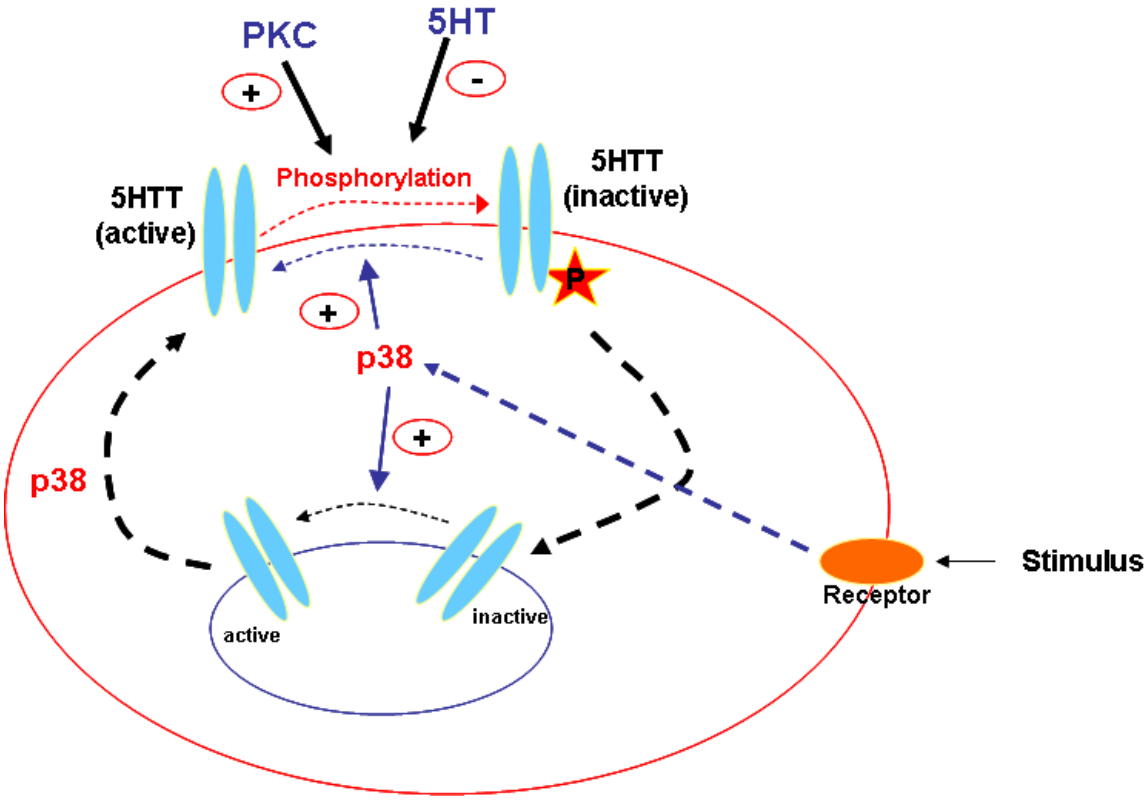

Figure 1.12 Post-translational regulation of the activity of the 5HT transporter (5HTT).

Activation of protein kinase C (PKC) results in the phosphorylation and subsequent inactivation and internalisation of 5HTT. The presence of 5HT inhibits 5HTT phosphorylation retaining active 5HTT at the plasma membrane. p38 MAP kinase is required for the trafficking of active 5HTT back to the plasma membrane. Furthermore the activation of p38 by cell surface receptors such as the adenosine receptor is important in maintaining 5HTT in its active state, a process that is dependent on protein phosphatase 2A (PP2A).

1.5 ROLE OF 5HT AND 5HTT IN PULMONARY HYPERTENSION

In the lungs, 5HT is locally released from pulmonary neuroendocrine cells and neuroepithelial cell bodies distributed throughout the airways. The lungs play an important role in the removal of 5HT from the circulation, with as much as 95% being taken up or inactivated (Gaddum et al., 1953, Thomas and Vane, 1967, Wiersma and Roth, 1980). Under normal circumstances, pulmonary tissue is exposed to low levels of 5HT as it is taken up and stored in platelets, thus removing it from the circulation. However, under hypoxic conditions (Johnson and Georgieff, 1989) and during situations involving mechanical strain (Pan et al., 2006) large amounts of 5HT are secreted. In the pulmonary circulation 5HT is thought to play a role in promoting SMC proliferation, vasoconstriction and thrombosis, processes involved in the development of PAH.

A large body of evidence now exists implicating 5HT in the development of the condition. For instance, several patients with PAH have elevated plasma levels of 5HT, in addition to a decrease in the levels of 5HT stored in platelets (Herve et al., 1995). Moreover, 5HT was also implicated in the development of PAH in patients following treatment with anorexigens such as aminorex and fenfluramine derivatives. Aminorex increases plasma levels of 5HT by inducing its release from platelets and attenuating its breakdown by inhibiting MAO (Zheng et al., 1997, Fishman, 1999). Fenfluramine derivatives also increase levels of circulating 5HT. These anorexigens interact with 5HTT, stimulating the release of 5HT from platelets and inhibit its reuptake (Buczko et al., 1975, Fristrom et al., 1977). Furthermore, in PAECs from PAH patients, increased TPH1 expression and 5HT synthesis has been observed and this was thought to contribute to increased SMC proliferation (Eddahibi et al., 2006). Another study found that the effects of chronic hypoxia were markedly attenuated in mice deficient in TPH, with reductions in right ventricular pressure (RVP) and vascular remodelling reported (Morecroft et al., 2007). Together, these observations confirm an important role for 5HT in vascular remodelling during PAH.

Both 5HT receptors and 5HTT contribute to the actions of 5HT in the pulmonary hypertensive process. 5HT is a potent vasoconstrictor and in the human pulmonary arteries mediates its effects *via* the $5HT_{1B}$ receptor (Morecroft et al., 1999), whilst the $5HT_{2A}$ receptor is important in the contractile response in other species, including rats (Chand and Altura, 1980, MacLean et al., 1996). However, in rats maintained under chronic hypoxic conditions, contractile responses to 5HT are enhanced and this process is mediated by both $5HT_{2A}$ and $5HT_{1B}$ receptors (MacLean et al., 1996, Keegan et al., 2001). $5HT_{1B}$ receptors have also been found to be upregulated in experimental PAH (Rondelet et al., 2003). In addition to the role of 5HT receptors in vasoconstriction, they also play a role in mediating vascular remodelling. In chronically hypoxic rats, administration of a $5HT_{1B}$ antagonist significantly reduced hypoxia-induced right ventricular hypertrophy (RVH) and vascular remodelling (Keegan et al., 2001). Furthermore, in $5HT_{1B}$ knock-out mice, chronic hypoxia-induced PAH was also markedly attenuated (Keegan et al., 2001). The $5HT_{2B}$ receptor has also been reported to facilitate the development of PAH. In one study, chronically hypoxic mice with inactive $5HT_{2B}$ receptors failed to develop PAH (Launay et al., 2002). This study also observed an increase in $5HT_{2B}$ receptor transcript in patients with idiopathic PAH. Additionally, treatment with $5HT_{2A}$ receptor-selective antagonists have been found to have beneficial effects, improving survival and reducing vascular remodelling in monocrotaline-induced PAH (Hironaka et al., 2003). Furthermore, several cellular studies have highlighted the mitogenic effects of 5HT in the pulmonary cells and therefore its potential contribution to vascular remodelling. In some cell types the mitogenic effects are mediated *via* 5HT receptors. In rat pulmonary artery fibroblasts for example, inhibition of the $5HT_{2A}$ receptor markedly reduced 5HT-induced proliferation (Welsh et al., 2004). Similar effects have also been observed in other fibroblast cell lines (Lee et al., 1999). However, it is the 5HTT that has attracted most attention in recent times and several lines of evidence suggest a major role for 5HTT in vascular remodelling.

5HTT expression has been found to be elevated in the lungs of many patients with PAH. This increased expression has been associated with polymorphisms in the 5HTT gene (Eddahibi et al., 2003). In a study carried out by Eddahibi *et*

al (2001), 65% of patients with idiopathic PAH were found to be homozygeous for the L-allelic variant, conferring increased expression of 5HTT, compared to only 27% of controls. Subsequently, in patients with the LL-genotype, PAH was found to be more severe than those with LS or SS-genotypes who expressed lower levels of 5HTT (Eddahibi et al., 2003). Various animal models also support the role of 5HTT in the development of PAH and its contribution to vascular remodelling. Such studies have shown that administration of 5HTT-selective inhibitors protect against both hypoxia- and monocrotaline-induced PAH (Guignabert et al., 2005, Marcos et al., 2003). Furthermore, mice deficient in 5HTT display a marked reduction in RVH and vascular remodelling in response to hypoxia compared with control (Eddahibi et al., 2000a). Conversly, mice over-expressing 5HTT display increased RVP under normoxic conditions, and have notably exaggerated responses to hypoxia compared to control animals, with elevated vascular remodelling and RVH (MacLean et al., 2004).

The levels of 5HTT, as well as $5HT_{1B}$, $5HT_{2A}$ and $5HT_{2B}$ receptors have all been found to be elevated in pulmonary arteries of patients with PAH (Marcos et al., 2004). However, only 5HTT is upregulated in PASMCs (Marcos et al., 2004), suggesting it may contribute specifically to SMC hyperplasia and play a role in mediating vascular remodelling. In fact, in mice engineered to over-express 5HTT in SMCs only, increases in RVP, RVH and vascular remodelling were observed to occur spontaneously by 8 weeks of age, with these effects worsening with time (Guignabert et al., 2006). Furthermore, augmented proliferative capabilities of PASMCs from patients with PAH have been associated with increased expression levels of 5HTT (Marcos et al., 2004, Eddahibi et al., 2001). The enhanced proliferation of these cells to 5HT or serum can be abolished by 5HTT inhibitors such as citalopram and fluoxetine (Marcos et al., 2004). In further support of its role in vascular remodelling, the increase in 5HTT expression observed in PAH patients was located mainly in the medial layer of remodelled vessels (Eddahibi et al., 2001). Hypoxia has also been found to result in increased 5HTT expression and transport activity, augmenting mitogenic responses in PASMCs (Eddahibi et al., 1999). Therefore, through the actions of 5HTT and its receptors, 5HT appears to play a major role in the development of pulmonary hypertension, contributing to both vasoconstriction and vascular remodelling.

1.6 ERK MAP KINASE PATHWAY

The extracellular-signal regulated kinase (ERK) MAP kinase pathway is crucial in mediating the mitogenic effects of 5HT (Lee et al., 1999, Lee et al., 2001, Suzuki et al., 2003, Liu et al., 2004). MAP kinases (MAPKs) are a family of well-conserved proteins expressed in all eukaryotic cells. Three major classes of MAPKs have been identified: ERK, p38 and c-jun N-terminal kinase (JNK). MAPKs are serine/threonine kinases and are activated by phosphorylation on a Thr-X-Tyr motif. ERK has the dual phosphorylation motif Thr-Glu-Tyr, JNK has Thr-Pro-Tyr and the Thr-Gly-Tyr motif is present on p38 (Davis, 1995). The overall sequence identity among ERK, p38 and JNK is 40-45%. p38 and JNK are activated by various stress stimuli, including cytokines, osmotic shock and hypoxia (Welsh et al., 2001b, Davis, 2000, Zarubin and Han, 2005) (Figure 1.13).

Currently eight ERK MAPKs have been identified, termed as ERK1-8 (Bogoyevitch and Court, 2004). ERK1 and ERK2 were the first identified members and are the most extensively studied. They are expressed ubiquitously and have 90% sequence identity (Boulton et al., 1991). The Ras/Raf/MEK/ERK cascade is a well conserved pathway that is involved in the control of many fundamental cellular processes such as proliferation, differentiation and apoptosis (Lewis et al., 1998, Pearson et al., 2001). A wide range of extracellular stimuli have been found to activate ERK, *via* the stimulation of tyrosine receptor kinases and GPCRs, through Ras-dependent and Ras-independent pathways (Lewis et al., 1998). The Ras/Raf/MEK/ERK pathway conveys signals in the form of a cascade of phosphorylation events. Receptor-mediated activation of Ras, a small GTPase, at the plasma membrane promotes its binding to the N-terminus of Raf kinases (Raf-1, A-Raf, B-Raf), recruiting Raf to the membrane and subsequently activating them. Raf activation is also dependent on phosphorylation at multiple sites. In the case of Raf-1, phosphorylation sites important for its activation at Ser338, Tyr341, Thr491 and Ser 949 have been identified (Chong et al., 2001). Once active Raf, then phosphorylates MEK (mitogen activated protein kinase kinase) within its activation loop at two serine residues (Ser 217 and Ser 221) (Alessi et al.,

1994). MEK may then subsequently activate ERK by phosphorylating tyrosine and threonine residues located within its kinase activation loop. In the case of human ERK1, phoshorylation occurs at Tyr202 and Thr 204, while human ERK2 is phosphorylated on Tyr185 and Thr197. Unlike MEK, significant ERK activation requires phosphorylation at both sites, with Tyr phosphorylation preceeding that of Thr (Ferrell and Bhatt, 1997). Active ERK is then able to mediate many cellular processes by acting on substrates in the nucleus and cytoplasm (Kolch, 2005) (Table 1.2).

In quiescent cells ERK is localised in the cytoplasm (Torii et al., 2004). Under these circumstances, ERK forms a complex with MEK, which retains it in the cytoplasm due to the presence of a nuclear export sequence in the amino-terminal domain of MEK (Fukuda et al., 1996). Activation of ERK leads to the dissociation of the MEK/ERK complex allowing ERK to translocate to the nucleus where it can phosphorylate multiple transcription factors (refer to Table 1.2), modulating gene transcription and mediating many physiological responses (Torii et al., 2004, Brunet et al., 1999, Lewis et al., 1998, Pearson et al., 2001) (Figure 1.14). ERK activity is terminated by dephosphorylation of the tyrosine and threonine groups within its activation loop. Dual specificity phosphatases (DUSPs), which display differing levels of specificity for MAPKs, play an important regulatory role by dephosphorylating and inactivating ERKs. At least nine DUSPs have been isolated in mammalian cells and it is thought that these phosphatases participate in the negative feedback control of MAPK activation (Keyse, 2000). Nuclear accumulation of ERK is transient and inactivated ERK must relocalise to the cytoplasm, a process which is critical for further stimulation to occur. It has been suggested that the relocalisation of inactive ERK occurs by a MEK-dependent transport system, whereby MEK transiently enters the nucleus and binds inactive ERK, exporting it back to the cytoplasm (Adachi et al., 2000).

The ERK MAPK cascade is regulated at many levels. A number of scaffolding proteins regulate signalling through this pathway (Kolch, 2005). Kinase suppressor of Ras-1 (KSR1) acts as a scaffold and has been found to bind all kinase members of the ERK cascade (Morrison, 2001, Kolch, 2005). For example, KSR promotes MEK activation by presenting MEK to activated Raf (Muller et al., 2001). Exogenous inhibitors of the ERK/MAPK pathway also

exist. One such inhibitor RKIP (Raf kinase inhibitor protein) bind both Raf and MEK preventing their interaction and thus MEK phosphorylation and activation (Yeung et al., 1999).

β-arrestins, which are known to play a role in desensitizing and internalising GPCRs, also act as scaffolding proteins for the ERK cascade. β-arrestins consist of two 46kDa proteins, β-arrestin-1 and β-arrestin-2 (Luttrell and Lefkowitz, 2002). Following agonist binding and subsequent GPCR phosphorylation by GPCR kinases (GRKs), β-arrestins directly interact with the phosphorylated GPCR at the cell surface. β-arrestin binding uncouples the receptor from heterotrimeric G proteins and targets it to clathrin-coated pits for removal from the plasma membrane by endocytosis (Ceresa and Schmid, 2000). β-arrestin can simultaneously bind various other signalling proteins, including components of the ERK cascade. All three ERK pathway components have been observed in GPCR/β-arrestin complexes. In rat kidney epithelial cells, protease-activated receptor 2 (PAR2) stimulation results in the formation of complexes containing internalised receptor, β-arrestin-1, Raf-1 and activated ERK (DeFea et al., 2000). Similarly, in COS-7 and HEK293 cells, activation of the angiotensin type 1a receptor (AT1aR) leads to the formation a receptor, β-arrestin-2, Raf-1, MEK1 and ERK2 complex (Tohgo et al., 2002).

β-arrestins not only bind ERK cascade components but also contribute to GPCR-mediated ERK activation. For instance, overexpression of β-arrestin has been found to significantly increase angiotensin-mediated ERK activation in COS-7 cells (Tohgo et al., 2002). Furthermore, levels of active ERK bound to β-arrestin complexes are increased when Raf-1 is overexpressed and markedly reduced when a kinase-inactive MEK protein is expressed, indicating that the β-arrestin scaffold acts as a platform for signal transmission from Raf to MEK and ERK (Luttrell et al., 2001). In addition to this, β-arrestins appear to target ERK activity to a pool of cytoplasmic substrates. In the case of the AT1aR, β-arrestin facilitates GPCR-mediated ERK activation but inhibits ERK-dependent transcription by binding to active ERK and retaining it in the cytoplasm (Tohgo et al., 2002). Furthermore, the stability of the GPCR/ β-arrestin interaction determines the level of β-arrestin-bound ERK, thus influencing the subcellular localization of activated ERK and the physiological consequences of ERK activation (Tohgo et al., 2003).

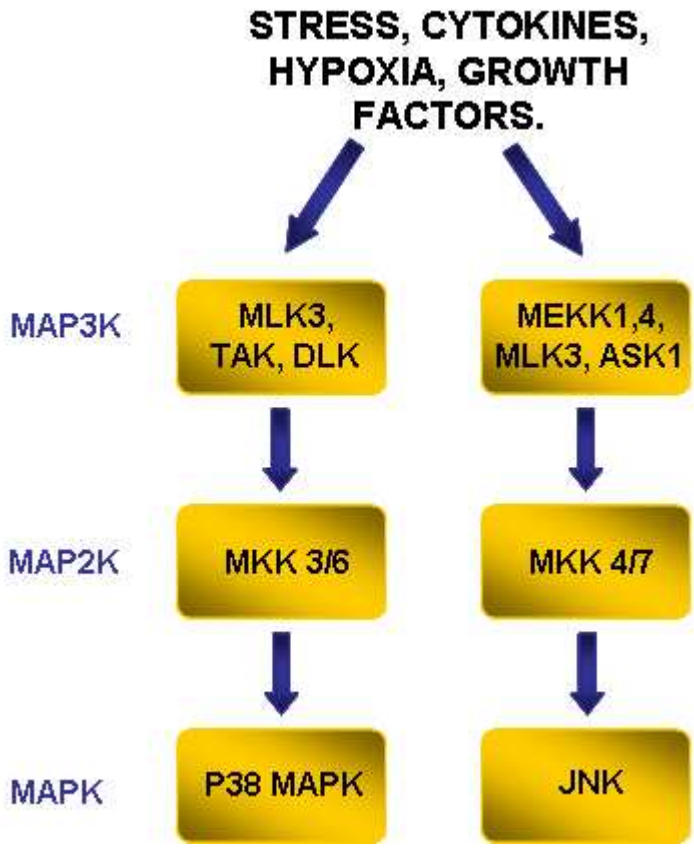

Figure 1.13 p38 and JNK signalling cascades.

The activation of p38 and c-Jun N-terminal kinase (JNK) requires a 3-tiered cascade. A MAP kinase (MAPK) is activated by a MAP kinase kinase (MAP2K), which it turn is also activated by phosphorylation by a MAP kinase kinase kinase (MAP3K). MAPKs can be activated by a least 2 MAP2Ks and several MAP3Ks. MAP2Ks display substrate specificity, while MAP3Ks can activate multiple MAPK cascades. p38 has 4 isoforms, α,β,γ and δ, while JNK has 3 isoforms, JNK 1,2 and 3 (Raman and Cobb, 2003).

Transcription factors	*Kinases and phospatases*	*Cytoskeletal proteins*	*Signaling proteins*	*Apoptotic proteins/ proteinases*	*Other proteins*
ALM1	DAPK	Annexin XI	EGFR	Bad	Amphiphysin 1
Androgen receptor	ERK1/2	Caldesmon	ENaCβ/γ	Bim-EL	CPSII/CAD
ATF2	FAK1	CENP-E	Fe65	Calpain	CR16
BCL6	GRK2	Connexin	FRS2	Caspase 9	GRASP55
BMAL1	Inhibitor-2	Cortactin	Gab1	EDD	GRASP65
CBP	Lck	Crystallin	Gab2	IEX1	HABP1
CEBPβ	MAPKAP3	DOC1R	GAIP	MCL-1	Histone H
CRY1/2	MAPKAP5	Dystrophin	Grb10	TIS2	HnRNP-K
E47	MEK1/2	Lamin B2	IRS1	TNFR CD120a	KIP
Elk1	MKP1/2	MAP1	LAT		MBP
ER81	MKP3	MAP2	LIFR		PHAS-I
ERF	MKP7	MAP4	MARCKS		CPLA2
Estogen receptor	MLCK	MISS	Naf1α		Rb
c-Fos	MNK1/2	NF-H	PDE4		SAP90/PDS95
Fra1	MSK1/2	NF-M	PLCγ		Spinophilin
GATA1/2	PAK1	Paxillin	PLCβ		Topoisomerase II
HIF1α	PTP2C	Stathmin	K_V4.2		Tpr
HSF1	Raf1	SWI/SNF	KSR1		TTP(Nup47)
ICER	B-Raf	Synapsin 1	Rab4		Tyrosine hydroxylase
c-Jun	RSK1-4	Tau	SH2-B		Vif
Microphthalmia	S6K	Vinexin β	ShcA		Vpx
c-Myc	Syk	Calnexin	Sos1		
N-Myc			Spin90		
Net (Sap2)			TSC2		
NFATc4					
NF-IL6					
NGF1-B/TR3/Nur77					
Pax6					
PPARγ					
p53					
Progesteron receptor					
RNA Pol II					
PUNX2					
Sap1					
Smad1					
Smad2/3					
SP1					
SRC1					
SREBP1/2					
STAT1/3					
STAT5a					
TAL1/SCL					
TFII-I					
TFIIIB					
TGIF					
TIF1A					
Tob					
UBF					

Table 1.2 ERK Substrates.

Once activated ERK is able to phosphorylate its substrates, with different activating stimuli resulting in the phosphorylation of different substrates. More than 150 cytoplasmic and nuclear ERK substrates have been identified, including protein kinases, protein phosphatases, transcription factors, scaffolding proteins, signalling molecules, cytoskeletal proteins, receptor and apoptosis-related proteins. Table adapted from Lu and Xu (2006).

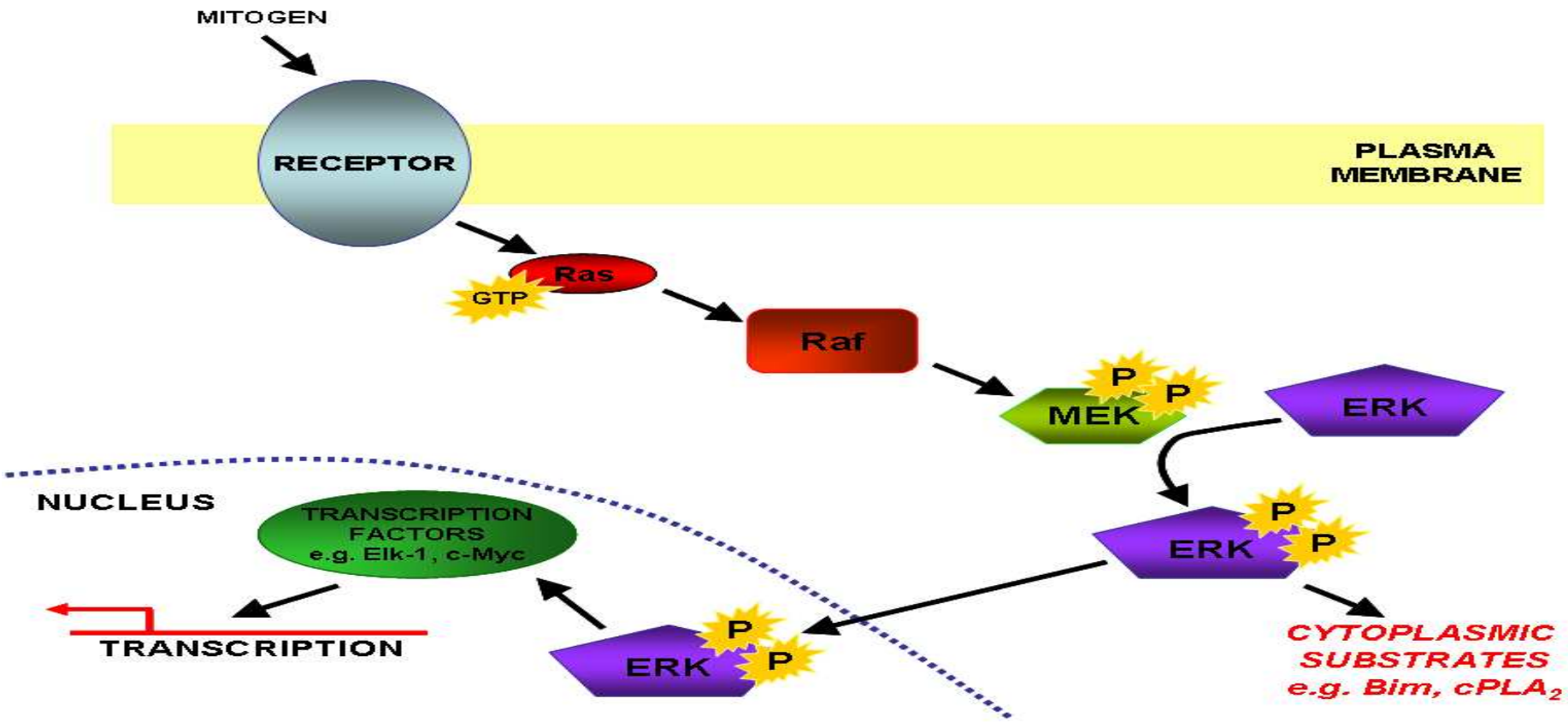

Figure 1.14 ERK1/2 MAP kinase signalling pathway.

Generally, ERK1/2 is activated by a cascade initiated by activation of the small G protein Ras, followed by activation of a Raf family member and subsequent activation of MEK1/2. Raf can also be activated independently of Ras resulting in ERK activation (Lu and Xu, 2006). Once active, ERK can then activate substrates in the nucleus, resulting in the transcription of target genes or act on substrates in the cytoplasm (refer to Table 1.2 for examples of substrate).

1.7 Rho AND Rho EFFECTORS

Rho and its effectors have been implicated in the signalling pathways that contribute to vasoconstriction and vascular remodelling during the development of PAH and may also be involved in signal transduction downstream of 5HT.

1.7.1 SMALL G-PROTEINS

Rho belongs to the small GTP-binding protein superfamily of monomeric G proteins with molecular masses in the region of 20-40kDa. Currently, more than one hundred small G proteins have been identified in eukaryotic cells, which can be characterised into five major families: Ras, Rho, Rab, Sar/Arf and Ran (Takai et al., 2001). Small GTP-binding proteins play an important role in signal transduction, with the Ras family involved in gene expression, playing an important role in the activation of the ERK MAPK cascade. Cytoskeletal regulation is mediated *via* the Rho family, whereas the Rab and Sar1/Arf family are involved in regulating vesicular trafficking. Finally, the Ran family regulate nucleocytoplasmic transport and microtubule dynamics involved in cell cycle progression (Takai et al., 2001). The Rho family has around 20 distinct members including, Rho, Rac, Cdc42, RhoD, RhoG, TC10, Rnd and TTF, which share 50-55% identity (Hall and Nobes, 2000). In particular, Rho has 3 isoforms A, B and C, with Rho A the most extensively studied. All Rho isoforms can be selectively inhibited by the C3 transferase (C3) enzyme from *Clostridium Botulinum* by ADP-ribosylation (Aktories et al., 1989).

1.7.2 REGULATION OF Rho-GTPases

Rho-GTPases, like other small G proteins, act as molecular switches by cycling between inactive GDP-bound and active GTP-bound states (Schmidt and Hall, 2002, Jaffe and Hall, 2005). These interactions take place at the plasma membrane and Rho-GTPases interact with the membrane *via* a twenty-carbon chain geranylgeranyl lipid residue attached to their C-terminus (Seabra, 1998). Guanine nucleotide exchange factors (GEFs) facilitate the exchange of GDP for

GTP, resulting in activation of the Rho-GTPase and subsequent downstream effector pathways. GTPase activating proteins (GAPs) accelerate the intrinsic GTPase of Rho family members, resulting in their inactivation. Guanine nucleotide dissociation inhibitors (GDIs) also play a role in regulating the activity of these small G proteins. GDIs interact with GDP bound Rho-GTPases, sequestering them in the cytosol, inhibiting GDP dissociation and controlling cycling between the membrane and cytosol (Schmidt and Hall, 2002, Jaffe and Hall, 2005) (Figure 1.15). Lysophosphatidic acid (LPA) was the first agonist identified to activate Rho (Ridley et al., 1992). Since then several other ligands acting on both GPCRs and receptor tyrosine kinases (RTKs) have been found to activate the small GTPase. GEFs are critical mediators of Rho-GTPase activity and extracellular stimuli acting on membrane receptors are thought to activate GEFs, subsequently resulting in the activation of Rho-GTPase and downstream signalling pathways (Schmidt and Hall, 2002). Some GEFs are highly specific towards a single GTPase, for example, p115RhoGEF is selective for Rho (Hart et al., 1996), whilst others such as Vav1 regulate the activity of Cdc42, Rac and Rho (Olson et al., 1996).

1.7.3 PHYSIOLOGICAL FUNCTIONS OF Rho

The Rho-GTPase family mediates a variety of biological responses. In addition to the major role regulating assembly of the actin cytoskeleton (Hall, 1998), Rho-GTPases also participate in a variety of other functions mediated by a wide variety of effector proteins. These process include cell polarity, gene transcription, vesicular transport, cell cycle progression, enzyme regulation and microtubule dynamics (Etienne-Manneville and Hall, 2002, Jaffe and Hall, 2005).

Rho itself mediates many diverse biological functions. Implicated in the regulation of neuronal development, activation of Rho has been found to inhibit neurite extension and the formation of dendritic spines (Luo, 2000, Li et al., 2000, Wong et al., 2000). Rho also plays an important role in cell contraction. In the cardiovascular system for instance, vasoconstriction and vasodilation, process that control blood flow and are also important to normal physiological function can be regulated by Rho. In aortic smooth muscle, numerous agonists

have been shown to activate Rho, which *via* its effector protein ROCK induces myosin light chain phosphorylation thereby promoting contraction (Fukata et al., 2001, Sakurada et al., 2001). Futhermore, Rho is also involved in the control of barrier function in vascular endothelial cells, which controls functions such as the extravasation of circulating lymphocytes into underlying tissues (van Nieuw Amerongen et al., 2000). The contractile forces generated by Rho activation are thought to destabilise endothelial cell-cell junctions (Wojciak-Stothard et al., 2001). Moreover, during migration, the small GTPase regulates contractile forces required at the rear of the cell and is also associated with focal adhesion assembly (Raftopoulou and Hall, 2004). In addition, Rho plays a role in phagocytosis, mediating type II phagocytosis by macrophages *via* the complement receptor (Caron and Hall, 1998). A role in cell proliferation and cell cycle progression has also been described. In fibroblasts Rho plays two important roles in this process, inhibiting expression of cyclin/cyclin dependent kinase (Cdk) inhibitor $p21^{Waf/Cip1}$ and also inducing cyclin D1 expression in mid-G1 phase (Olson et al., 1998, Welsh et al., 2001a). Rho also participates in the secretion of mediators such as histamine from immune cells (Norman et al., 1996, Pinxteren et al., 2000). Furthermore, Rho modulates gene expression by regulating transcription factors such as serum response factor (SRF) and NFκB (Hill et al., 1995, Perona et al., 1997). The large and diverse array of responses elicited by Rho activation are regulated by its many effector proteins.

1.7.4. Rho EFFECTOR PROTEINS

Several cellular targets of Rho have been identified. These effectors interact specifically with the GTP-bound form of the GTPase at specific sites (Bishop and Hall, 2000). The Rho effectors protein kinase N1 (PKN1) and PKN2 are involved in endosomal trafficking. Other Rho effectors, mammalian diaphanous protein 1 (mDia1), mDia 2 and mDia 3 mediate both microtubule stabilisation and actin polymerisation. Citron, another mediator is a kinase that is critical for cytokenesis and has also been implicated in other elements of cell cycle progression. One of the most widely studied Rho effector proteins is ROCK, which plays an important role in mediating cell contractility.

1.7.5. RHO ASSOCIATED KINASE (ROCK)

ROCK, a 160kDa serine/threonine kinase, was the first identified substrate of Rho and was initially characterised for its role in mediating stress fibre formation and focal adhesions (Leung et al., 1996, Somlyo and Somlyo, 2000). Two isoforms have been identified, ROCK1 and ROCK2, which have 65% overall sequence identity. The kinase domain of these proteins is highly conserved, exhibiting 92% identity (Nakagawa et al., 1996). ROCK1 is widely expressed in a variety of tissues including, the heart, lung, kidney, pancreas and skeletal muscle, with little expression detected in the brain. ROCK2 however, is highly expressed in the brain, with low levels also detected in the lung (Amano et al., 2000). Structurally, ROCK is composed of an N-terminal kinase domain, a coiled-coil domain and an auto-inhibitory C-terminus. The auto-inhibitory region contains the Rho-binding (RB) and pleckstrin homology (PH) domains. In its resting state, both RB and PH domains can bind independently to the amino terminal kinase domain, inhibiting kinase activity. Binding of Rho-GTP with the RB domain alters the conformation of ROCK, disrupting the interaction between auto-inhibitory and kinase regions, freeing the kinase domain and thus activating ROCK (Amano et al., 2000, Riento and Ridley, 2003) (Figure 1.16). ROCK can also be activated independently of Rho by some lipids, especially arachadonic acid (AA) (Feng et al., 1999). Furthermore, other small GTPases, including Gem and RhoE, have been shown to bind to ROCK and have an inhibitory effect. RhoE inhibits ROCK1 by binding to its amino-terminal region, encompassing the kinase domain, and attenuates the ability of ROCK to phosphorylate its downstream targets (Riento et al., 2003). Gem on the other hand, binds the coiled-coil domain of ROCK adjacent to the RB domain. This has been suggested to modify the substrate specificity of ROCK, as it inhibits ROCK-mediated phosphorylation of myosin light chain but not LIM kinase (Ward et al., 2002).

1.7.6. ROCK EFFECTOR PROTEINS

ROCK activates a variety of proteins to mediate its effects. Most notably, ROCK interacts with and phosphorylates both myosin light chain (MLC) and the myosin-binding subunit of MLC phosphatase (Amano et al., 1996, Kawano et al., 1999). MLC phosphatase is inhibited by phosphorylation, resulting in increased MLC phosphorylation. This, in addition to the direct phosphorylation of MLC by ROCK, stimulates the actin-activated ATP-ase activity of myosin and promotes the assembly of actin-myosin filaments and mediates stress fibre formation (Amano et al., 1996, Kawano et al., 1999, Bresnick, 1999). Activation of these substrates also increases tension generation and induces contraction in muscular cells (Fukata et al., 2001, Sakurada et al., 2001). Another ROCK target is LIM kinase (LIMK) which, when phosphorylated by ROCK, subsequently phosphorylates and inactivates cofilin, resulting in stabilisation of actin filaments (Bamburg et al., 1999, Maekawa et al., 1999). ROCK also activates a ubiquitous Na^+/H^+ exchange protein (NHE1), which also contributes to stress fibre formation as well as focal adhesion formation (Tominaga and Barber, 1998, Tominaga et al., 1998). Other ROCK substrates that contribute to actin assembly are adducin and the ERM (ezrin/radaxin/moesin) family proteins. Phosphorylation of adducin by ROCK may be necessary for membrane ruffling, while activation of ERM is thought to be involved in microvilli formation (Amano et al., 2000). Furthermore, the ROCK-mediated activation of collapsin response mediator protein-2 (CRMP2) participates in the process of neuronal growth cone collapse (Arimura et al., 2000). ROCK also phosphorylates the intermediate filament proteins, vimentin, neurofilament and glial fibrillary acidic protein (GFAP) to induce depolymerisation, a function important in cytokinesis (Kosako et al., 1997, Goto et al., 1998, Hashimoto et al., 1998, Yasui et al., 1998). In summary, it can be seen that ROCK activates a wide range of effector proteins necessary to tranduce signals initiated by Rho activation (Figure 1.17). Activation of the Rho/ROCK pathway has been implicated in variety of pathological condition such as hypertension, vascular inflammation, atherosclerosis and cerebral ischemia (Rikitake and Liao, 2005). ROCK may also play a role in the development of PAH, which is discussed extensively in the introduction to Chapter 4.

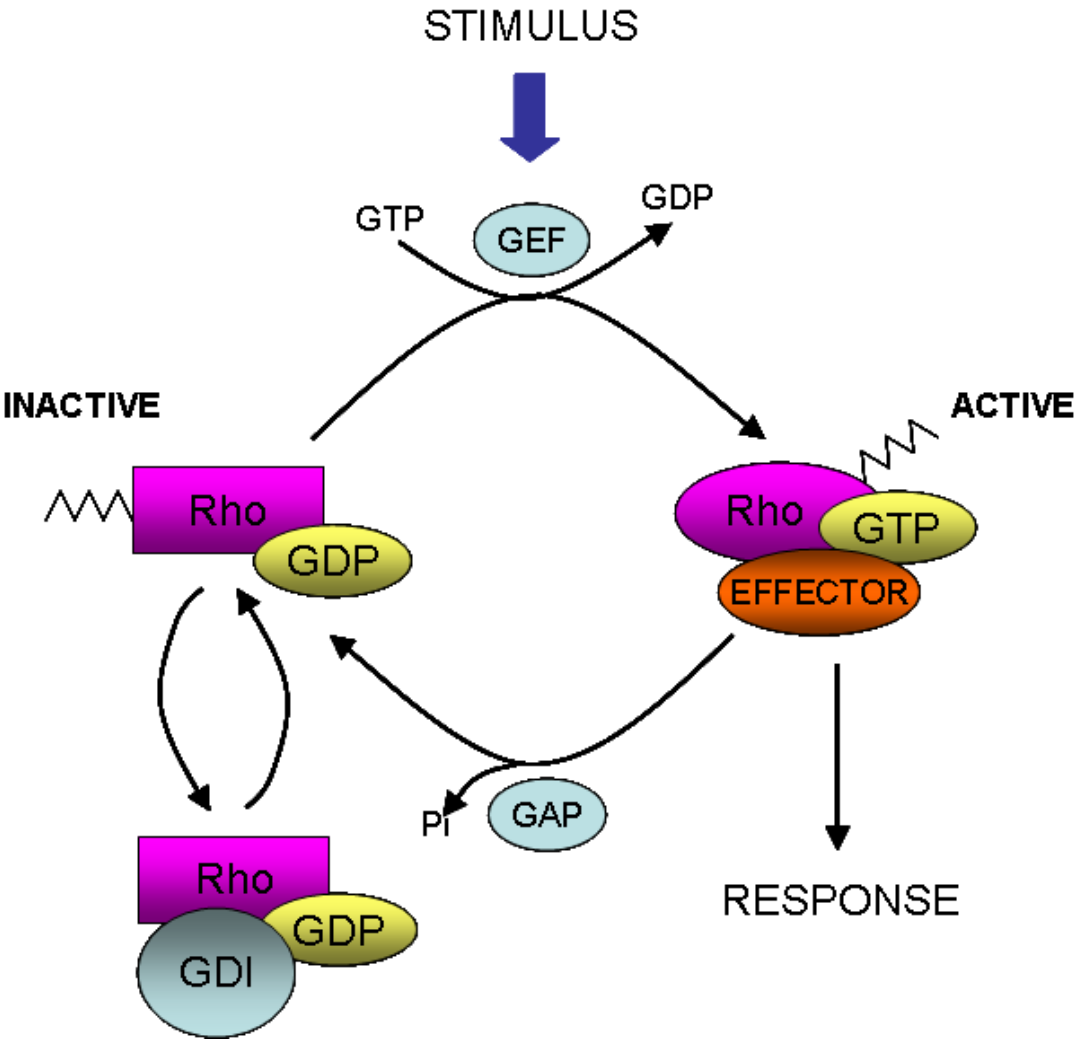

Figure 1.15 Regulation of Rho-GTPase activation.

Small GTPases such as Rho cycle between an inactive GDP-bound form and an active GTP-bound form. Activation is controlled by guanine nucleotide exchange factors (GEFs), which facilitate exchange of GDP for GTP. GTPase activating proteins (GAPs) increase intrinsic GTPase activity of Rho family members thereby deactivating them. Guanine nucleotide dissociation inhibitors (GDIs) also regulate activity by sequestering Rho-GTPases in the cytosol.

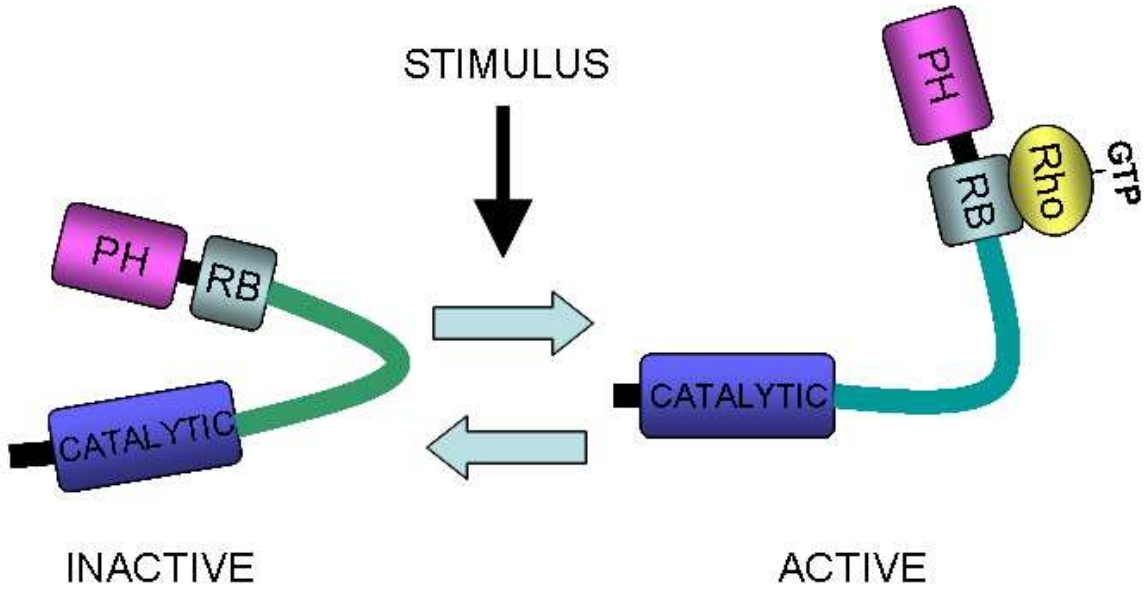

Figure 1.16 Regulation of Rho-kinase (ROCK) activity.

Binding of Rho-GTP to the Rho-binding domain (RB) of ROCK disrupts an autoinhibitory intramolecular interaction, freeing the catalytic kinase domain and allowing it to interact with ROCK substrates. ROCK remains active until GTP hydrolysis of Rho occurs. (PH, pleckstrin homology domain). Figure adapted from Amano *et al* (2000).

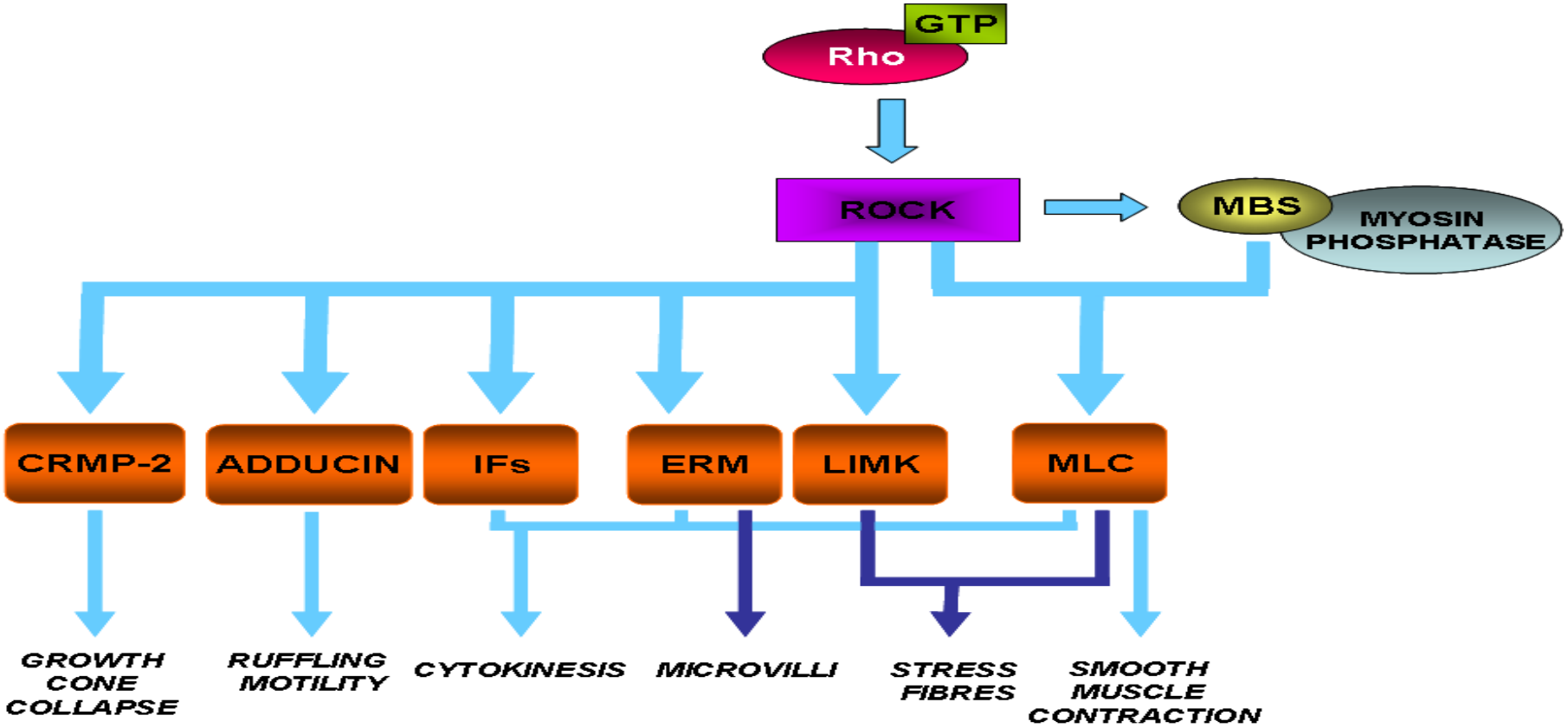

Figure 1.17. Summary of ROCK effectors and functions.
The activation of ROCK by the small G protein Rho results in the phosphorylation of downstream substrates that have multiple physiological effects. ROCK substrates include collapsin response mediator protein 2 (CRMP2), adducin, intermediate filament proteins (IFs), LIM kinase (LIMK), ERM family proteins (ezrin/radixin/moesin), myosin light chain (MLC) and the myosin binding subunit of myosin phosphatase (MBS). Figure adapted from Amano *et al* (2000).

1.8 5HT-INDUCED SIGNALLING PATHWAYS INVOLVED IN VASCULAR REMODELLING

Although 5HTT appears to be the major player in mediating the mitogenic effects of 5HT in human PASMCs (Marcos et al., 2004, Eddahibi et al., 1999), 5HT receptors have also been found to contribute to the signalling process required for cellular proliferation even if they do not directly induce proliferation in their own right. Much of the work on 5HT-induced mitogenic signalling has been carried out in bovine PASMCs. Briefly, these studies suggest that formation of reactive oxygen species (ROS) by the activation of NADPH oxidase, following 5HT entry *via* 5HTT, results in the activation of ERK, a process that is pivotal in mediating the proliferative effects of 5HT (Lee et al., 1999, Lee et al., 2001, Lee et al., 1998b). Once in the nucleus, activated ERK can phosphorylate transcription factors including GATA-4 (Suzuki et al., 2003), Elk-1 and Erg-1, as well as inducing cyclin D1 expression (Liu et al., 2004) and thus promoting proliferation. In this cell type, 5HT receptors have also been found to contribute to the proliferative effects of 5HT independently of ERK activation. Stimulation of the $5HT_{1B/1D}$ receptor has been shown to result in the activation of the small G-protein Rho and the resulting activation of ROCK, its downstream mediator, facilitates the translocation of ERK to the nucleus where it can mediate its mitogenic effects (Liu et al., 2004). Futhermore, the $5HT_{2A}$ receptor is also involved in the mitogenic response. This receptor appears to activate the phosphatidylinositol-3-kinase (PI3K)/protein kinase B (PKB) pathway, resulting in the downstream activation of the mammalian target of rapamycin (mTOR) and subsequent p70 ribosomal S6 kinase (S6K1) activation (Liu and Fanburg, 2006). Reactive oxygen species were also found to be a requirement for PKB activation in this instance. Furthermore, inhibition of MEK by various means blocked 5HT-induced S6K phosphorylation but not that of PKB suggesting another pathway may be involved (Liu and Fanburg, 2006). Therefore, in this cell type, diverse, independent signalling pathways are activated by 5HT, *via* 5HT receptors and 5HTT, resulting in PASMCs proliferation (Figure 1.18).

The signalling pathways utilised by 5HT appear to be cell type-specific. In a study using commercially available human PASMCs, 5HT was found to induce proliferation by a different mechanism for that seen in bovine PASMCs, however this model also highlighted the requirement for both 5HTT and 5HT receptors in the mitogenic process. In this case, the $5HT_{2A}$ receptor mediated ERK activation in response to 5HT, while 5HT transported into the cell *via* 5HTT was subsequently broken down by MAO-A to produce reactive oxygen species required to facilitate the translocation of ERK to the nucleus (Lawrie et al., 2005) (Figure 1.19). A role for MAO has also been suggested in 5HT signal transduction in cardiac myocytes. In these cells, the $5HT_{2B}$ receptor was found to contribute to the proliferative effects of 5HT, as inhibition of this receptor resulted in the reduction of 5HT-mediated ERK activation. However, inhibition of 5HTT had a more marked effect, inhibiting ERK activation by more than 80%. As suggested in human PASMCs, entry of 5HT into the cell *via* 5HTT, resulted in the generation of reactive oxygen species by the action of MAO-A. In this instance, the generation of ROS were required to induce ERK activation (Bianchi et al., 2005). Various 5HT receptors has been demonstrated to coupled positively to ERK activation, including $5HT_{1A}$ (Cloez-Tayarani et al., 2004, Adayev et al., 2003), $5HT_{2A}$ (Gooz et al., 2006), $5HT_{2B}$ (Nebigil et al., 2003, Nebigil et al., 2000b), $5HT_4$ (Norum et al., 2003), and $5HT_7$ (Lin et al., 2003, Norum et al., 2003). In the case of the $5HT_{2B}$ receptor, activation of ERK promotes cell survival in addition to progression through the cell cycle (Nebigil et al., 2003, Nebigil et al., 2000b). Taken together, these studies suggest that ERK activation is required in order to mediate the mitogenic effects of 5HT and also highlight the importance of 5HTT and 5HT receptors in this process.

In addition to its role in mitogenesis, 5HT has also been found to promote cell migration. In human aortic endothelial cells, 5HT has been shown to potently enhance cell migration through a RhoA- and ERK-dependent pathway mediated by $5HT_1$ receptors and 5HTT (Matsusaka and Wakabayashi, 2005a). Similar effects were also observed in human aortic SMC, although in this cell type migration was mediated *via* the $5HT_{2A}$ receptor (Matsusaka and Wakabayashi, 2005b). Likewise, 5HT induces PASMC migration, a process that may be important in vascular remodelling given the extension of PASMCs into non-muscular pulmonary artery during PAH. Day *et al* (2006) reported that in bovine

PASMC, 5HT stimulated migration and cytoskeletal reorganisation through activation of the $5HT_4$ receptor, a process that required the elevation of cAMP and activation of a chloride channel (Day et al., 2006). In this cell type, ERK activation is also required for migration to occur, although this was not mediated by either the $5HT_4$ receptor subtype or elevation of cAMP. Regulation of motility by ERK may therefore occur in parallel to 5HT-induced cytoskeletal rearrangments and be mediated by another receptor (Day et al., 2006).

5HT also plays a role in PAH by influencing other pathways important in the development and maintanence of the condition. For example recently, 5HT was found to transactivate PDGFRβ in PASMCs and this process was dependent on 5HTT. The transactivation process described was important in mediating SMC proliferation and migration (Liu et al., 2007). Furthermore, 5HT has also been observed to interact with the BMP signalling pathway and promote the development of PAH. In a study using BMPR2-deficient mice, treatment with 5HT exaggerated the pulmonary hypertensive effects of chronic hypoxia compared to wild-type littermates. It was proposed this effect occurred due to the ability of 5HT to inhibit BMPR2 signalling, attenuating Smad 1/5 phosphorylation and the transcription of BMP/Smad target genes (Long et al., 2006). It can therefore be seen that 5HT stimulates diverse signalling pathways, mediated *via* 5HTT and various 5HT receptors, and also modulates other signalling pathways resulting in the promotion of vascular remodelling and thus contributing to the development of PAH.

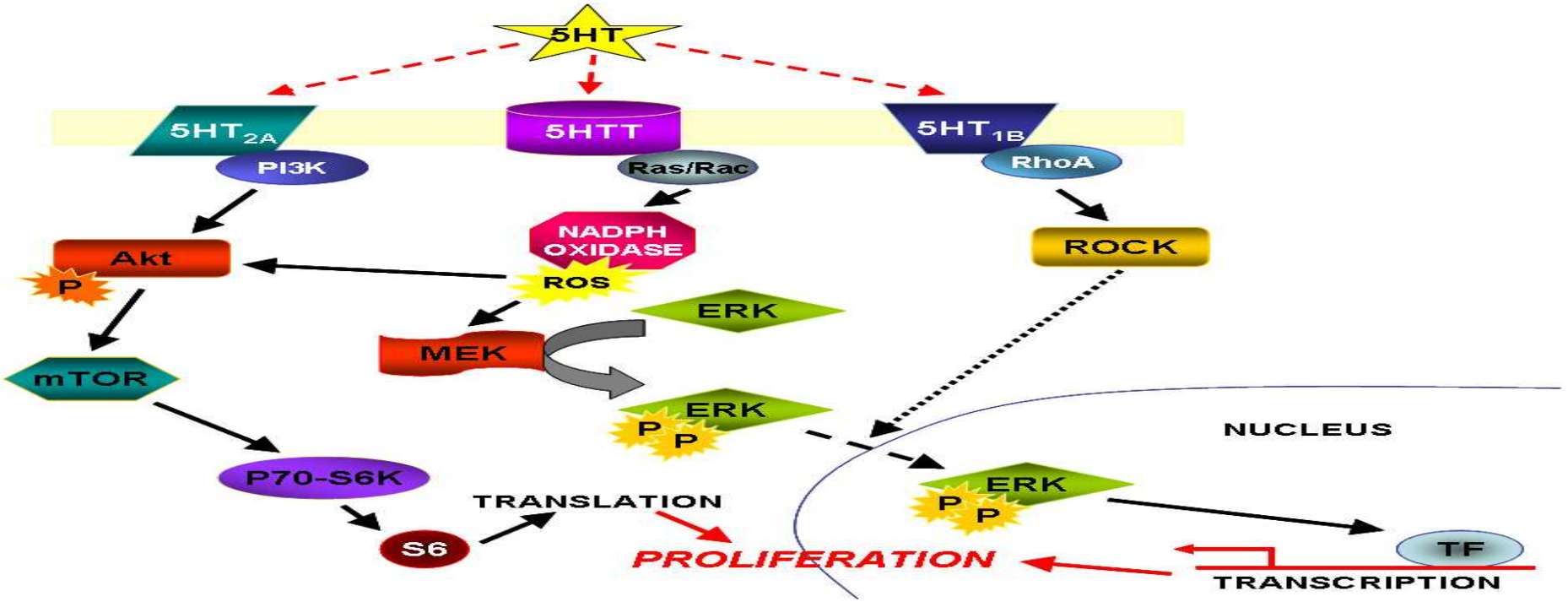

Figure 1.18 5HT-induced mitogenic signalling transduction pathways in bovine pulmonary artery smooth muscle cells (PASMCs).

In bovine PASMCs 5HT-induced proliferation requires co-operation between ERK, ROCK and PI3K pathways. The action of 5HT on the 5HT transporter (5HTT) results in the activation of the ERK pathway, a process that is dependent on the production of reactive oxygen species (ROS) by NADPH oxidase. The $5HT_{1B}$ receptor is involved in mediating the activation of ROCK, which is required to facilitate the translocation of active ERK to the nucleus. Additionally, activation of the PI3K/Akt pathway by the $5HT_{2A}$ receptor is also important in the proliferative response. Figure adapted from Liu and Fanburg (2006).

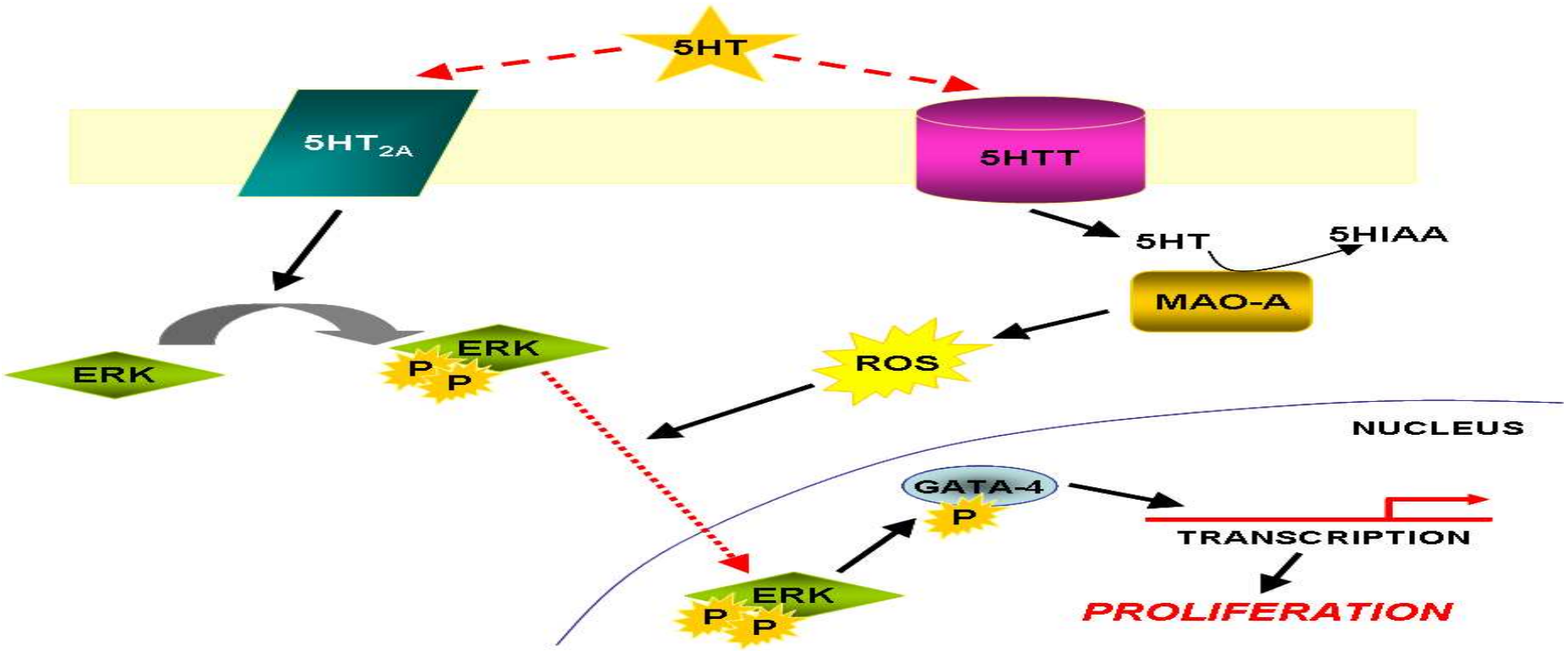

Figure 1.19 5HT-induced mitogenic signal transduction pathways in human PASMCs.

In human PASMCs the $5HT_{2A}$ receptor and the 5HT transporter (5HTT) co-operate to mediate the proliferative effects of 5HT. The $5HT_{2A}$ receptor is involved in the process of ERK activation, while transport of 5HT into the cell *via* 5HTT results in the formation of reactive oxygen species (ROS) due to 5HT breakdown by the enzyme monoamine oxidase-A (MAO-A). ROS produced are required for the translocation of ERK to the nucleus, where it activates the transcription factor GATA-4 and transcription of mediators that result in cellular proliferation. Figure adapted from Lawrie *et al* (2005).

1.9 AIM

The monoamine and mitogen, 5HT has been implicated in the development of PAH. However, the signal transduction pathways utilised by 5HT to induce cell proliferation and thus contribute to pulmonary vascular remodelling are not fully understood. Therefore, the aim of this thesis is to characterise cellular signalling pathways that contribute to the mitogenic effects of 5HT and determine a role for these pathways *in vivo*.

Chapter 2

MATERIALS AND METHODS

2.1 Materials

Abcam Ltd, Cambridge, UK:

Anti-SP1 antibody (ab13370), anti-GAPDH antibody (ab8245).

Amersham Biosciences UK Ltd, Buckinghamshire, UK:

[methyl-^{3}H]Thymidine (specific activity 2.0Ci/mmol), Rainbow Markers (14.4-220 kDa).

Biorad Laboratories Ltd, Hemel Hempstead, UK:

Bradford's reagent.

Calbiochem, Merck Biosciences Ltd, Nottingham UK:

Anti-cyclinD1(Ab-3) mouse mAb (CC12), conconavalin A from* Conconavalin ensiformis *(conA), phorbol-12-myristate-13-acetate (PMA), U0126, SB203580.

Cambrex Bio Science, Berkshire, UK:

Dulbecco's Modified Eagle Medium with 4.5g/l glucose (DMEM), Dulbecco's Phosphate Buffered Saline without Ca^{2+} and Mg^{2+} (PBS).

Cell Signaling Technology Inc, Beverly, MA, USA:

Phospho-MEK1/2 (Ser217/221) antibody (9121), p44/42 MAP Kinase antibody (9102), phospho-p44/42 MAP Kinase (Thr202/Tyr204)(E10) mouse mAb (9106), phospho-p38 MAP Kinase (Thr180/Tyr182) antibody, p38 MAP Kinase antibody (9212).

Cytoskeleton Inc, Denver, CO, USA:

Cell permeable C3 transferase from *Clostridium botulinum*.

Fisher Scientific, Loughborough, Leicestershire, UK:

4-2-hydroxyethyl-1-piperazineethanesulphonic acid (HEPES), glycine, hydroxymethyl-aminomathane (Tris) base, sucrose, sodium hydroxide, ammonium persulphate, concentrated hydrochloric acid, sodium carbonate, sodium hydrogen carbonate, sodium dihydrogen ortho-phosphate, disodium hydrogen ortho-phosphate.

Inverclyde Biologicals, Bellshill, Lanarkshire, UK

Protan nitrocellulose membrane (Scleicher and Schuell; pore size 0.2µm).

Invitrogen Ltd, Paisley, UK:

AlexaFluor®594 -conjugated phalloidin, Trizol reagent.

McQuilkin Laboratory Supplies:

Skimmed Milk

Melford Laboratories Ltd, Ipswich, Suffolk, UK:

Dithiothreitol (DTT).

Meniel-Glaser, Braunschweig, Germany:
Microscope slides, coverslips.

Perkin Elmer, Boston, MA, USA:

Western Lightning™Chemiluminescence Reagent Plus, glass fibre filter mat A, sample bags, Betaplate Scint for Betaplate.

Pierce, Rockford, IL, USA:

Western blot stripping solution.

Riedel-de Haen, Germany:

Ethylenediaminetetra-acetic acid (EDTA), ethyleneglycol-bis(2-aminoethyl)-N,N,N',N-tetra acetic acid (EGTA), glycerol, methanol.

Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA:

Phospho-cofilin (Ser3) antibody (sc12912).

Sigma-Aldrich Ltd, Poole, Dorset, UK:

Cytochalasin D from* Zygosporium mansonii *(Cyto D), latrunculin B (Lat B), monoclonal anti-α tubulin antibody (T9026), goat anti-mouse IgG (whole molecule) peroxidase conjugate (A4416), methiothepin (mesylate salt), iproniazid (phosphate salt), anisomycin (AN), 5-hydroxytryptamine (serotonin)(creatine sulphate salt) (5HT), foetal bovine serum (FBS), L-glutamine (200mM), penicillin streptomycin solution (10,000 units penicillin and 10mg streptomycin per ml in 0.9% NaCl), N-acetyl-L-cysteine (NAC), goat anti-rabbit IgG peroxidase conjugate (A8275), anti-sheep IgG peroxidase conjugate (A3415), monodansylcadaverin (MDC), 30% (w/v) acrylamide/0.8% (w/v) bis-acrylamide, trypsin-EDTA, Bromophenol Blue, phenylmethylsulphonyl fluoride (PMSF), benzamidine, soybean trypsin inhibitor, Tween-20, Triton X-100, bovine serum albumin (BSA), 4,4 dicarboxy-2, 2 biquinoline disodium salt, sodium deoxycholate, sodium potassium tartrate, copper (II) sulphate, N,N,N',N'-tetramethylethylenediamine (TEMED), paraformaldehyde, nonident P-40 (NP-40).

Tocris Bioscience, Bristol, UK:

Y27632 dihydrochloride, citalopram hydrobromide, GR55562 dihydrochloride, ketanserin tartrate, fluoxetine hydrochloride, α-methyl-5-hydroxytryptamine, CP94253.

Upstate Biotechnology, Lake Placid, NY, USA:

Anti-phospho-MYPT1 (Thr 696)(rabbit polyclonal IgG)(07-251), anti-MYPT1 (sheep immunoaffinity purified IgG)(07-159).

VWR International Ltd, Poole, UK:

Sodium chloride, sodium dodecyl sulphate (SDS), potassium hydroxide, potassium chloride.

2.2 METHODS

2.2.1 Cell Culture.

CCL-39 Chinese hamster lung fibroblast cells were cultured in T-75 flasks in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 2mM L-glutamine, 100U/ml penicillin, 100µg/ml streptomycin and 10% (v/v) foetal bovine serum (FBS). Cells were maintained at 37^{o}C in a humidified 5% (v/v) CO_2 atmosphere (5% CO_2/95% air) until confluent. Once confluent the cell monolayer was washed with Ca^{2+}- and Mg^{2+} - free phosphate buffered saline (PBS). The cells were then detached by addition of 2 ml trypsin, followed by incubation at 37^{o}C. 6ml of media were then added to the flask and cells resuspended by gentle pipetting. Thereafter, cells were either passaged into T-75 flasks to maintain the cell line or seeded into dishes for experimental analysis.

2.2.2 Preparation of Cell Extracts for Immunoblotting.

Cells for immunoblotting were grown to confluence in 6-well tissue culture plates. Following treatment with the appropriate stimuli, reactions were ceased by transferring to ice. Media was then removed and the cell monolayer washed three times with 2 ml ice-cold PBS. Cells were then lysed by scraping into 200µl of RIPA buffer (50mM HEPES pH 7.5, 150mM NaCl, 1% (v/v) Triton X-100, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 0.01M sodium phosphate, 5mM EDTA, 0.1mM PMSF, 1µg/ml soybean trypsin inhibitor, 1µg/ml benzamidine) and transferred to ice-cold micro-centrifuge tubes, then allowed to solublise for 30 minutes with occasional vortexing. The insoluble cellular debris was then removed by centrifugation (20,000 g for 15 min, 4^{o}C). 150µl of supernatant were then taken for assay to determine protein concentration and analysis by SDS-PAGE and immunoblotting.

2.2.3 Determination of protein concentration by bicinchoninic acid (BCA) protein assay.

BCA assays were carried out in a 96-well plate format with duplicate bovine serum albumin (BSA) standards ranging in concentration from 0-2 mg/ml in a volume of 10µl. The same volume of each sample of unknown protein concentration was also added in duplicate. Each well was then supplemented with 200µl of BCA solution (1% (w/v) 4,4 dicarboxy-2, 2 biquinoline disodium salt, 2% (w/v) sodium carbonate, 0.16% (w/v) sodium potassium tartrate, 0.4% (w/v) sodium hydroxide, 0.95% (w/v) sodium dicarbonate (pH 11.25), 0.08% (w/v) copper (II) sulphate) and incubated at room temperature for 10-15 minutes. Protein concentration was then determined by measuring absorbance of samples at 492nm (A_{492}) using a MRX-TCII plate reader (Dynex Technologies). The absorbance of the known standards was used to generate a best-fit straight-line plot of A_{492} *versus* protein concentration, from which the protein concentration of the unknown samples could be deduced.

2.2.4. Determination of protein concentration by Bradford's protein assay.

Bradford protein assays were carried out in a 96 well plate with duplicate BSA standards ranging in concentration from 0-2 mg/ml in a volume of 10µl. The same volume of each sample with unknown protein concentration was also added in duplicate. Each well was then supplemented with 50µl of Bradford's reagent which had been diluted 1:4 in distilled deionised water. Samples were then incubated for 10 minutes at room temperature and protein concentrations determine by measuring absorbance at 630nm (A_{630}). A best-fit straight-line plot of A_{630} *versus* protein concentration was then constructed using data obtained from the standards. This plot was then used to deduce the unknown protein concentration of the experimental test samples.

2.2.5 SDS-PAGE and Immunoblotting Analysis.

The protein concentrations of samples prepared for SDS-PAGE were determined by bicinchoninic acid (BCA) protein assay, unless otherwise stated. Samples were then equalised for protein concentration and volume, and 2x SDS-PAGE sample buffer (50mM Tris, pH 6.8 at room temperature, 10% (v/v) glycerol, 12% (w/v) SDS, 10mM dithiothreitol, 0.0001% (w/v) bromophenol blue) added. Samples then underwent fractionation by SDS-PAGE using a 10% acrylamide resolving gel (10% (w/v) acrylamide, 0.3% (w/v) bisacrylamide, 0.4M Tris (pH 8.8), 0.1% (w/v) SDS, 0.01% (w/v) ammonium persulphate and 0.001% (v/v) TEMED) and 3% acrylamide stacking gel (3% (v/v) acrylamide, 0.1% (v/v) bisacrylamide, 0.1M Tris (pH 6.8), 0.1% SDS, 0.01% ammonium persulphate and 0.001% (v/v) TEMED) unless otherwise stated. Electrophoresis of samples was carried out in the presence of pre-stained protein markers (Rainbow Markers 14.3 - 220 kDa, Amersham Biosciences) in order to estimate the molecular mass of immunoreactive proteins. Using Biorad Mini-Protean III gel electrophoresis systems, electrophoresis was carried out at 150V in running buffer (27.4mM Tris, 0.19M glycine, 0.1% (w/v) SDS) until the bromophenol blue dye front reached the bottom edge of the gel. Fractionated proteins were then transferred to nitrocellulose membrane at 400mA for 45 minutes in transfer buffer (24.7mM Tris, 0.19M glycine and 20% (v/v) methanol). Following transfer, the nitrocellulose membrane was washed briefly in Tris Buffered Saline-Tween (TBST) (20mM Tris pH 7.5 at room temperature, 150mM NaCl, 0.1% Tween 20), before blocking for one hour at room temperature in Blotto (5% (w/v) skimmed milk in TBST). Membranes were then incubated overnight with 1:1000 dilution of primary antibody in 5% BSA (w/v) in TBST at 4^{o}C. Subsequently, membranes were washed three times in TBST for 10 minutes each wash. The appropriate HRP-conjugated secondary antibody diluted 1:1000 in Blotto was then incubated with membranes for 1 hour at room temperature before three further 10-minute washes in TBST. Each membrane was then treated with ECL reagents (Perkin Elmer) as per the manufacturer's instructions and immunoreactive proteins were then visualised by exposure to X-ray film.

Densitometry was performed on each resulting film exposure to determine normalized levels of the protein of interest using Total Lab version 2.0 imaging software.

2.2.6 [^{3}H]-Thymidine incorporation assay of DNA synthesis

Cells were plated out at a density of $2x10^4$ cell/ml into 96-well plates and grown in full media for 24 hours before serum starving for a further 24 hours. The media was then replaced with fresh media containing no serum and the appropriate stimuli in a final volume of 200µl. Each experimental condition was carried out in triplicate. After an incubation period of 18 hours, 0.5µCi/well [^{3}H]-thymidine was added. Proliferation was stopped after a further 6 hours by harvesting cells onto glass fibre filter mats using a Betaplate 96-well harvester (Wallac). The glass fibre filter mats used are printed on both sides with a grid to aid alignment with the wells of 96-well plates. During the harvesting process the 96-well plate harvester washes each well with distilled water three times, dislodging the cell monolayer. The contents of the aspirate are then transferred directly to the corresponding grid square of the glass fibre filter mat by vacuum filtration. Filter mats are then left to dry before being sealed in clear plastic sample bags with 10 ml of scintillation fluid (Betaplate Scint for Betaplate). Filters were then placed in a 96-format filtermat cassette and incorporated [^{3}H]-thymidine assessed by liquid scintillation counting, using a 1205 Betaplate Liquid Scintilation Counter (Wallac) and results expressed as counts per minute (cpm).

2.2.7 Preparation of cytosolic and nuclear cell fractions for nuclear translocation experiments.

Cells were seeded into 10cm tissue culture dishes and grown to confluence. Media was then replaced with serum free media and incubated overnight. Following this, cells were treated with the appropriate stimuli in the presence or absence of inhibitors and the reaction stopped by transferring onto ice. Subsequent steps were also performed on ice. Media was aspirated and the

cell monolayers washed twice with ice cold PBS. Cells were then gently scraped from each tissue culture dish in a volume of 1ml PBS and transferred to ice cold micro-centrifuge tubes. Samples were centrifuged at a temperature of 4^{o}C for 4 minutes at ~2000g. The resulting pellet was then resuspended in 400µl of nuclear extraction buffer A (10mM HEPES (pH 7.9 with KOH), 10mM KCl, 0.1mM EDTA, 0.1mM EGTA, 1mM DTT) and left to incubate on ice for 15 minutes, after which, 25µl of 10% (v/v) NP-40 were added and samples vortexed. Subsequently, samples were briefly centrifuged and the resulting supernatant containing the cytosolic cellular fraction removed and transferred to another micro-centrifuge tube.

The remaining pellet was then washed 4 times with 500µl buffer A, with brief centrifugation (20,000g for 15 seconds) between each wash and the supernatant discarded. After the final wash the pellet was resuspended in nuclear extraction buffer B (20mM HEPES (pH 7.9 with NaOH), 450mM NaCl, 1mM EDTA, 1mM EGTA 1mM DTT), vortexed and incubated on ice with occasional agitation for 15 minutes. Finally, samples were centrifuged at 20,000g for 15 minutes and the resulting supernatant, containing the nuclear fraction removed and transferred to a fresh micro-centrifuge tube. Protein concentrations were measured using Bradfords assay and samples analysed by SDS-PAGE and immunoblotting.

2.2.8 Staining of Actin Cytoskeleton

Cells were seeded in 6-well plates (approx 2 x 10^5 cells/ml) onto sterile glass coverslips and grown for 16-24 hours, prior to serum starvation for a further 16 hours. Cells were then treated with agonist in the presence or absence of other agents as described for each individual experiment.

The following steps were carried out at room temperature. Firstly, the media was removed and discarded and coverslips washed three times in 2 ml PBS. In order to fix the cell monolayer, coverslips were then incubated for 15 minutes in 2 ml of 4% (w/v) paraformaldyde in a 5% (v/v) sucrose/PBS solution. Subsequently coverslips were washed a further twice with 2 ml PBS and the cell monolayer permeabilised by the addition of 2 ml 0.1% (v/v) Triton X-100 in PBS

for 2 minutes. Coverslips were then washed twice in 2 ml PBS and blocked for 30 minutes in 5% (w/v) BSA in PBS. 100µl of Alexa Fluor 594-conjucated phalloidin diluted 1:20 in a 5% (w/v) BSA/PBS were then added to each coverslip and incubated fro 20-30 minutes. Finally, cells were washed twice in PBS and mounted on glass slides in 40% (v/v) glycerol/PBS. Fluorescent proteins were visualised on a Zeiss fluorescent microscope using x 40 objective and pictures obtained using Axiovision AC version 4.4 software.

2.3 In Vivo METHODS

2.3.1 In Vivo Experimental Design

Experiments were conducted in accordance with the United Kingdom Animals (Scientific Procedures) Act 1986. $5HTT^+$ mice that overexpress 5HTT, previously generated as described by Jennings *et al* 2006, were used in this study. These mice were generated from the C567BL/6 X CBA wildtype (WT) strain, which were also used as controls.

Briefly, the transgene used to develop the $5HTT^+$ was 500 kb yeast artificial chromosome (YAC35D8) containing the human 5HTT (h5HTT) gene flanked by a 150 kb sequence towards the 5' end and a 300kb towards the 3' end, with the short allele of the 5HTTLPR in the promoter region and a 10-repeat allele of the VNTR in intron 2. The yeast artificial chromosome was modified to include a hemagglutinin epitope tag at the C-terminus of the 5HT protein and a LacZ reporter gene downstream of an internal ribosomal entry site as described previously (Shen et al., 2000). Analysis by *in situ* hybridisation showed that h5HTT mRNA was expressed in a pattern that closely resembled the endogenous mouse 5HTT gene (Jennings et al., 2006).

For the purposes of this study both WT and $5HTT^+$ female mice aged 5-6 months were maintained in either normoxic (~ 21% (v/v) oxygen) or hypoxic/hypobaric conditions for a period of 14 days. To achieve hypoxic conditions, animal were housed in a hypobaric chamber that was initially

depressurised slowly by 50 mbar every 15 minutes until the pressure reached 550 mbar (~10% O_2). Animals were then maintained at 550 mbar for 14 days. During this period mice were dosed daily with either vehicle (water) or 30mgkg^{-1} Y27632 (a ROCK inhibitor) by oral gavage.

2.3.2 ***In Vivo*** **Haemodynamic Measurements**

After 14 days of drug dosage, anaesthesia was induced by 3% (v/v) halothane and body weight of mice determined. Anaesthesia was then maintained with halothane (1% (v/v) to 1.5% (v/v)) and a mixture of nitrous oxide and oxygen (1:6) using a face mask. Systemic arterial blood pressure (SAP) was measured by cannulation of the carotid artery. In order to do so, the right carotid artery was first isolated and tied off distally using surgical thread. Following this a small artery clip was placed around the proximal end of the artery to prevent blood flow. A small incision was made in the artery and the cannula (0.75mm OD, Portex) advanced in the proximal direction. The cannula was then tied in place and the artery clip removed to allow measurement of SAP and heart rate (HR).

Right ventricular pressure was measured using a 25 gauge needle mounted on a micromanipulator. The tip of the needle was aligned with the mid point of the sternum and advanced through the diaphragm into the right ventricle. Entry of needle into the right ventricle was confirmed by the morphology of the pressure trace obtained. Both cannula and needle were attached to pressure transducers (Elcomatic E751A) connected to a MP100 data acquisition system (BIOPAC Systems Inc, Santa Barbra, CA).

Results were analysed using an AcquiKnowledge 3.5 software package.

Following the measurement of haemodynamics, heart and lungs were removed from each animal for analysis.

2.3.3 Meaurment of Right Ventricular Hypertrophy

Hearts removed following haemodynamic measurements were carefully dissected. The atria were first removed, the right ventricular free fall was then dissected from the left ventricle plus septum. The right ventricle free wall and

LV plus septum were weighed separately and the ratio of right ventricle to left ventricle calculated.

2.3.4 TaqMan Reverse Transcription-Polymerase Chain Reaction

RNA was extracted from mouse whole lung tissue using Trizol reagent. Real-time fluorogenic reverse transcription-polymerase chain reaction (PCR) was then performed by TaqMan® Gene Expression Assay (Applied Biosystems, CA, USA) using gene expression probes for mouse ROCK 1 and ROCK 2 (Mn00485745_m1 and Mm00485761_m1 respectively) according to the manufacturers instructions. Relative abundance of ROCK 1 and ROCK 2 mRNA was determined by using the comparative delta CT method with 18s ribosomal RNA as an internal control.
This work was carried out by Dr J Sheward, University of Edinburgh.

2.3.5 Lung Histology

Three sagittal sections were obtained from left lungs. Sections were stained with Elastica-Van Gieson stain and pulmonary arteries (<80 μm) microscopically assessed in a blinded fashion for muscularization. Arteries were considered muscularized if they processed a distinct double-elastic lamina visible for at least half the diameter in the vessel cross section. At least 100 arteries were counted per lung section. The percentage of vessels containing double-elastic lamina was calculated as the number of muscularized vessels/total number of vessels counted x 100.
This work was carried out by Dr I Morecroft, University of Glasgow.

2.3.6 Statistical Analysis.

Statistical analyses were carried out using either Students two-tailed unpaired *t*-test as described in Graphpad Prism 4 software. Where appropriate, one-way analysis of variance (ANOVA) with Newman-Keuls multiple comparison post test was applied, unless otherwise stated.

Chapter 3
Characterisation of 5HT-stimulated Mitogenic Signalling Pathways

3.1 INTRODUCTION

5HT plays an important role in the etiology of PAH, contributing to remodelling and vasoconstriction of the pulmonary circulation. As described previously, 5HT and the expression and activity of 5HTT have been reported to play a critical role in the disease process. For instance, 5HT and 5HTT have been implicated in pulmonary vascular smooth muscle cell hyperplasia and vascular proliferation in both experimental hypoxic PAH and the human condition (Eddahibi et al., 2001; Eddahibi et al., 2000b). Furthermore, polymorphisms in the promoter region of the 5HTT gene resulting in increased 5HTT expression have been reported in around 65% of patients with familial PAH (Eddahibi et al., 2003).

At a cellular level several studies have highlighted the ability of 5HT to induce proliferation in a variety of cell types including, cardiac myocytes, endothelial cells and PASMCs (Bianchi et al., 2005; Pakala et al., 1999; Liu and Fanburg, 2006). The ability of 5HT to mediate cellular proliferation may be an important contributing factor to pulmonary artery remodelling during pulmonary hypertension. An array of intracellular pathways has been proposed to describe the signalling mechanisms utilised by 5HT to result in cellular proliferation. A common theme of these hypotheses is the ability of 5HT to induce ERK1/2 activation, a process which appears to be key to the proliferative response (Lee et al., 1999; Lee et al., 2001).

5HT can mediate cell signalling by interacting with several 5HT receptor subtypes or through 5HTT, which transports 5HT across the plasma membrane using a Na^+/Cl^- gradient. A large body of evidence exists suggesting the mitogenic effects of 5HT are largely mediated *via* 5HTT. Much of this work has been carried out in bovine PASMCs. In this cell type, signal transduction initiated by 5HT involves 5HTT-dependent generation of reactive oxygen species (ROS) and activation of the MEK/ERK pathway (Lee et al., 1998b; Lee et al., 1999; Lee et al., 2001). In short, the 5HT-induced signalling response includes the sequential activation of Ras/Rac-1, NADPH oxidase activation, generation of ROS and finally the phosphorylation and activation of ERK MAP kinase (Lee et al., 1999; Suzuki et al., 2003). Downstream transcriptional components of this pathway have also been characterised. For instance, in

PASMCs, stimulation with 5HT has been found to induce the ERK-dependent DNA binding of transcription factors, Erg-1, GATA-4, as well as phosphorylation of Elk-1 (Liu et al., 2004; Suzuki et al., 2003).

5HT receptors also appear to be important in the proliferative response, with a number of studies reporting the combined action of receptors and transporter are required to mediate the effects of 5HT. For example, in rat pulmonary artery fibroblasts, the $5HT_{2A}$ receptor and 5HTT are both involved in 5HT-induced proliferation (Welsh et al., 2004). In another fibroblast cell line, 5HTT, $5HT_{1B/1D}$ and $5HT_{2A}$ are all thought to be involved in 5HT-induced ERK activation and proliferation (Lee et al., 1999). Interactions between 5HTT and receptors have also been highlighted in PASMCs, where stimulation of $5HT_{1B/1D}$ and $5HT_2$ receptors result in the activation of distinct signalling pathways required to facilitate the activation of ERK *via* 5HTT (Liu et al., 2004; Liu and Fanburg, 2006).

The role of the p38 MAP kinase cascade in 5HT-mediated signalling is unclear. In certain cell types 5HT has been shown to activate p38 (Lieb et al., 2005, Welsh et al., 2004) and also play a role in 5HT-mediated contraction (Tasaki et al., 2003). On the other hand, several studies have also shown that 5HT has no effect on p38 and is unable to induce its activation (Cloez-Tayarani et al., 2004; Lee et al., 2001). In addition to this, p38 is thought to play an important role in the regulation of 5HTT (Prasad et al., 2005; Zhu et al., 2005).

Another possible mechanism for 5HT induced proliferation has been suggested. In this model 5HT entry into the cell may result in proliferation *via* receptor-independent reactive oxygen species generation. It has been reported recently that 5HT is able to induce hypertrophy in cultured rat cardiac myocytes (Bianchi et al., 2005; Vindis et al., 2000). This process appears to require the production of hydrogen peroxide, generated by the breakdown of 5HT by the enzyme MAO A. Furthermore, the reactive oxygen species formed are required for ERK activation, which in turn contributes to the myocardial remodelling. MAO B has also been shown to generate hydrogen peroxide and that this leads to ERK1/2 dependent mitogenesis (Vindis et al., 2000).

Other factors may also be pivotal in regulating 5HT-induced ERK activation. For instance, components of the endocytotic pathway may be involved. The scaffolding proteins β-arrestins have been shown to bind ERK1/2 and thus

regulate its activity (Tohgo et al., 2003; Tohgo et al., 2002). In addition to this, it has been shown that the $5HT_{1A}$ receptor must undergo calcium/calmodulin-dependent receptor endocytosis in order to activate ERK (Della Rocca et al., 1999). Furthermore, several other scaffolding proteins have been shown to regulate the ERK pathway including MEKK1 and MP1 (Morrison and Davis, 2003). These proteins could potentially be involved in mediating 5HT-induced ERK activation.

In summary, several potential signalling pathways required for 5HT-induced mitogenesis have been suggested. However, much of this work has been carried out in PASMCs. As mentioned previously, each cell type within the pulmonary artery contributes to the remodelling process that occurs during PAH. It would therefore be beneficial to investigate the role of 5HT and the signalling mechanisms utilised in other cell types. In this chapter, 5HT induced mitogenesis was investigated in a fibroblast cell line (Chinese hamster lung fibroblasts (CCL-39s)) in order to elucidate any novel signalling mechanisms that may be involved in the remodelling process.

3.2 RESULTS

Prior to identifying components involved in 5HT mitogenic signalling, the timecourse of 5HT-induced ERK1/2 activation was first characterised. Quiescent CCL-39 cells were stimulated with 5HT (1µM) over a 24 hour period. Subsequent immunoblotting using a phospho-specific ERK1/2 antibody revealed that addition of 5HT rapidly and transiently induced ERK1/2 activation, with maximal activation occurring around 2 minutes ($p<0.01$ *versus* vehicle, n=3) (Figure 3.1). Levels returned to that of basal after 30 minutes with no further activation noted during the 24 hour period. 5HT was also found to have a mitogenic effect in CCL-39 cells, inducing a dose dependent increase in proliferation as determined by [^{3}H]-thymidine incorporation (Figure 3.2). Inhibition of the ERK1/2 MAP kinase signalling cascade using U0126 (1µM), a MEK inhibitor, markedly attenuated the effects of 5HT, reducing the maximal proliferation by 80±5% (Figure 3.2). Furthermore, inhibition of MEK significantly reduced 5HT-induced ERK1/2 activation by around 73±3% ($p<0.001$ *versus* 5HT alone, n=3) (Figure 3.3). Under these circumstances responses to PMA were also significantly decreased ($p<0.05$, n=3), indicating U0126 was effective at the concentration used.

Under certain conditions the p38 MAP kinase cascade has been implicated in signal transduction *via* 5HT receptors (Welsh et al., 2004) and in the regulation of 5HTT (Zhu et al., 2005). To investigate the involvement of p38 in 5HT-induced signalling in CCL-39 cells, cells were stimulated over a period of time with 5HT (1µM). Treatment with 5HT was found to have no effect on the levels of phosphorylated p38 (p-p38) during the timecourse studied, suggesting 5HT is unable to activate p38 (Figure 3.4). In addition to this, the role of p38 in 5HT-induced proliferation was assessed (Figure 3.5). Treatment with p38 inhibitor SB203580 at a concentration of 5µM had no effect on the ability of 5HT to induce proliferation. In contrast, SB203580 at a concentration of 10µM markedly reduced 5HT-induced proliferation, resulting in levels of [^{3}H]-thymidine incorporation similar to those witnessed under vehicle conditions. SB203580 (5µM) was effective in inhibiting p38 as pre-treatment with the inhibitor resulted in a significant attenuation in the response to anisomycin, a known p38 activator (63.7±3% reduction, $p<0.001$, n=3) (Figure 3.6).

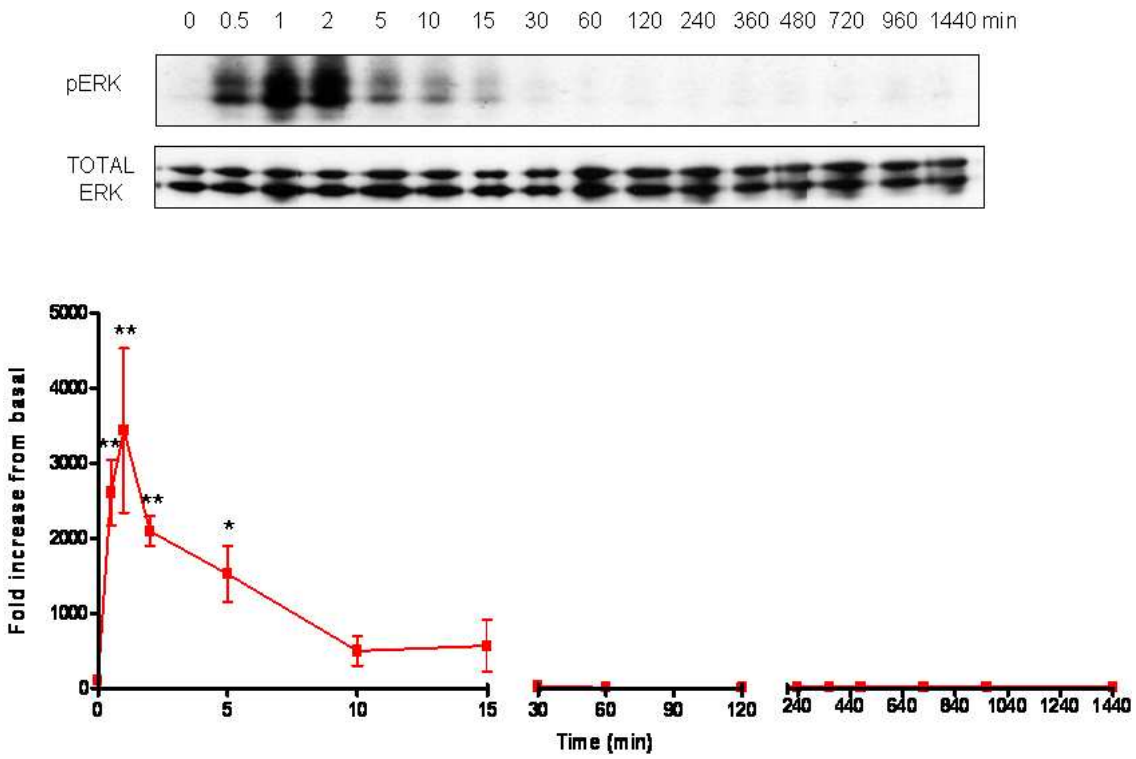

Figure 3.1 Timecourse of 5HT-induced ERK activation.

CCL-39 cells were serum starved for 16 hours and then stimulated with 5HT (1µM) for 0-24 hours. Cell lysates were prepared and equalised for protein concentration by BCA assay. Samples were then analysed by SDS-PAGE and immunoblotting using phospho-specific and total ERK antibodies. Blots shown are representative. Results are expressed as mean ± SEM (n=3). * $p<0.05$, ** $p<0.01$ *versus* vehicle using Newman-Keuls multiple comparison test.

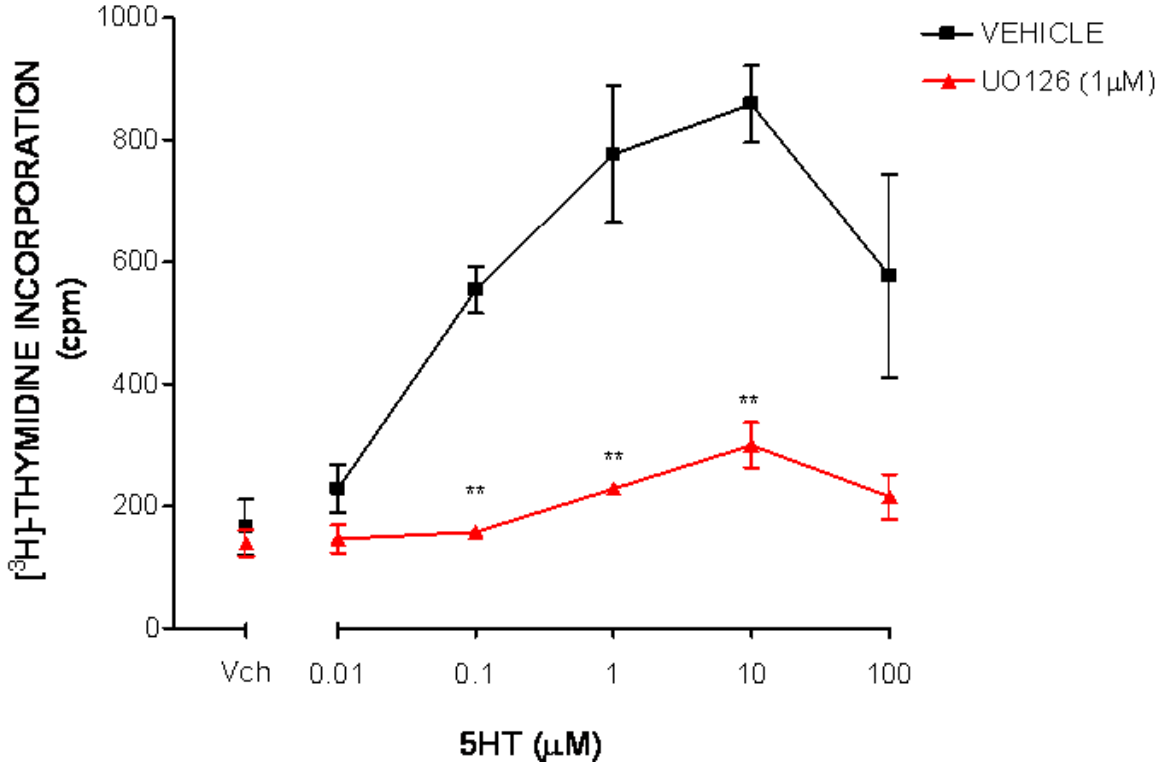

Figure 3.2 Effect of MEK inhibition by UO126 on 5HT-induced proliferation.

CCL-39 cells were serum starved for 24 hours then incubated with 0-100µM 5HT in the presence or absence of UO126 (1µM) (MEK inhibitor) for a further 24 hours, with 0.5µCi/well [^{3}H]-thymidine added for the final 6 hours of this incubation. Cell were then harvested onto a glass fibre filter mat and [^{3}H]-thymidine incorporation assessed by liquid scintillation counting. Data shown are representative of n=3. Results expressed as mean ± SEM of triplicate samples. ** $p<0.01$ *versus* vehicle using an unpaired, two tailed t-test.

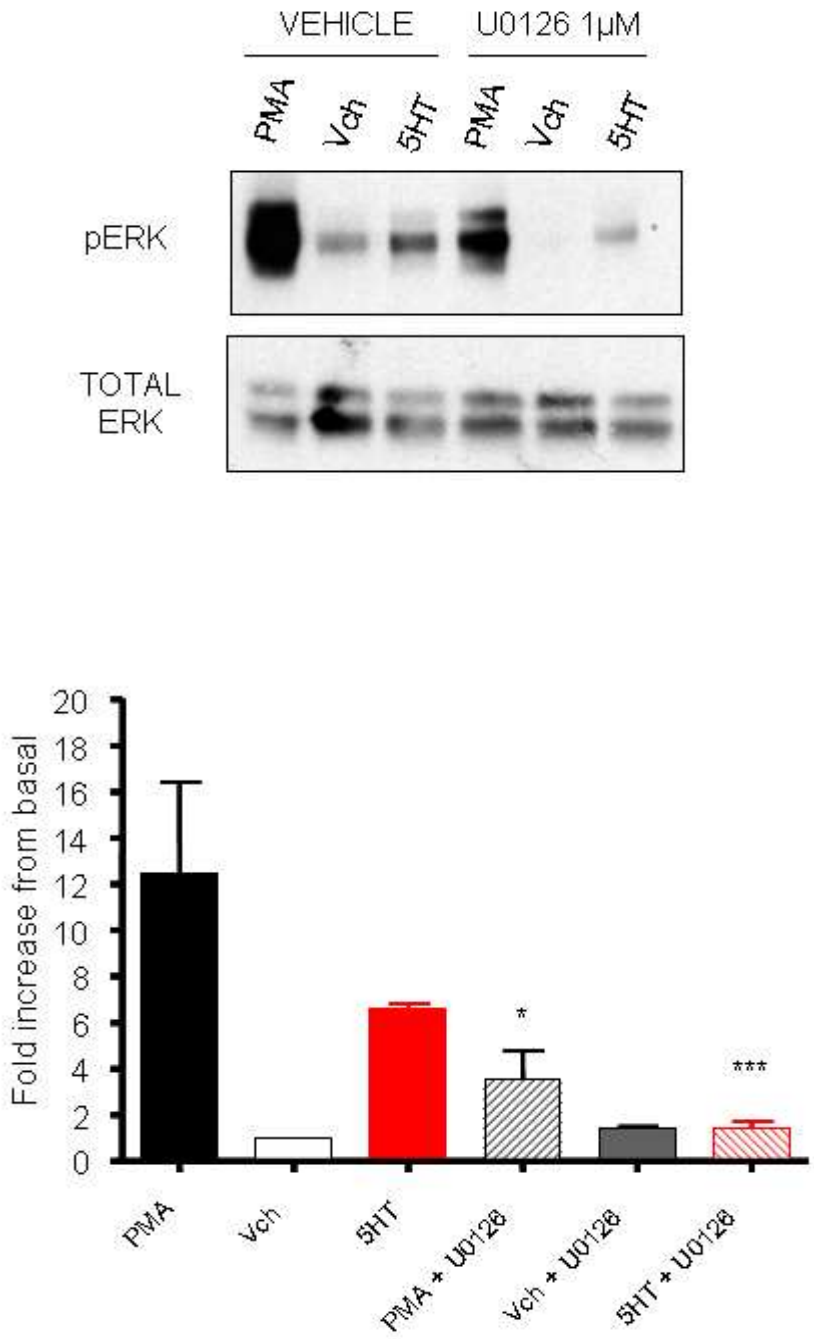

Figure 3.3 Effects of MEK inhibition with UO126 on ERK activation.

After serum starvation for 16 hours, CCL-39 cells were pre-treated with either vehicle or UO126 (1µM) for 30 minutes prior to stimulation with 5HT (1µM) or PMA (2µM) for 5 minutes. Cell lysates were prepared and equalised for protein concentration by BCA assay. Samples were then analysed by SDS-PAGE and immunoblotting using phospho-specific and total ERK antibodies. Blots shown are representative. Results are expressed as mean ± SEM (n=3). * $p<0.05$ versus PMA, *** $p<0.001$ *versus* 5HT using Newman-Keuls multiple comparison test.

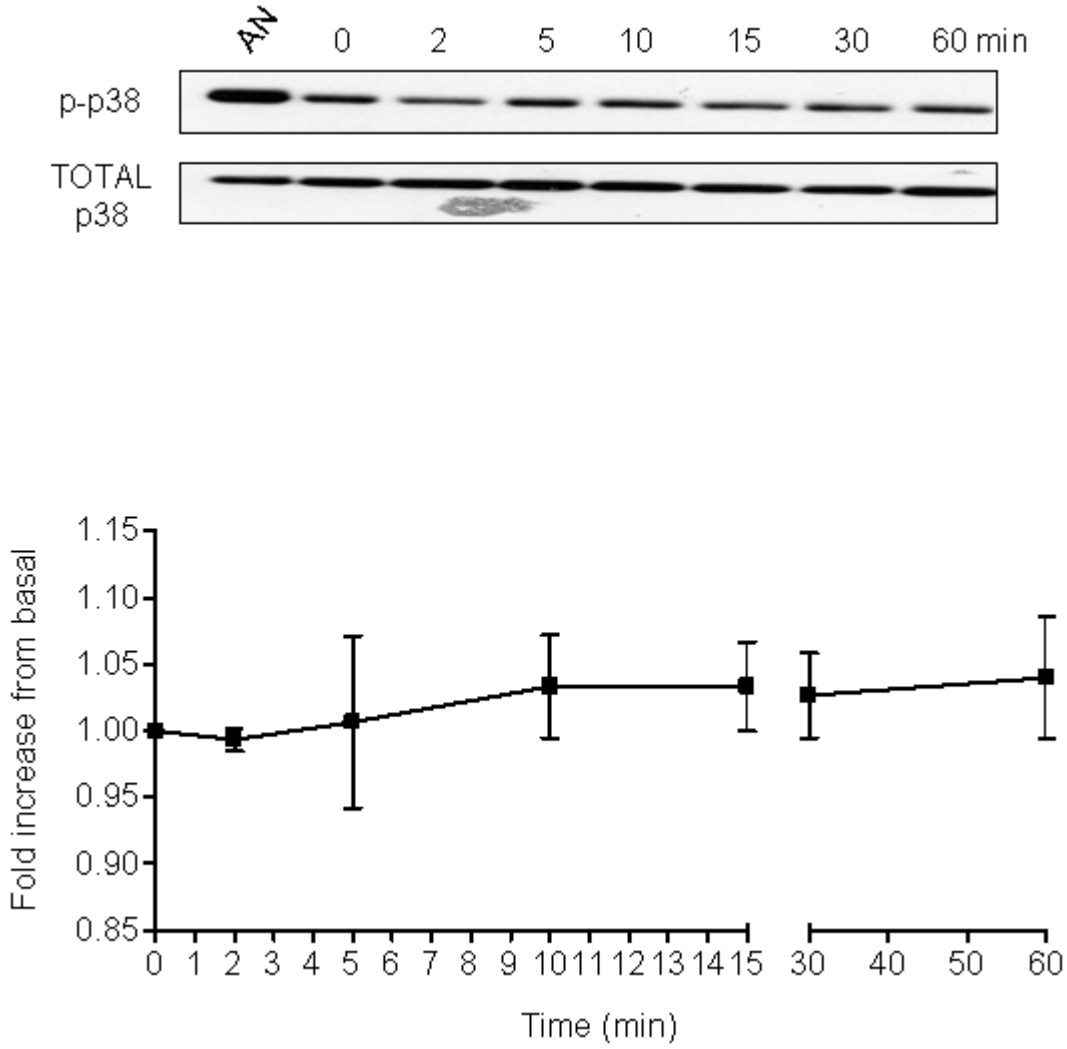

Figure 3.4 Timecourse of the effects of 5HT on p38 activation.

Following serum starvation for 16 hours, CCL-39 cells were stimulated with 5HT (1µM) for 0-120 minutes or with anisomycin (1µg/ml) for 5 minutes. Cell lysates were prepared and equalised for protein concentration by BCA assay. Samples were then analysed by SDS-PAGE and immunoblotting using phospho-specific and total p38 antibodies. Blots shown are representative. Results are expressed as mean ± SEM (n=3). Statistical analysis using Newman-Keuls multiple comparison test revealed no significant differences. Stimulation with anisomysin (1µg/ml) for 5 minutes resulted in a 1.8±0.3 fold increase from basal.

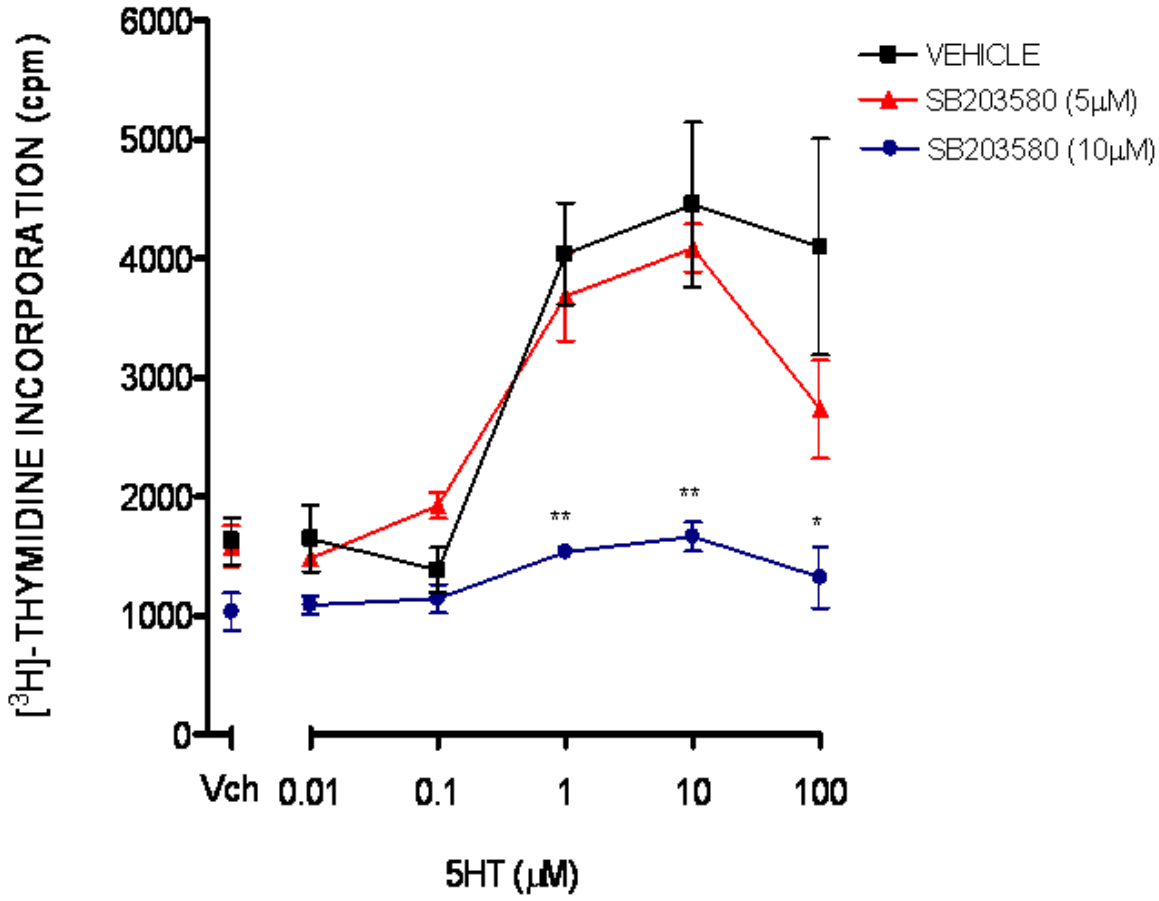

Figure 3.5 Effects of p38 inhibition by SB203580 on 5HT-induced proliferation.

CCL-39 cells were serum starved for 24 hours then incubated with 0-100µM 5HT in the presence or absence of SB203580 (5µM or 10µM) (p38 inhibitor) for a further 24 hours, with 0.5µCi/well [^{3}H]-thymidine added for the final 6 hours of this incubation. Cell were then harvested onto a glass fibre filter mat and [^{3}H]-thymidine incorporation assessed by liquid scintillation counting. Data shown are representative of n=3 assays. Results expressed as mean ± SEM of triplicate samples. * $p<0.05$, ** $p<0.01$ *versus* vehicle using an unpaired, two tailed t-test.

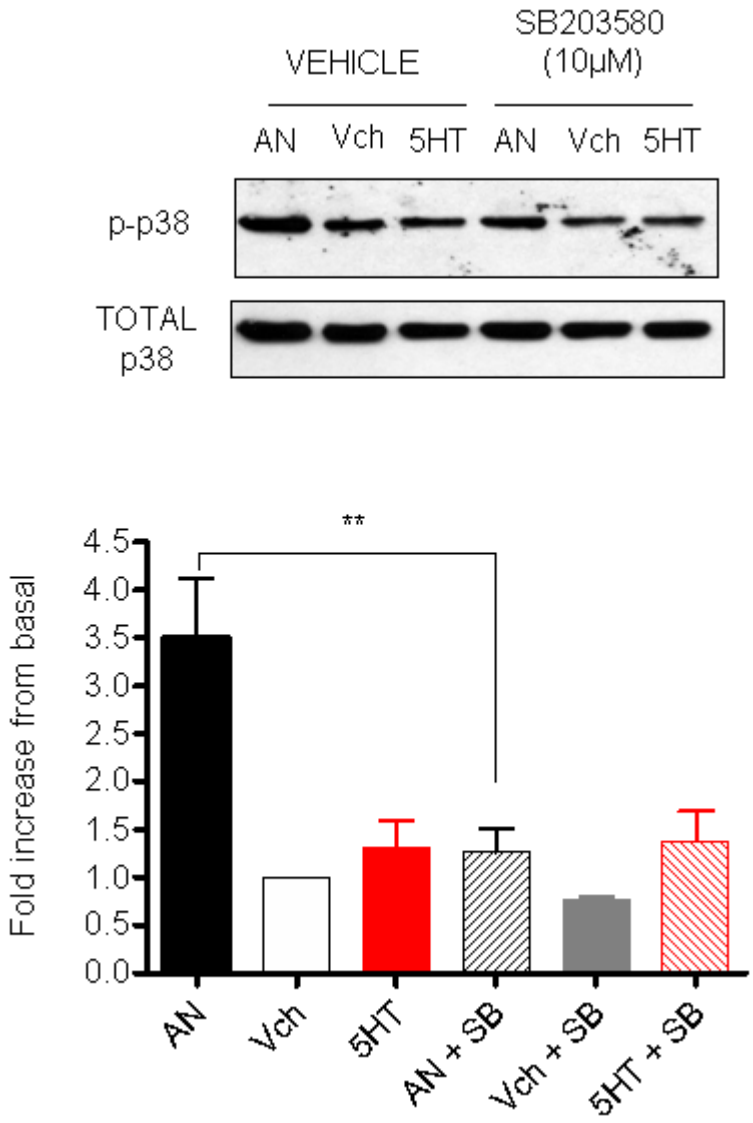

Figure 3.6 Effects of p38 inhibitor SB203580 on p38 activation.

After serum starvation for 16 hours, CCL-39 cells were pre-treated with either vehicle or SB203580 (10μM) for 30 minutes prior to stimulation with 5HT (1μM) or anisomysin (1μg/ml) for 5 minutes. Cell lysates were prepared and equalised for protein concentration by BCA assay. Samples were then analysed by SDS-PAGE and immunoblotting phospho-specific and total p38 antibodies. Blots shown are representative. Results are expressed as mean ± SEM (n=3). ** $p<0.01$ *versus* anisomycin using Newman-Keuls multiple comparison test.

However, the ability of SB203580 (10μM) to reduce 5HT-induced proliferation maybe owing to the fact, that at this concentration the p38 inhibitor also significantly reduced responses to PMA by 27±4% ($p<0.01$, n=3) (Figure 3.7), suggesting the ERK1/2 MAP kinase pathway may also be effected. A large body of evidence exists implicating 5HTT in the development of pulmonary hypertension. Therefore, the role of 5HTT in the pathways utilised by 5HT to induce its mitogenic effects were assessed. Using CCL-39 cells 5HTT was blocked using citalopram, a commonly used selective serotonin reuptake inhibitor (SSRI) and the effects on 5HT-induced ERK1/2 activation monitored. Pre-treatment with citalopram (1μM) was found to significantly attenuated the dose-dependent increase in ERK1/2 activation induced by 5HT, reducing the maximal 5HT response by 35±9% ($p<0.05$, n=3) (Figure 3.8). Moreover, treatment with citalopram also markedly decreased 5HT-induced proliferation by 82±2% of the maximal response (Figure 3.9).

5HT receptors, namely $5HT_{1B/1D}$ and $5HT_{2A}$ may also be involved in the 5HT-induced mitogenic response (Lee et al., 1999; Liu and Fanburg, 2006). Initially, the $5HT_{1/2}$ receptor antagonist methiothepin was used in order to determine if these 5HT receptors played any role in 5HT-induced ERK1/2 activation and proliferation in CCL-39 cells. Pre-treatment with methiothepin (1μM) was able to significantly attenuate the dose-dependent increase in ERK1/2 activation elicited by 5HT (Figure 3.10), with the maximal 5HT response reduced by 50±7% ($p<0.01$, n=3). Inhibition of $5HT_{1/2}$ receptors using methiothepin also almost completely abolished 5HT induced proliferation reducing the maximal response by 91±1 % (Figure 3.11).

The individual contribution of $5HT_{2A}$ and $5HT_{1B/1D}$ receptors was then assessed pharmacologically. Pre-treatment of cells with ketanserin (1μM), a $5HT_{2A}$ receptor antagonist, significantly reduced maximal 5HT-induced ERK1/2 activation by 47±9% ($p<0.05$, n=3) (Figure 3.12). Similarly, blockade of $5HT_{2A}$ receptors using ketanserin almost completely abolished 5HT-induced proliferation, resulting in levels of [^{3}H]-thymidine incorporation similar to those witnessed under vehicle conditions (Figure 3.13). The role of $5HT_{1B/1D}$ was also examined using GR55562, a selective $5HT_{1B/1D}$ receptor antagonist. Treatment with this compound also significantly reduced 5HT-induced ERK1/2 activation to 57±7% of the maximal response ($p<0.001$, n=3) (Figure 3.14). In addition to

this, $5HT_{1B/1D}$ receptor antagonism abolished the dose-dependent increase in proliferation induced by 5HT (Figure 3.15). Moreover, simultaneous inhibition of 5HTT and 5HT receptors using a combination of citalopram (1μM) and methiothepin (1μM) did not completely abolish 5HT induced ERK1/2 activation. At the maximal response, inhibition of 5HTT and $5HT_{1/2}$ receptors reduced 5HT-induced ERK1/2 activation by 57±5% ($p<0.001$, n=3) (Figure 3.16). It should also be noted that the SSRI and 5HT antagonists used in this study had no effect on the ability of PMA to activate ERK, indicating the reductions in ERK activation observed are specific to the 5HT signalling pathway. Taken together, these results suggest 5HTT, $5HT_{1B/1D}$ and $5HT_{2A}$ receptors all play a role in 5HT-induced ERK1/2 activation and proliferation, with inhibition of any one of these components resulting in the attenuation of the mitogenic effects of 5HT.

The role of receptor internalisation in 5HT-mediated ERK1/2 activation was also studied. Endocytosis is known to play an important role in the regulation of 5HT receptors and GPCRs in general (Bhatnagar et al., 2001; Le Roy and Wrana, 2005) as well as in regulating 5HTT signalling (Jayanthi et al., 2005). Using structurally unrelated compounds, the role of endocytosis in 5HT-induced ERK1/2 activation was investigated. Conconavalin A (Con A), a lectin and commonly used inhibitor of endocytotic processes (Pippig et al., 1995; Tang et al., 2000) was initially employed. Con A binds to α-D-mannosyl and α-D-glucosyl residues on cell surface proteins and lipid with high affinity, inhibiting their mobility and thus ability to be internalised (Zhao et al., 2002; Tang et al., 2000). Pre-treatment with this compound was found to significantly elevate basal levels of ERK1/2 activity within CCL-39 cells by 31±4% ($p<0.01$, n=3) (Figure 3.17). In addition to this, the maximal 5HT response was similarly increased in the presence of Con A (40±10% increase, $p>0.05$, n=3). Monodansylcadaverin (MDC), a compound previously shown to inhibit internalisation from the plasma membrane (Schutze et al., 1999; Davies et al., 1980, Chow et al., 1998) was also used (Figure 3.18). MDC is an inhibitor of transglutaminase, a membrane-bound enzyme that participates in the internalisation of receptors (Schutze et al., 1999; Davies et al., 1980; Chow et al., 1998). MDC was found to have no effect on either the basal levels of pERK1/2 or 5HT-induced ERK1/2 activation.

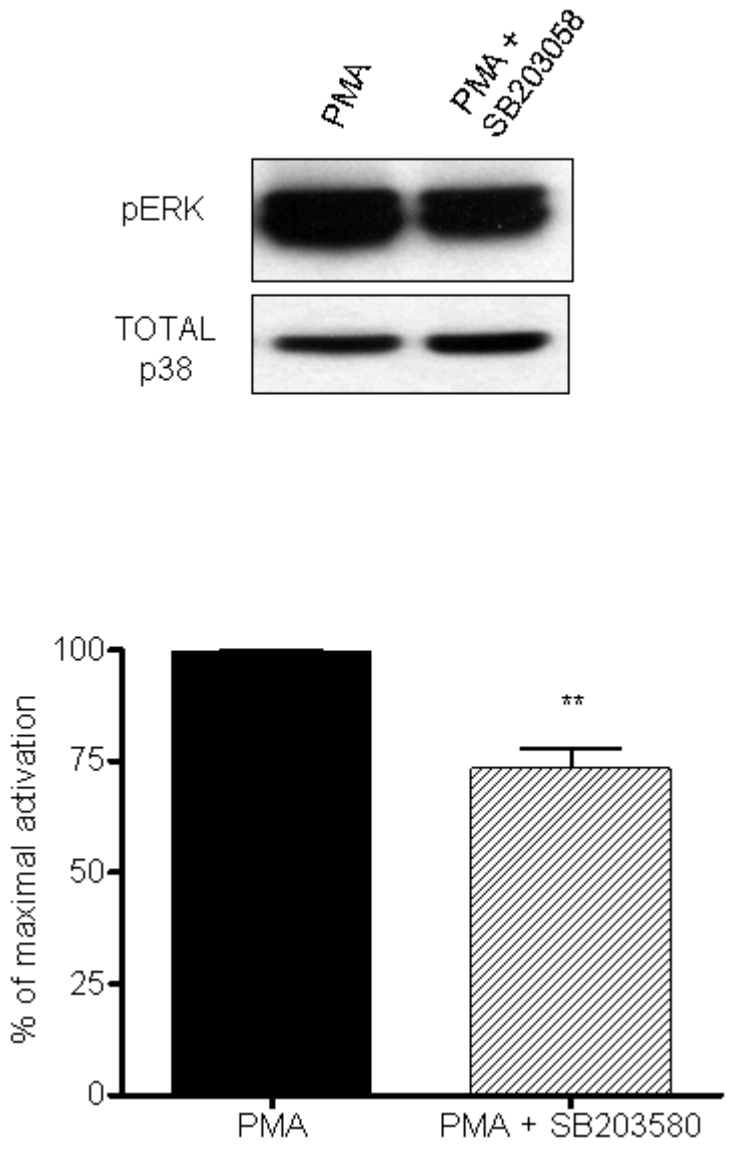

Figure 3.7 Effects of p38 inhibition by SB203580 on ERK activation.

After serum starvation for 16 hours, CCL-39 cells were pre-treated with either vehicle or SB203580 (10µM) for 30 minutes prior to stimulation with PMA (2µM) for 5 minutes. Cell lysates were prepared and equalised for protein concentration by BCA assay. Samples were then analysed by SDS-PAGE and immunoblotting using phospho-specific and total ERK antibodies. Blots shown are representative. Results are expressed as mean ± SEM (n=3). ** $p<0.01$ *versus* PMA using a two-tailed unpaired t-test.

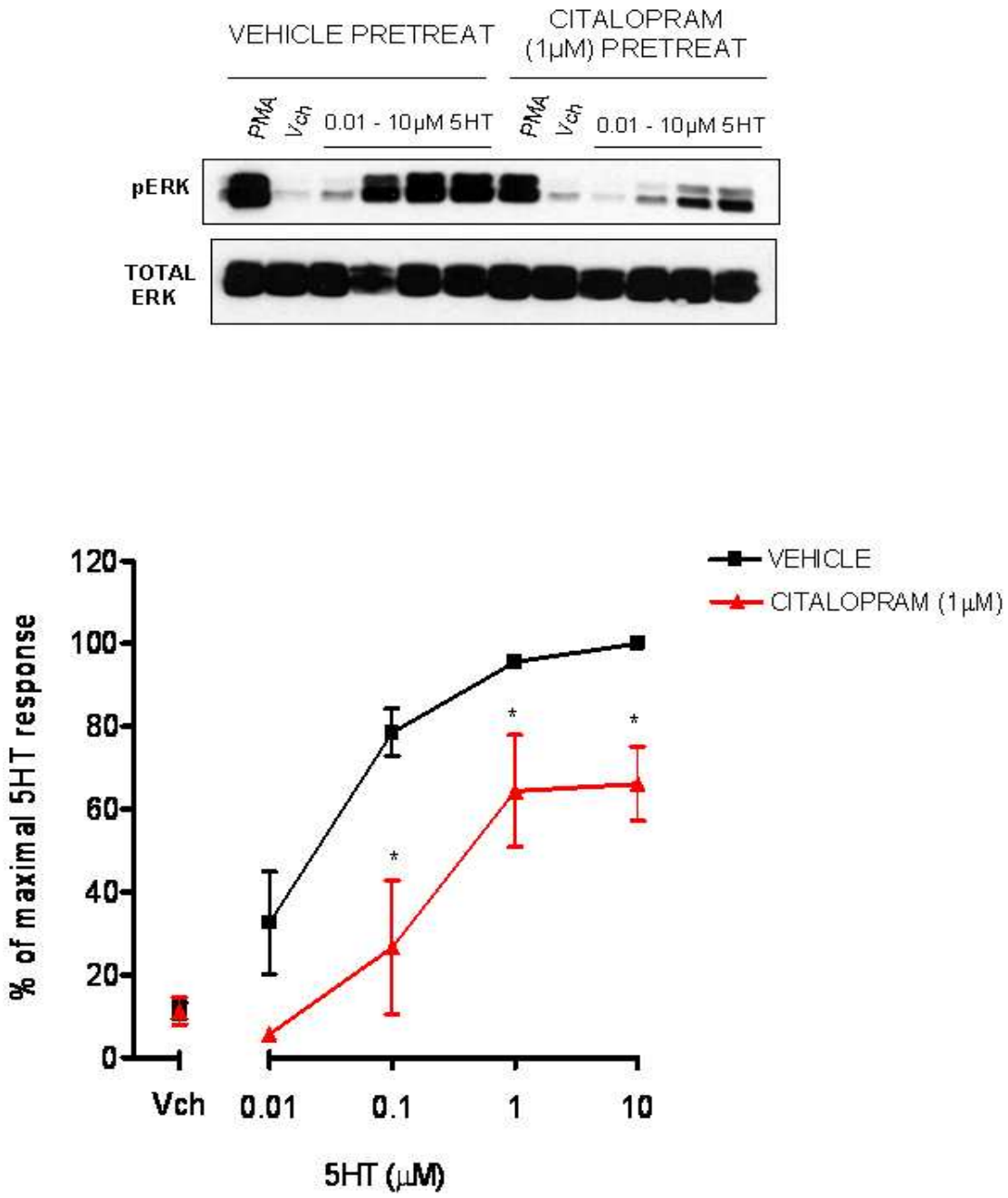

Figure 3.8 Effects of 5HTT inhibitor citalopram on 5HT-induced ERK activation.

After serum starvation for 16 hours CCL-39 cells were pre-treated for 1 hour with citalopram (1μM) (5HTT inhibitor). Cells were then stimulated for 5 minutes with increasing concentrations of 5HT (0-10μM) or PMA (2μM). Cell lysates were prepared and equalised for protein concentration by BCA assay. Samples were then analysed by SDS-PAGE and immunoblotting using phospho-specific and total ERK antibodies. Blots shown are representative. Results are expressed as mean ± SEM (n=3). * $p<0.05$ *versus* vehicle pre-treated cells using an unpaired, two tailed t-test.

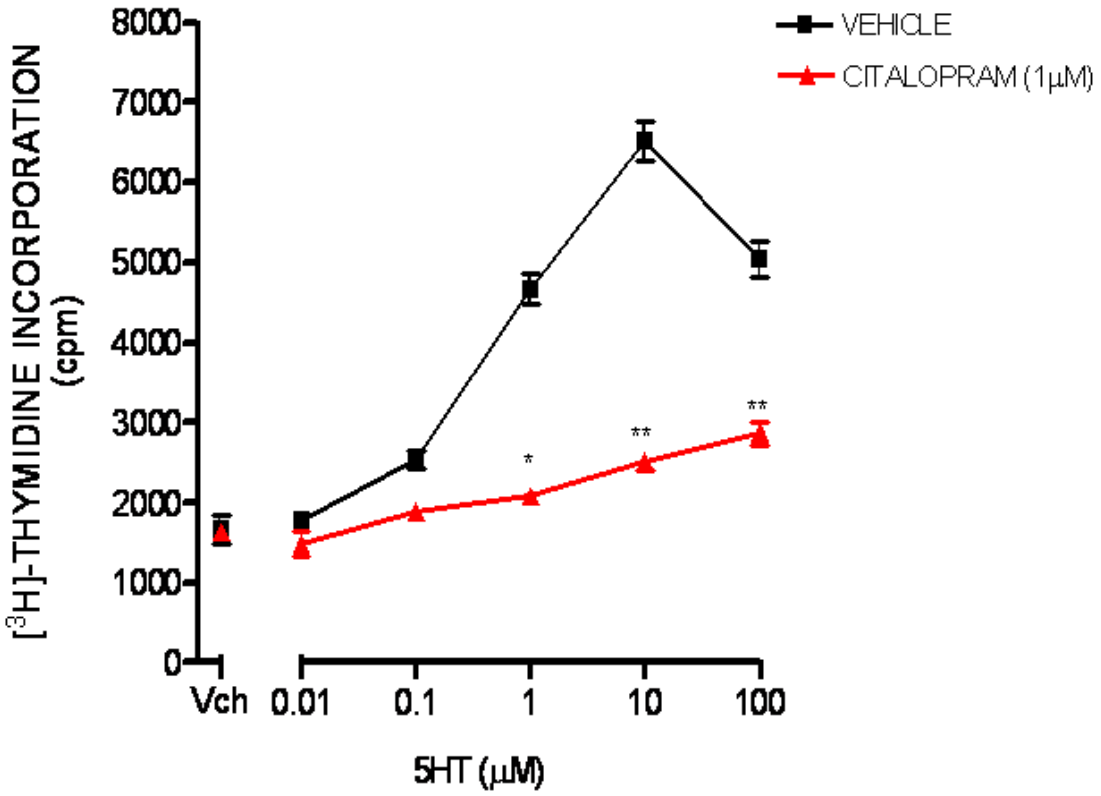

Figure 3.9 Effects of 5HTT inhibition by citalopram on 5HT-induced proliferation.

CCL-39 cells were serum starved for 24 hours then incubated with 0-100µM 5HT in the presence or absence of citalopram (1µM) (5HTT inhibitor) for a further 24 hours, with 0.5µCi/well [^{3}H]-thymidine added for the final 6 hours of this incubation. Cells were then harvested onto a glass fibre filter mat and [^{3}H]-thymidine incorporation assessed by liquid scintillation counting. Data show are representative of n=3 assays. Results expressed as mean ± SEM of triplicate samples. * $p<0.05$, ** $p<0.01$ *versus* vehicle using an unpaired, two tailed t-test.

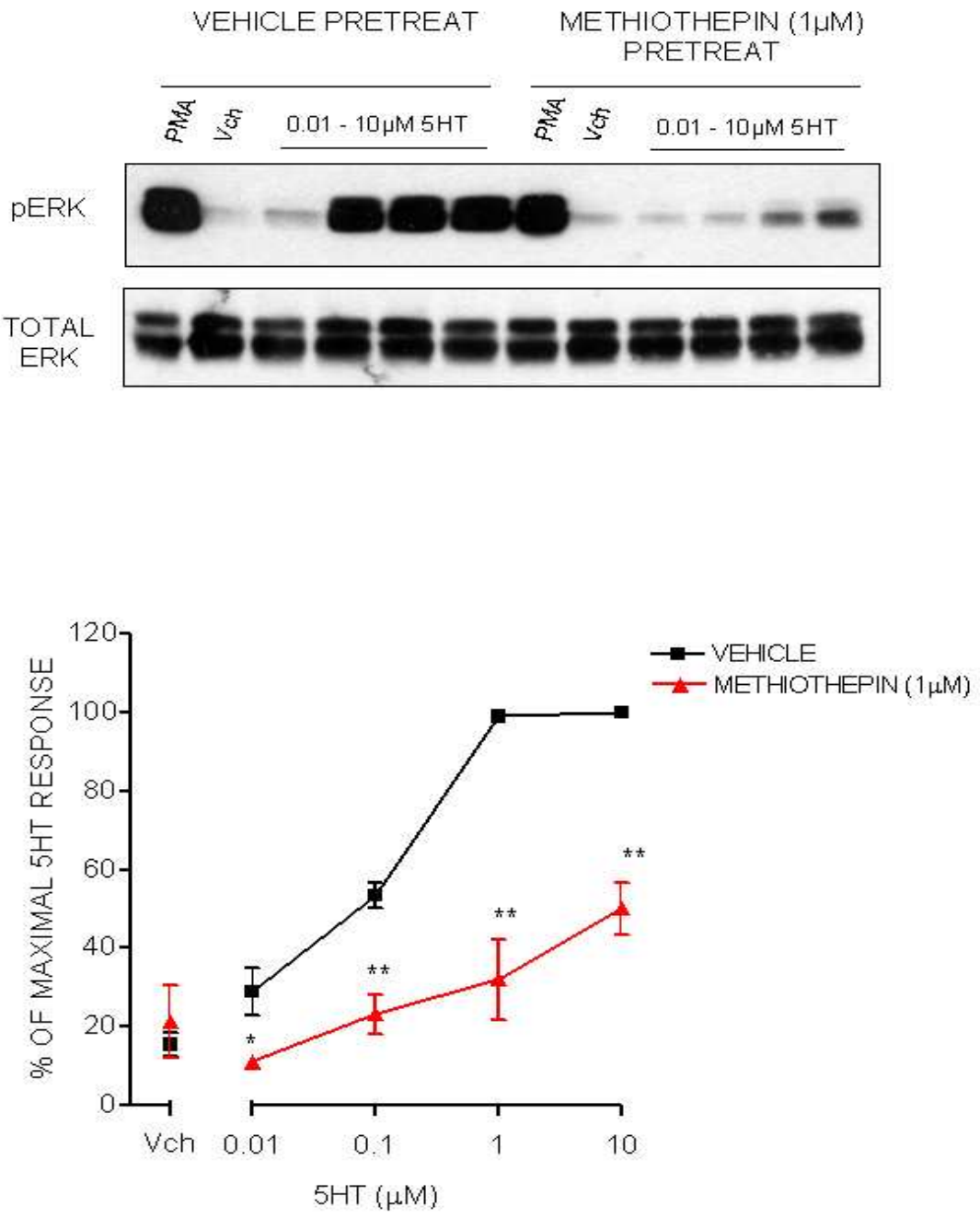

Figure 3.10 Effects of $5HT_{1/2}$ receptor antagonist methiothepin on 5HT-induced ERK activation.

After serum starvation for 16 hours CCL-39 cells were pre-treated for 1 hour with methiothepin (1μM) ($5HT_{1/2}$ receptor antagonist). Cells were then stimulated for 5 minutes with increasing concentrations of 5HT (0-10μM) or PMA (2μM). Cell lysates were prepared and equalised for protein concentration by BCA assay. Samples were then analysed by SDS-PAGE and immunoblotting using phospho-specific and total ERK antibodies. Blots shown are representative. Results are expressed as mean ± SEM (n=3). * $p<0.05$, ** $p<0.01$ *versus* vehicle pre-treated cells using an unpaired, two tailed t-test.

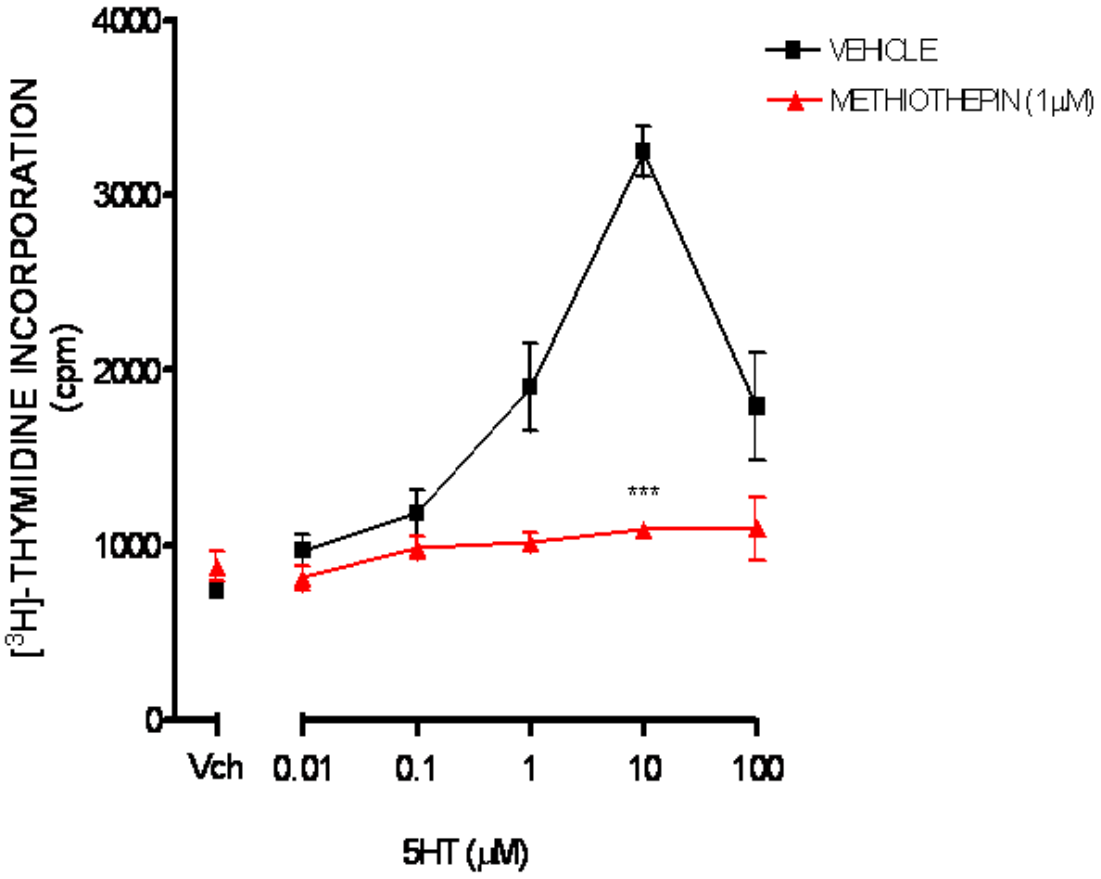

Figure 3.11 Effects of $5HT_{1/2}$ receptor antagonist methiothepin on 5HT-induced proliferation.

CCL-39 cells were serum starved for 24 hours then incubated with 0-100µM 5HT in the presence or absence of methiothepin (1µM) for a further 24 hours, with 0.5µCi/well [^{3}H]-thymidine added for the final 6 hours of this incubation. Cell were then harvested onto a glass fibre filter mat and [^{3}H]-thymidine incorporation assessed by liquid scintillation counting. Data shown are representative of n=3 assays. Results expressed as mean ± SEM of triplicate samples. *** $p<0.001$ *versus* vehicle using an unpaired, two tailed t-test.

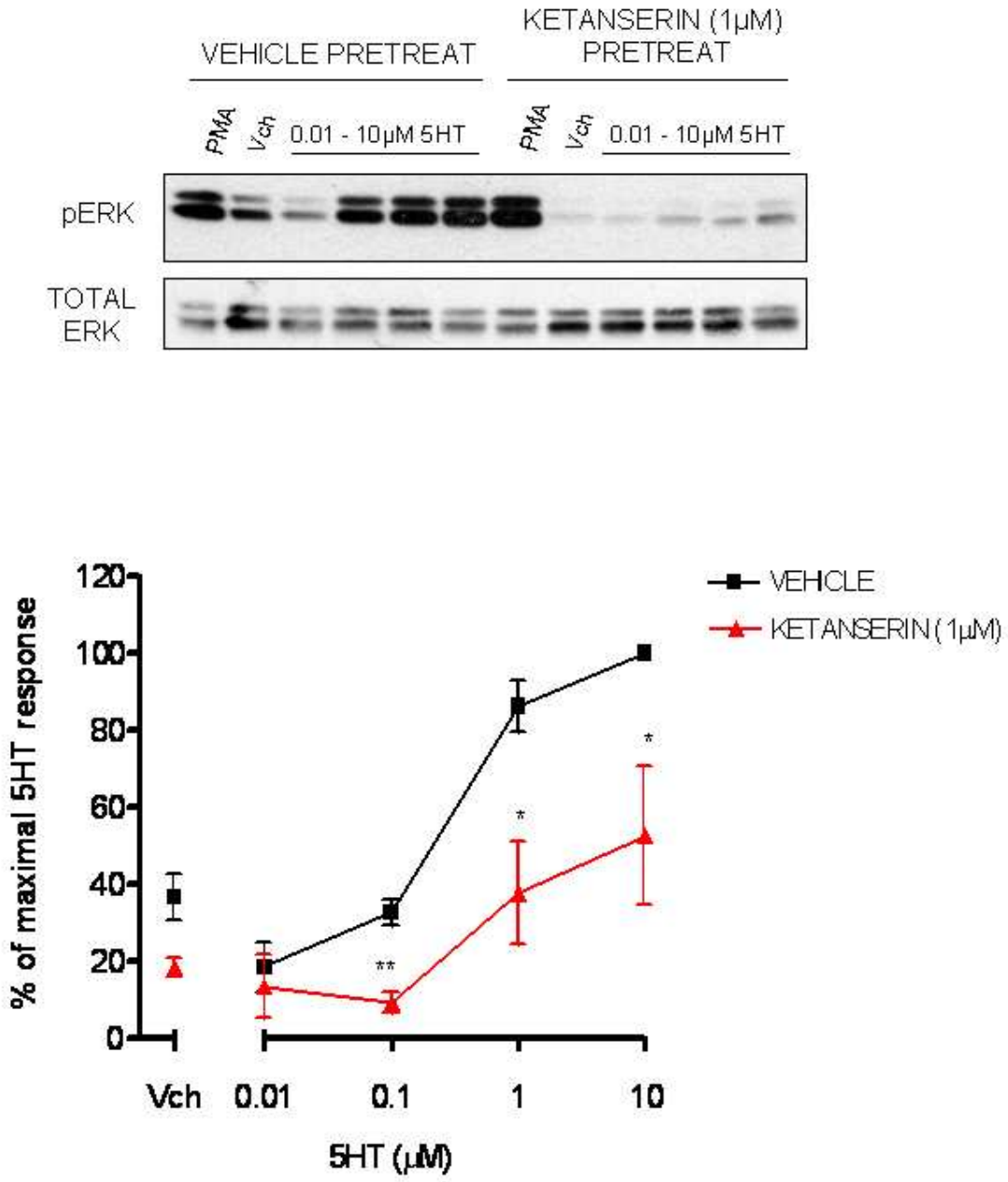

Figure 3.12 Effects of $5HT_{2A}$ receptor antagonist ketanserin on 5HT-induced ERK activation.

After serum starvation for 16 hours CCL-39 cells were pre-treated for 1 hour with ketanserin (1μM) ($5HT_{2A}$ receptor antagonist). Cells were then stimulated for 5 min with increasing concentrations of 5HT (0-10μM) or PMA (2μM). Cell lysates were prepared and equalised for protein concentration by BCA assay. Samples were then analysed by SDS-PAGE and immunoblotting using phospho-specific and total ERK antibodies. Blots shown are representative. Results are expressed as mean ± SEM (n=3). * $p<0.05$, ** $p<0.01$ *versus* vehicle pre-treated cells using an unpaired, two tailed t-test.

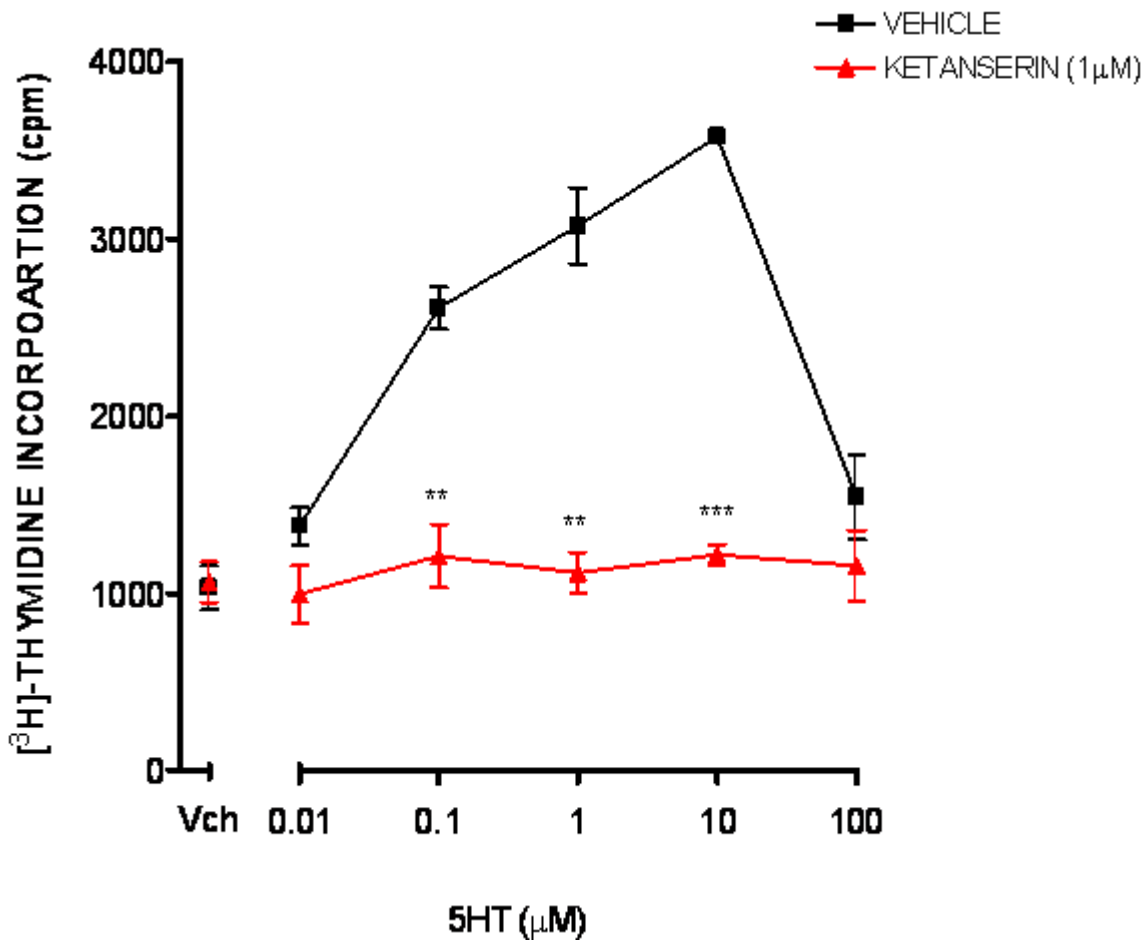

Figure 3.13 Effects of $5HT_{2A}$ receptor antagonist ketanserin on 5HT-induced proliferation.

CCL-39 cells were serum starved for 24 hours then incubated with 0-100μM 5HT in the presence or absence of ketanserin (1μM) for a further 24 hours, with 0.5μCi/well [^{3}H]-thymidine added for the final 6 hours of this incubation. Cell were then harvested onto a glass fibre filter mat and [^{3}H]-thymidine incorporation assessed by liquid scintillation counting. Data shown are representative of n=3 assays. Results expressed as mean ± SEM of triplicate samples. ** $p<0.01$, *** $p<0.001$ *versus* vehicle using an unpaired, two tailed t-test.

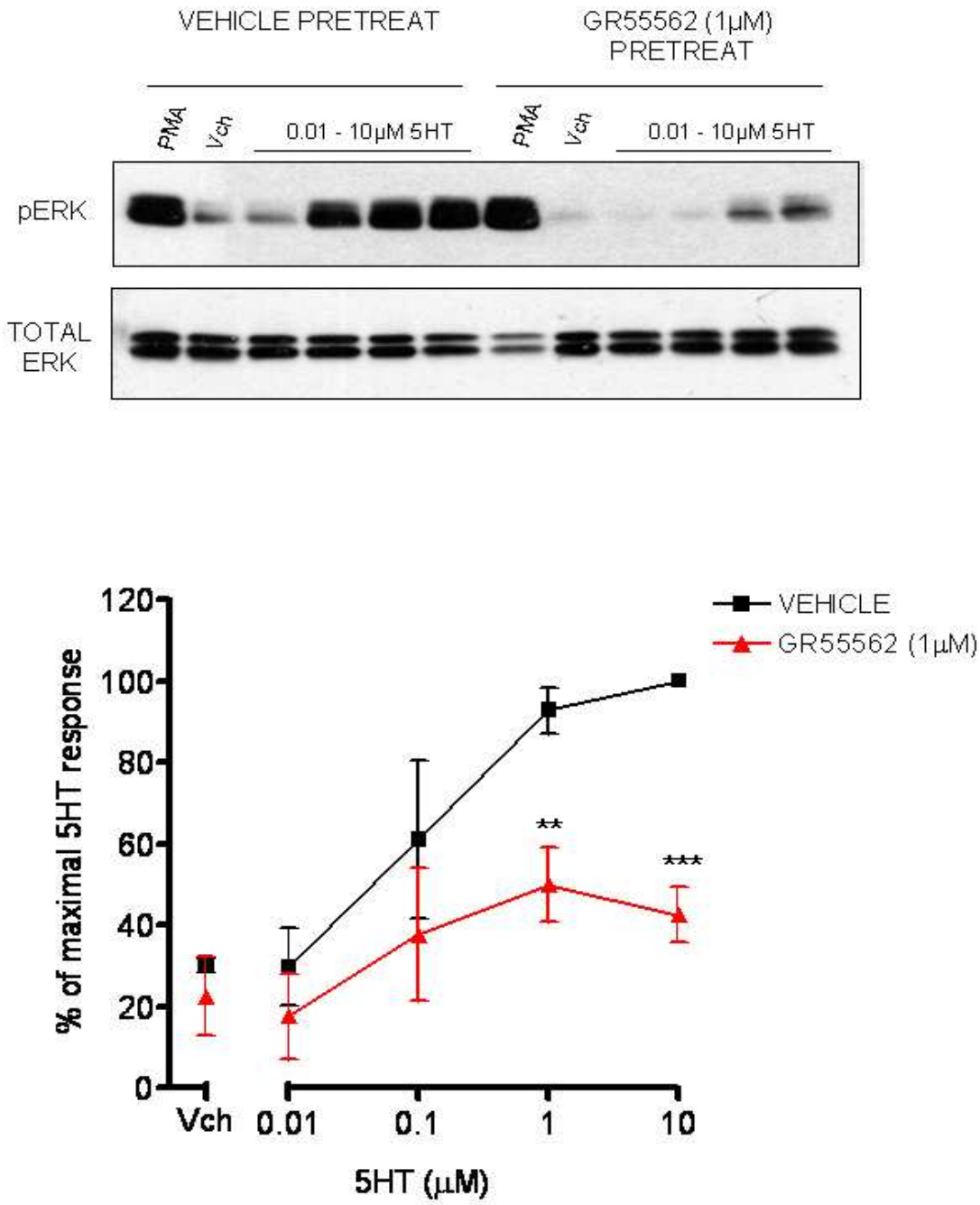

Figure 3.14 Effects of $5HT_{1B/1D}$ antagonist GR55562 on 5HT-induced ERK activation.

After serum starvation for 16 hours CCL-39 cells were pre-treated for 1 hour with GR55562 (1µM) ($5HT_{1B/1D}$ receptor antagonist). Cells were then stimulated for 5 minutes with increasing concentrations of 5HT (0-10µM) or PMA (2µM). Cell lysates were prepared and equalised for protein concentration by BCA assay. Samples were then analysed by SDS-PAGE and immunoblotting using phospho-specific and total ERK antibodies. Blots shown are representative. Results are expressed as mean ± SEM (n=3). ** $p<0.01$, *** $p<0.001$ *versus* vehicle pre-treated cells using an unpaired, two tailed t-test.

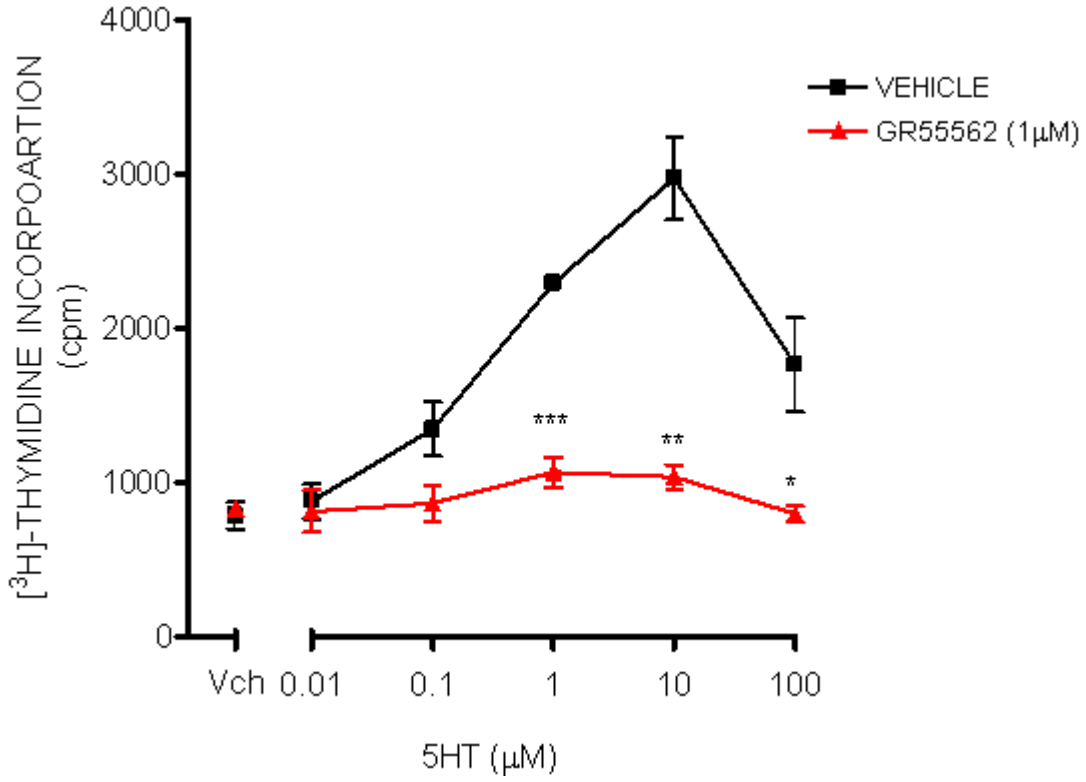

Figure 3.15 Effects of $5HT_{1B/1D}$ receptor antagonist GR55562 on 5HT-induced proliferation.

CCL-39 cells were serum starved for 24 hours then incubated with 0-100µM 5HT in the presence or absence of GR55562 (1µM) for a further 24 hours, with 0.5µCi/well [^{3}H]-thymidine added for the final 6 hours of this incubation. Cell were then harvested onto a glass fibre filter mat and [^{3}H]-thymidine incorporation assessed by liquid scintillation counting. Data shown are respresentative of n=3 assays. Results expressed as mean ± SEM (n=3) of triplicate samples. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ *versus* vehicle using an unpaired, two tailed t-test.

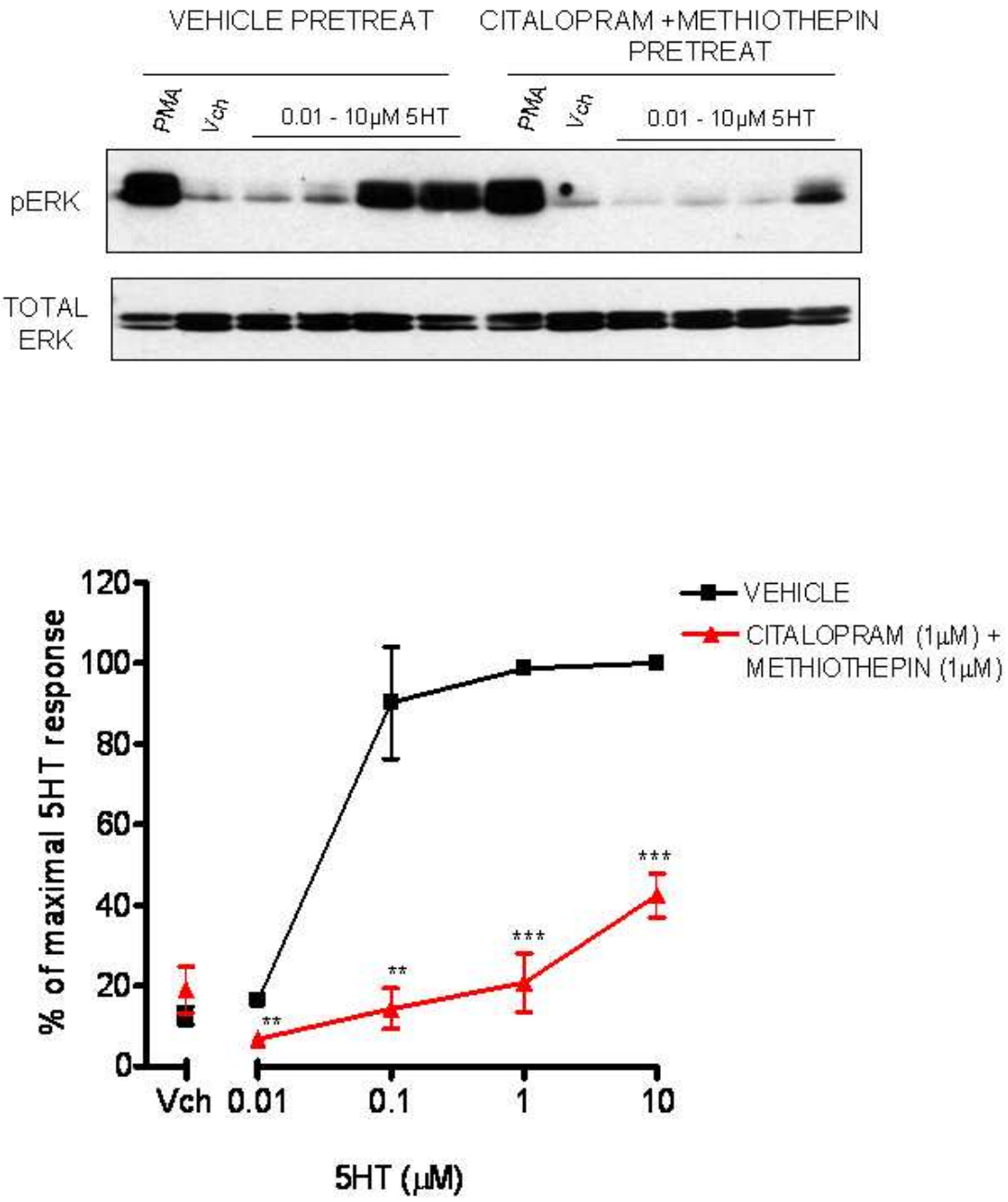

Figure 3.16 Effects of simultaneous $5HT_{1/2}$ receptor and 5HTT inhibition by methiothepin and citalopram on 5HT-induced ERK activation.

After serum starvation for 16 hours CCL-39 cells were pre-treated simultaneously for 1 hour with methiothepin (1µM) and citalopram (1µM). Cells were then stimulated for 5 minutes with increasing concentrations of 5HT (0-10µM) or PMA (2µM). Cell lysates were prepared and equalised for protein concentration by BCA assay. Samples were then analysed by SDS-PAGE and immunoblotting using phospho-specific and total ERK antibodies. Blots shown are representative. Results are expressed as mean ± SEM (n=3). ** $p<0.01$, *** $p<0.001$ *versus* vehicle pre-treated cells using an unpaired, two tailed t-test.

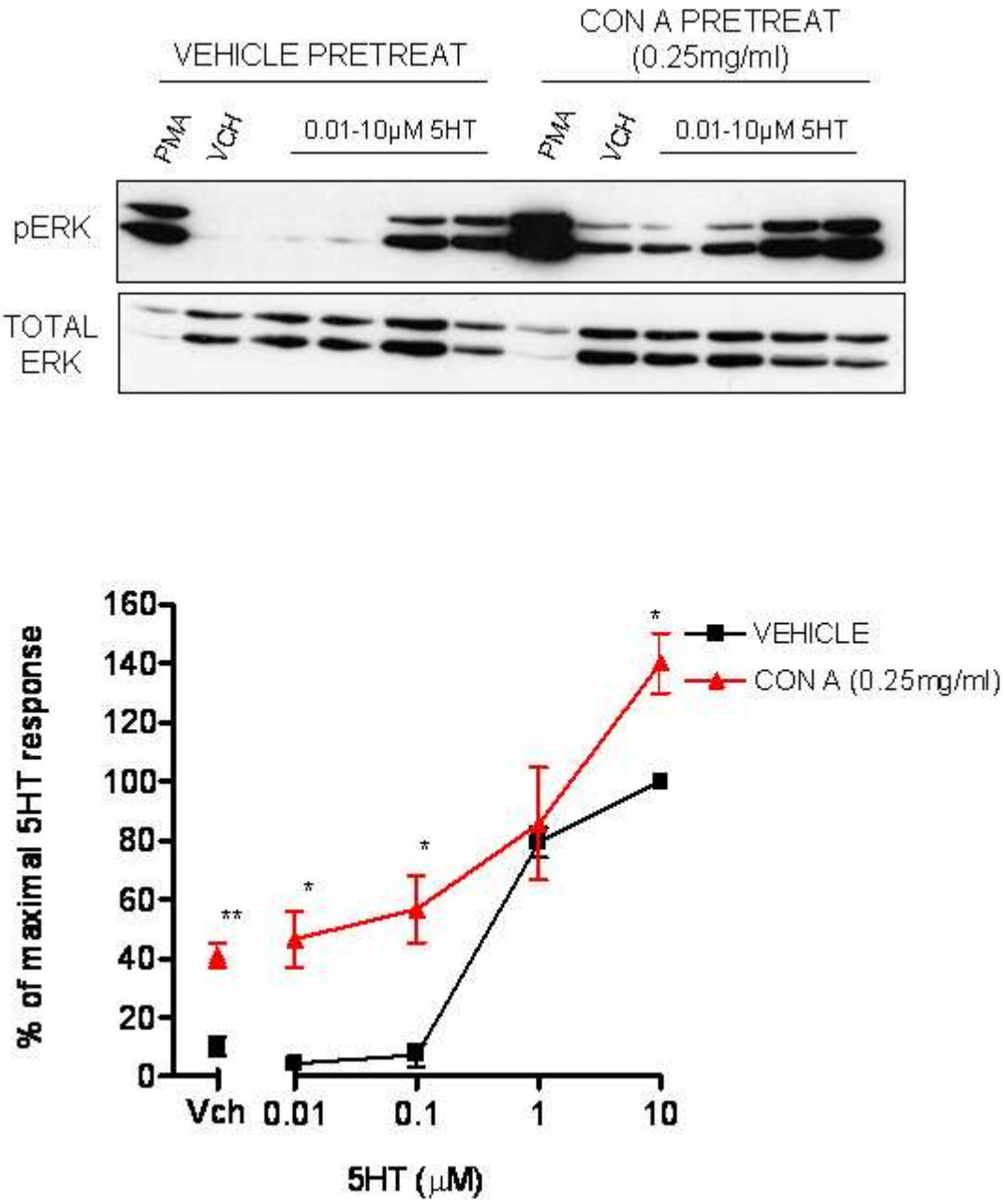

Figure 3.17 Effects of conconavalin A (conA) on 5HT-induced ERK activation.

After serum starvation for 16 hours CCL-39 cells were pre-treated for 30 minutes with conconavalin A (conA) (0.25mg/ml). Cells were then stimulated for 5 minutes with increasing concentrations of 5HT (0-10μM) or PMA (2μM). Cell lysates were prepared and equalised for protein concentration by BCA assay. Samples were then analysed by SDS-PAGE and immunoblotting using phospho-specific and total ERK antibodies. Blots shown are representative. Results are expressed as mean ± SEM (n=3). * $p < 0.05$, ** $p < 0.01$ *versus* vehicle pre-treated cells using an unpaired, two tailed t-test.

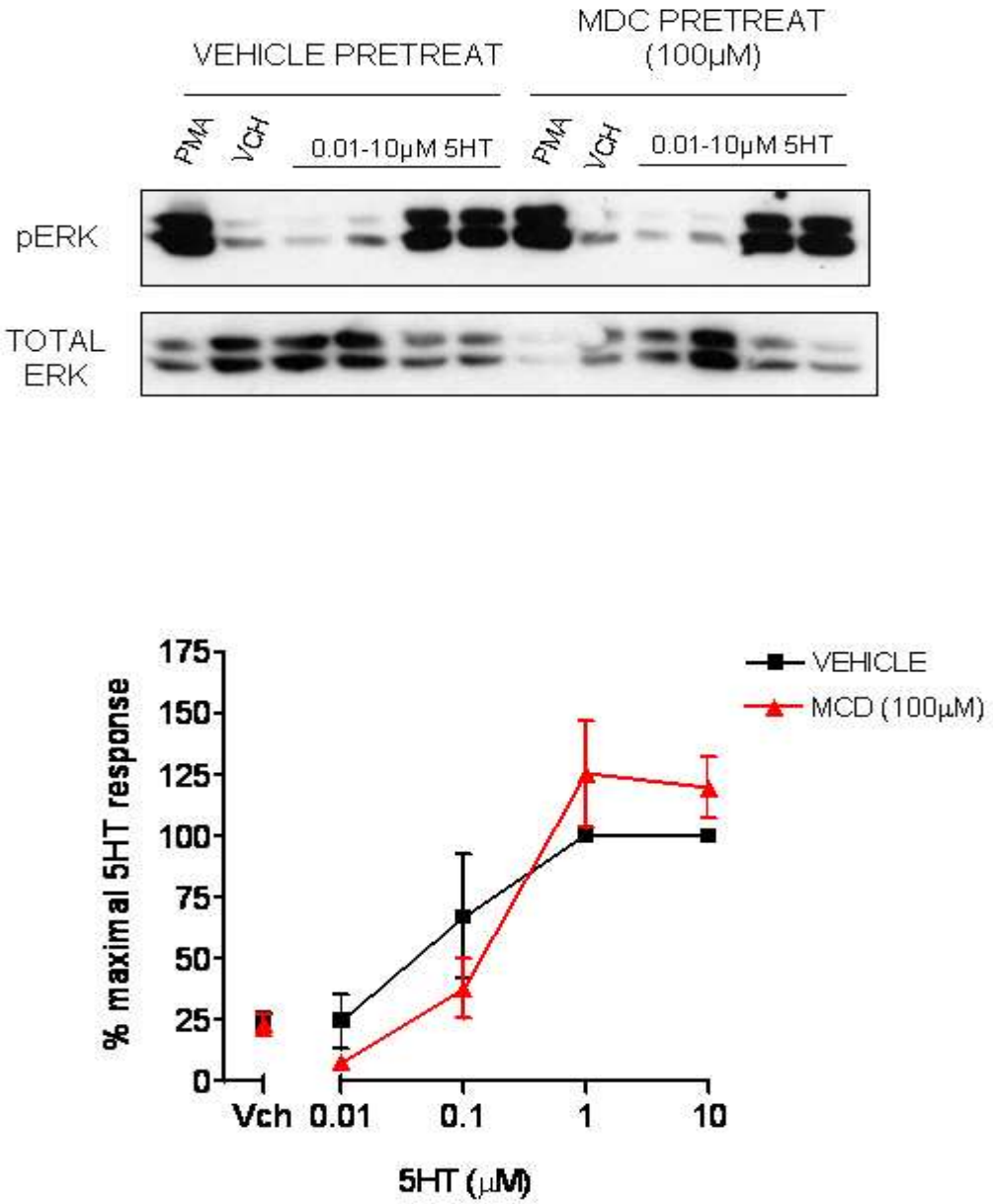

Figure 3.18 Effects of monodansylcadaverin (MDC) on 5HT-induced ERK activation.

After serum starvation for 16 hours CCL-39 cells were pre-treated for 30 minutes with monodansylcadaverin (MDC) (100µM). Cells were then stimulated for 5 minutes with increasing concentrations of 5HT (0-10µM) or PMA (2µM). Cell lysates were prepared and equalised for protein concentration by BCA assay. Samples were then analysed by SDS-PAGE and immunoblotting using phospho-specific and total ERK antibodies. Blots shown are representative. Results are expressed as mean ± SEM (n=3). Statistical analysis using an unpaired, two tailed t-test revealed no statistical differences between vehicle and MDC pre-treated cells.

In an attempt to distinguish between ERK1/2 activation elicited by 5HT receptors and 5HTT, selective inhibitors of each of these components were used to determine if endocytosis is important in either 5HT receptor or 5HTT-mediated responses. To investigate the role of the endocytotic pathway in 5HT receptor-mediated ERK1/2 activation, 5HTT was blocked using fluoxetine (1µM) so that 5HT applied to the cells would act only *via* 5HT receptors. In this instance, inhibition of 5HTT with fluoxetine resulted in a significant reduction in 5HT-induced ERK1/2 activation ($p<0.05$, n=3) (Figure 3.19). However, pre-treatment with both fluoxetine and MDC resulted in levels of ERK1/2 activation similar to that witnessed in vehicle pre-treated cells. Conversely, to determine the role played by the endocytotic pathway in 5HTT-mediated ERK1/2, $5HT_{2A}$ and $5HT_{1B/1D}$ receptors were blocked using ketanserin (1µM) and GR55562 (1µM) respectively. Inhibition of 5HT receptors was found to significantly reduce 5HT-mediated ERK1/2 activation ($p<0.01$, n=3) (Figure 3.20). Pre-treatment with both MDC and 5HT receptor antagonists resulted in levels of ERK1/2 activation similar to that induced by 5HT under vehicle pre-treated conditions. These findings suggest endocytosis is not a requirement for 5HT-induced ERK1/2 activation *via* either 5HTT or 5HT receptors in CCL-39 cells.

Possible downstream mediators of 5HTT and 5HT receptors were then investigated. Several studies have reported that the production of reactive oxygen species (ROS) may be pivotal in the ability of 5HT to produce its mitogenic response (Bianchi et al., 2005; Lawrie et al., 2005). In order to determine if ROS are involved in the 5HT-mediated response in CCL-39 cells, the antioxidant N-acetyl-cysteine (NAC) was employed. At 10mM, NAC was found to significantly attenuate 5HT-induced ERK1/2 activation, with maximal 5HT responses reduced by around 60±18% ($p<0.05$, n=3) (Figure 3.21). Reduction of ROS production using NAC also markedly reduced the proliferative effects of 5HT, with the maximal 5HT-induced response attenuated by 83±19% (Figure 3.22).

It has previously been suggested that on entry into cells the breakdown of 5HT by monoamine oxidases results in the production of ROS (Liu et al., 2004). Thus, to test this hypothesis, iproniazid, a monoamine oxidase (MAO) inhibitor was used and its effects on 5HT-induced ERK1/2 activation and proliferation studied. Pre-treatment with iproniazid (0.1mM) was found to have no effect on

the concentration dependent increase in ERK1/2 activation produced by 5HT (Figure 3.23). Likewise, selective inhibition of MAO did not effect the ability of 5HT to induce proliferation in this cell type (Figure 3.24).

ROCK has also been implicated in the development of PAH and may be a downstream mediator of the 5HT mitogenic response (Lee et al., 1999). In CCL-39 cells inhibition of ROCK using the selective inhibitor Y27632 (5μM) almost completely abolished 5HT-induced proliferation, attenuating the maximum response by 93±2% (n=3) (Figure 3.25). In addition to this, Y27632 also significantly attenuated the concentration-dependent increase in ERK1/2 activation mediated by 5HT. In this instance 5HT-induced ERK1/2 activation was reduced by 52±9% ($p<0.05$, n=3) (Figure 3.26). The effects of a cell permeable C3 transferase, which inhibits the function of ROCK by inactivating Rho GTPase, an upstream effector in the ROCK pathway, was also utilised. Inhibition of the ROCK pathway by this means produced a similar effect as that seen with Y27632. C3 transferase significantly attenuated the increase in ERK1/2 activation produced by 5HT by 43±15% ($p<0.05$, n=4) (Figure 3.27).

In order to further determine the role of ROCK in the 5HT-mediated response, cyclin D1, a downstream mediator in the ERK1/2 cascade was also monitored. 5HT (1μM) was found to increase levels of cyclin D1 present in CCL-39 cells in a biphasic manner over a 24 hour period (Figure 3.28). Stimulation with 5HT significantly increased cyclin D1 levels after 6 hours (62.5±5% increase from basal, $p<0.05$, n=5), subsequently levels of cyclin D1 decreased before peaking at 16 hours (107±15% increase from basal, $p<0.01$, n=5) and remained elevated at 24 hours (85±8% increase from basal, $p<0.01$, n=3). Inhibition of ROCK was able to abolish the elevation in levels of cyclin D1 induced by 6 hour stimulation with 5HT ($p<0.05$, n=3) (Figure 3.29). Taken together these results highlight the involvement for ROCK in the signalling pathway required for 5HT-induced ERK activation and proliferation.

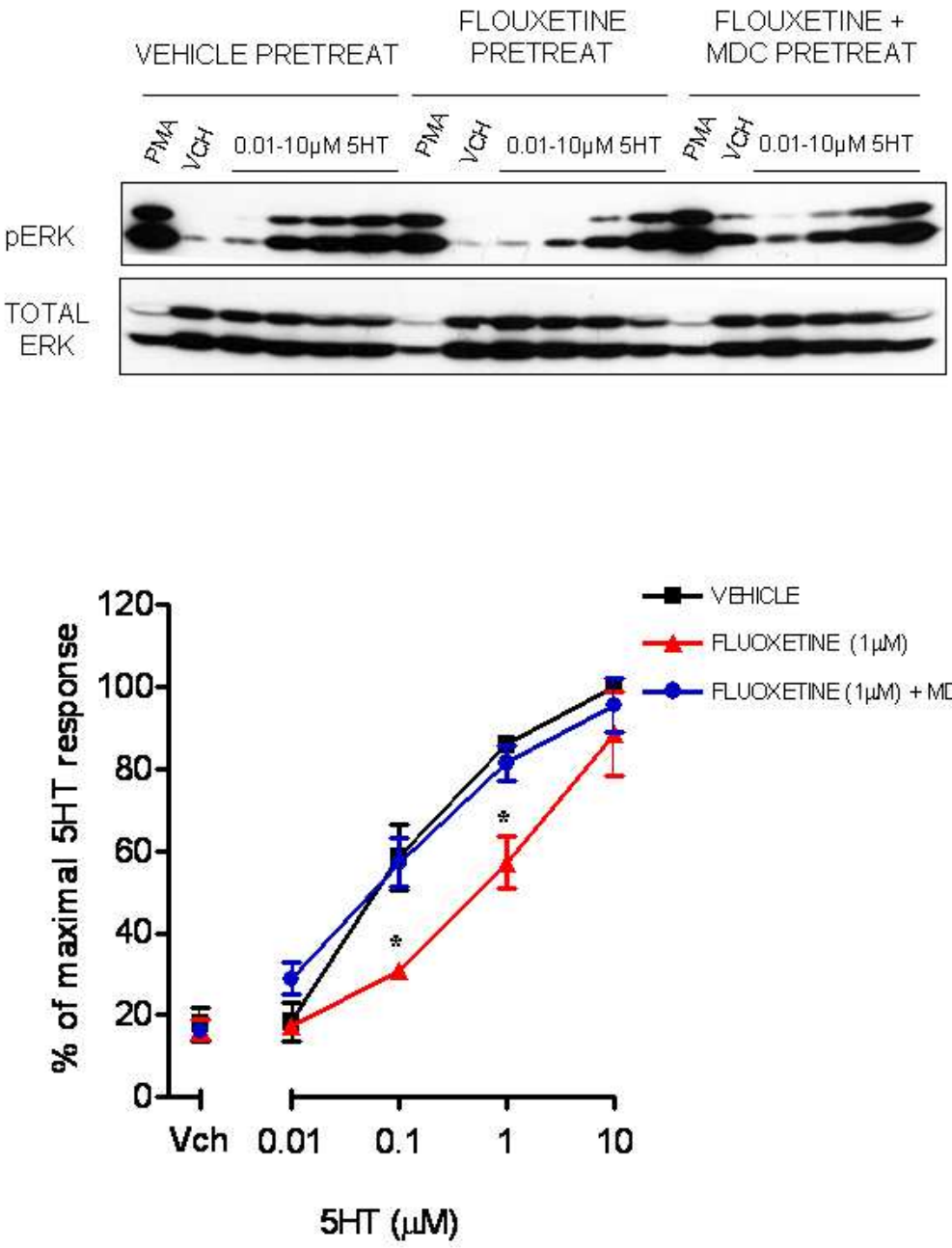

Figure 3.19 Effects of MDC on receptor mediated 5HT-induced ERK activation.

CCL-39 cells were serum starved for 16 hours. Following this, cells were treated with either fluoxetine (1μM) or MDC (100μM) alone or in combination for 30 minutes. Cells were then stimulated for 5 minutes with increasing concentrations of 5HT (0-10μM) or PMA (2μM). Cell lysates were prepared and equalised for protein concentration by BCA assay. Samples were then analysed by SDS-PAGE and immunoblotting using phospho-specific and total ERK antibodies. Blots shown are representative. Results are expressed as mean ± SEM (n=3). * $p < 0.05$ *versus* vehicle pre-treated cells using an unpaired, two tailed t-test.

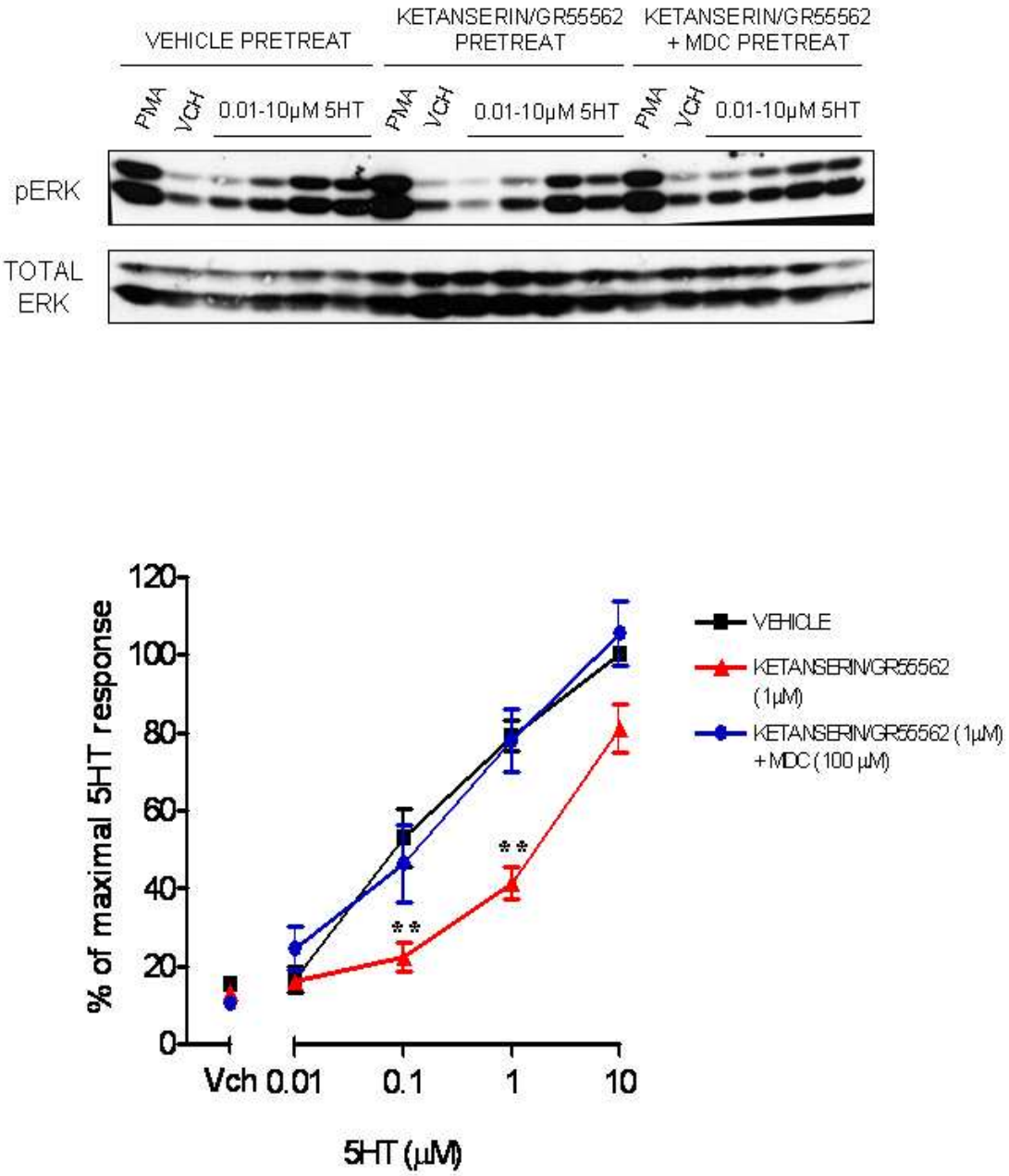

Figure 3.20 Effects of MDC on 5HTT mediated ERK activation.

CCL-39 cells were serum starved for 16 hours. Following this, cells were treated with either, ketanserin and GR55562 (1μM) or MDC (100μM) or a combination of both for 30 minutes. Cells were then stimulated for 5 minutes with increasing concentrations of 5HT (0-10μM) or PMA (2μM). Cell lysates were prepared and equalised for protein concentration by BCA assay. Samples were then analysed by SDS-PAGE and immunoblotting using phospho-specific and total ERK antibodies. Blots shown are representative. Results are expressed as mean ± SEM (n=3). ** $p < 0.01$ *versus* vehicle pre-treated cells using an unpaired, two tailed t-test.

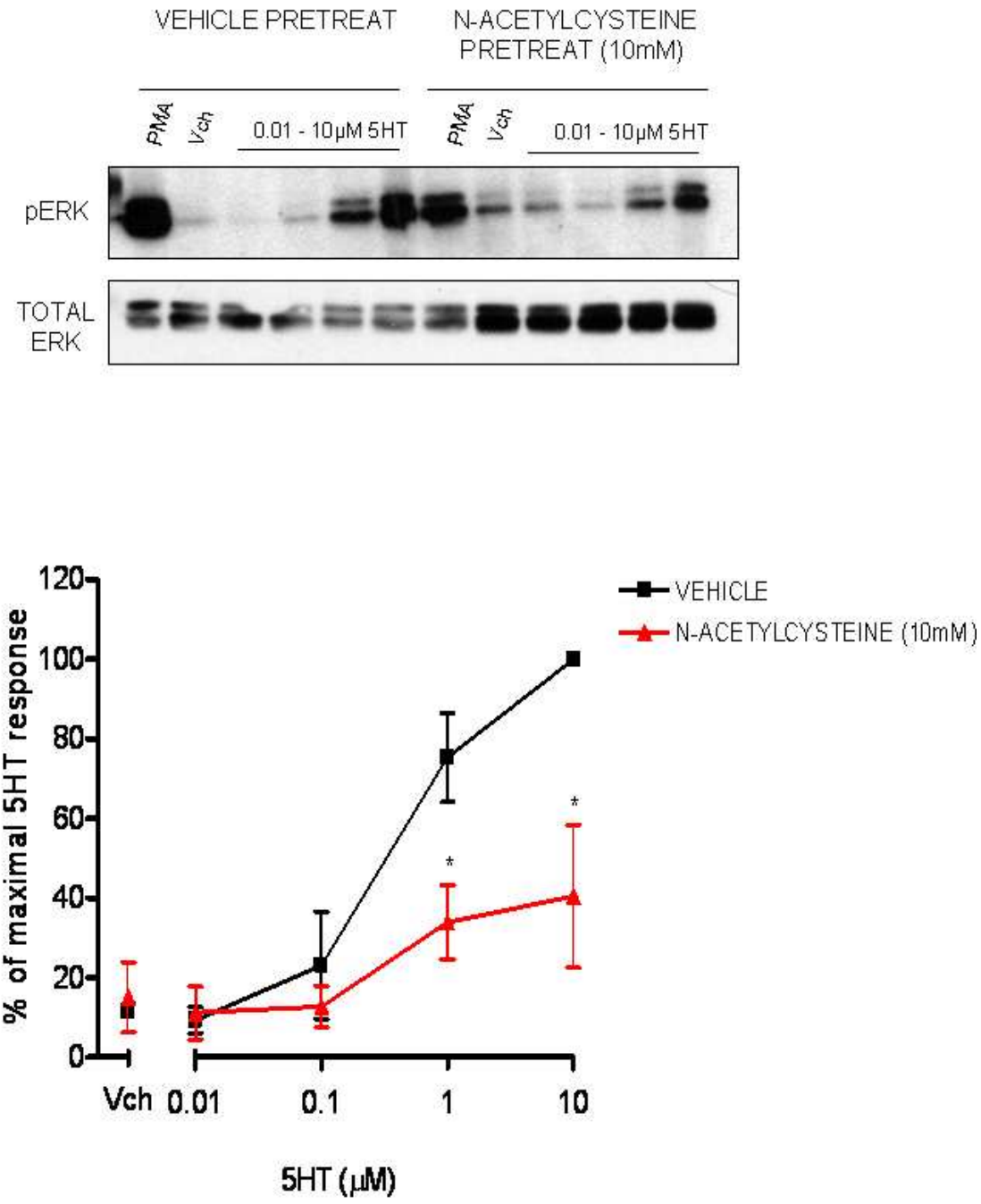

Figure 3.21 Effects of N-acetylcysteine (NAC) treatment on 5HT-induced ERK activation.

After serum starvation for 16 hours CCL-39 cells were pre-treated for 30 minutes with N-acetylcysteine (10mM). Cells were then stimulated for 5 minutes with increasing concentrations of 5HT (0-10μM) or PMA (2μM). Cell lysates were prepared and equalised for protein concentration by BCA assay. Samples were then analysed by SDS-PAGE and immunoblotting using phospho-specific and total ERK antibodies. Blots shown are representative. Results are expressed as mean ± SEM (n=3). * $p<0.05$ *versus* vehicle pre-treated cells using an unpaired, two tailed t-test.

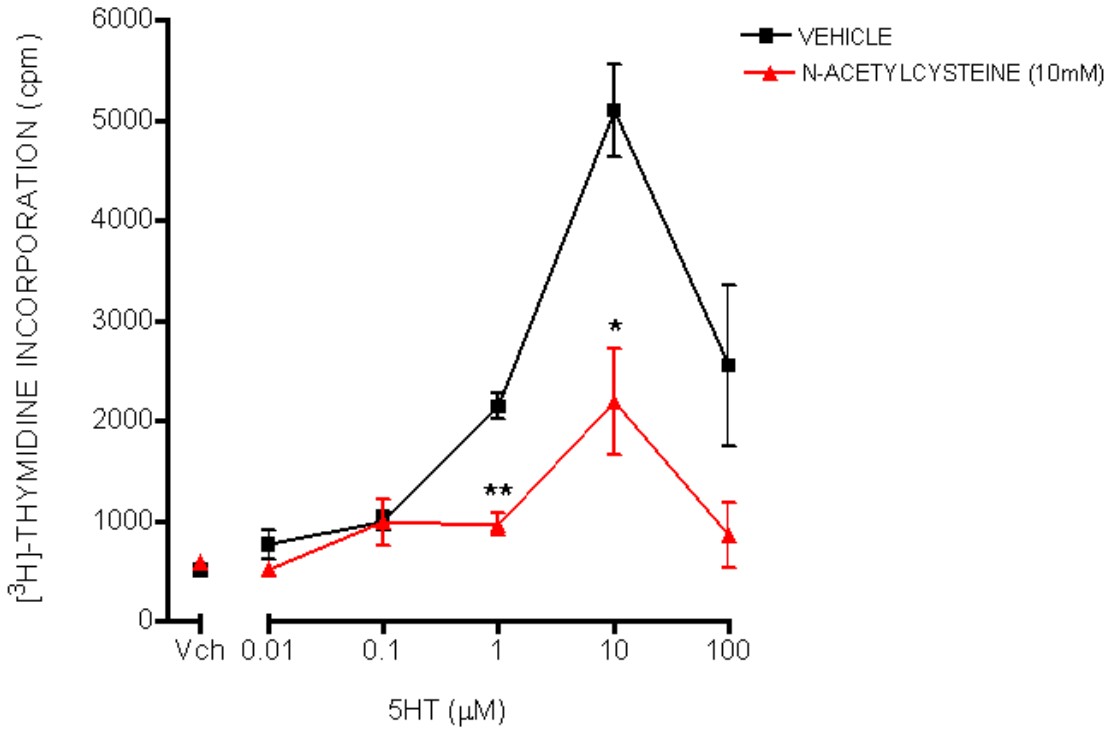

Figure 3.22 Effects of NAC treatment on 5HT-induced proliferation.

CCL-39 cells were serum starved for 24 hours then incubated with 0-100µM 5HT in the presence or absence of N-acetylcysteine (NAC) (10mM) for a further 24 hours, with 0.5µCi/well [^{3}H]-thymidine added for the final 6 hours of this incubation. Cell were then harvested onto a glass fibre filter mat and [^{3}H]-thymidine incorporation assessed by liquid scintillation counting. Data shown are representative of n=3 assays. Results expressed as mean ± SEM of triplicate samples. * $p<0.05$, ** $p<0.01$ *versus* vehicle using an unpaired, two tailed t-test.

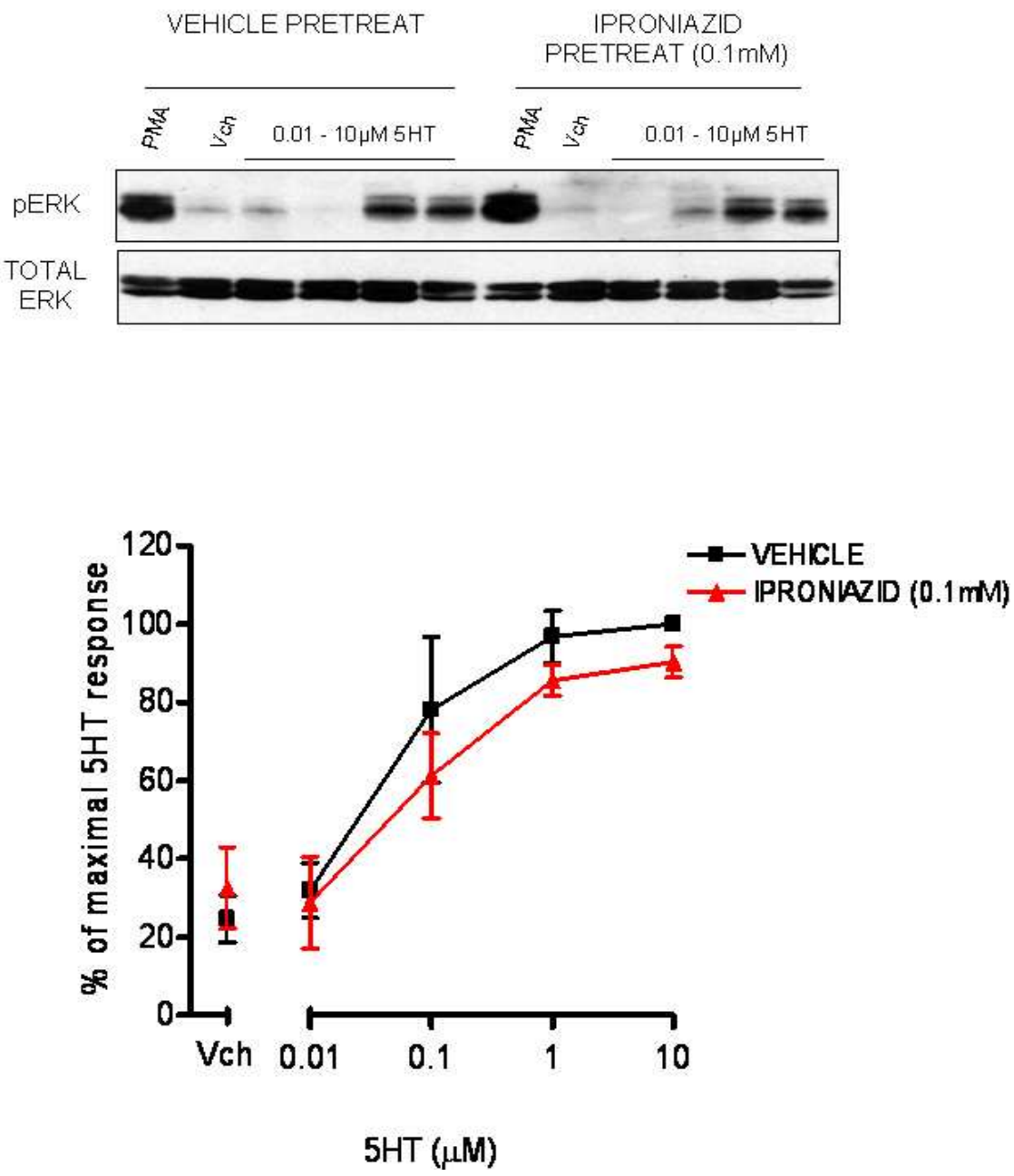

Figure 3.23 Effects of monoamine oxidase inhibition with iproniazid on 5HT-induced ERK activation.

After serum starvation for 16 hours CCL-39 cells were pre-treated for 1 hour with iproniazid (0.1mM) (monoamine oxidase inhibitor). Cells were then stimulated for 5 minutes with increasing concentrations of 5HT (0-10µM) or PMA (2µM). Cell lysates were prepared and equalised for protein concentration by BCA assay. Samples were then analysed by SDS-PAGE and immunoblotting using phospho-specific and total ERK antibodies. Blots shown are representative. Results are expressed as mean ± SEM (n=3). Statistical analysis using unpaired, two tailed t-tests revealed no significant differences between vehicle and iproniazid pre-treated cells.

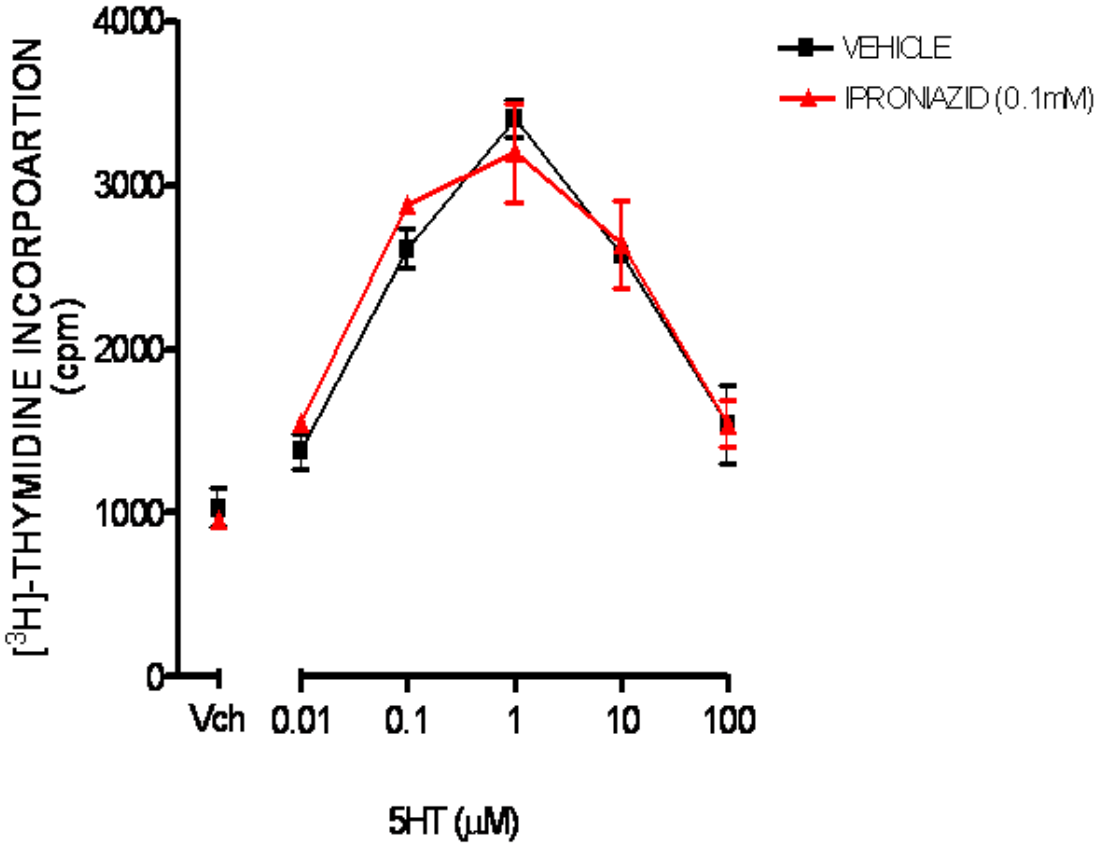

Figure 3.24 Effects of monoamine oxidase inhibition by iproniazid on 5HT-induced proliferation.

CCL-39 cells were serum starved for 24 hours then incubated with 0-100µM 5HT in the presence or absence of iproniazid (0.1mM) for a further 24 hours, with 0.5µCi/well [^{3}H]-thymidine added for the final 6 hours of this incubation. Cell were then harvested onto a glass fibre filter mat and [^{3}H]-thymidine incorporation assessed by liquid scintillation counting. Data shown are representative of n=3 assays. Results expressed as mean ± SEM of triplicate samples.

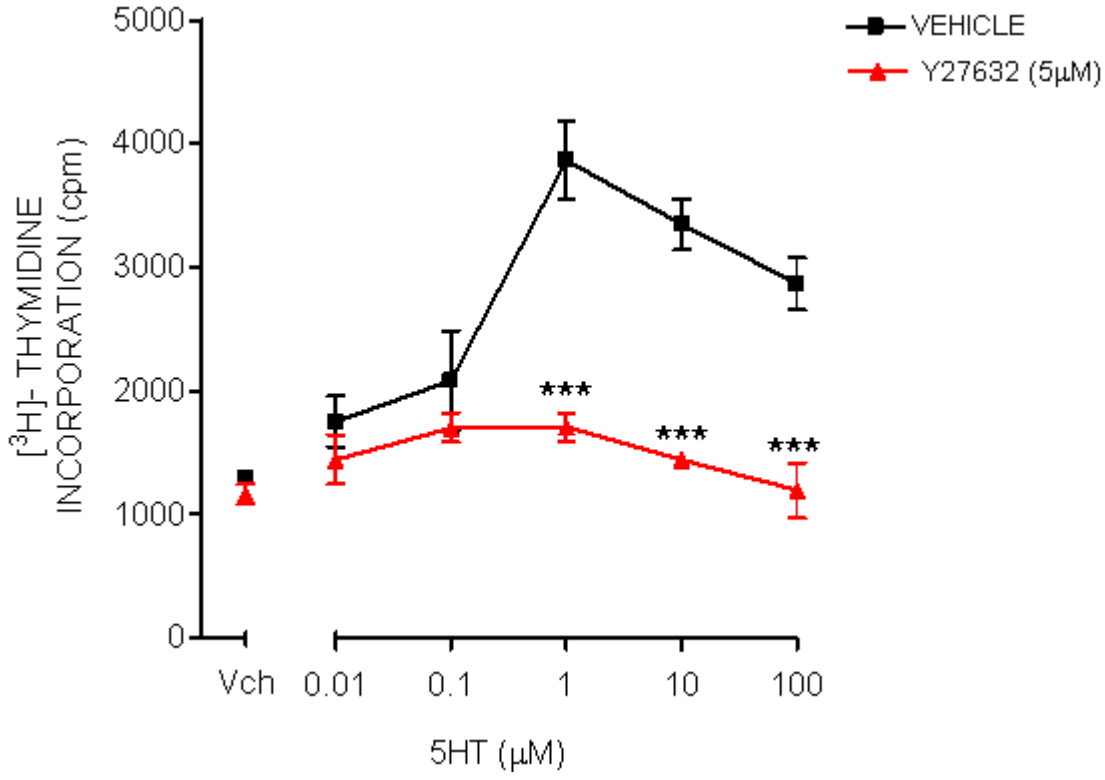

Figure 3.25 Effects of Rho-kinase inhibition by Y27632 on 5HT-induced proliferation.

CCL-39 cells were serum starved for 24 hours then incubated with 0-100µM 5HT in the presence or absence of Y27632 (5µM) (Rho-kinase inhibitor) for a further 24 hours, with 0.5µCi/well [^{3}H]-thymidine added for the final 6 hours of this incubation. Cell were then harvested onto a glass fibre filter mat and [^{3}H]-thymidine incorporation assessed by liquid scintillation counting. Data shown are representative of n=3 assays. Results expressed as mean ± SEM of triplicate samples. *** $p<0.001$ *versus* vehicle using an unpaired, two tailed t-test.

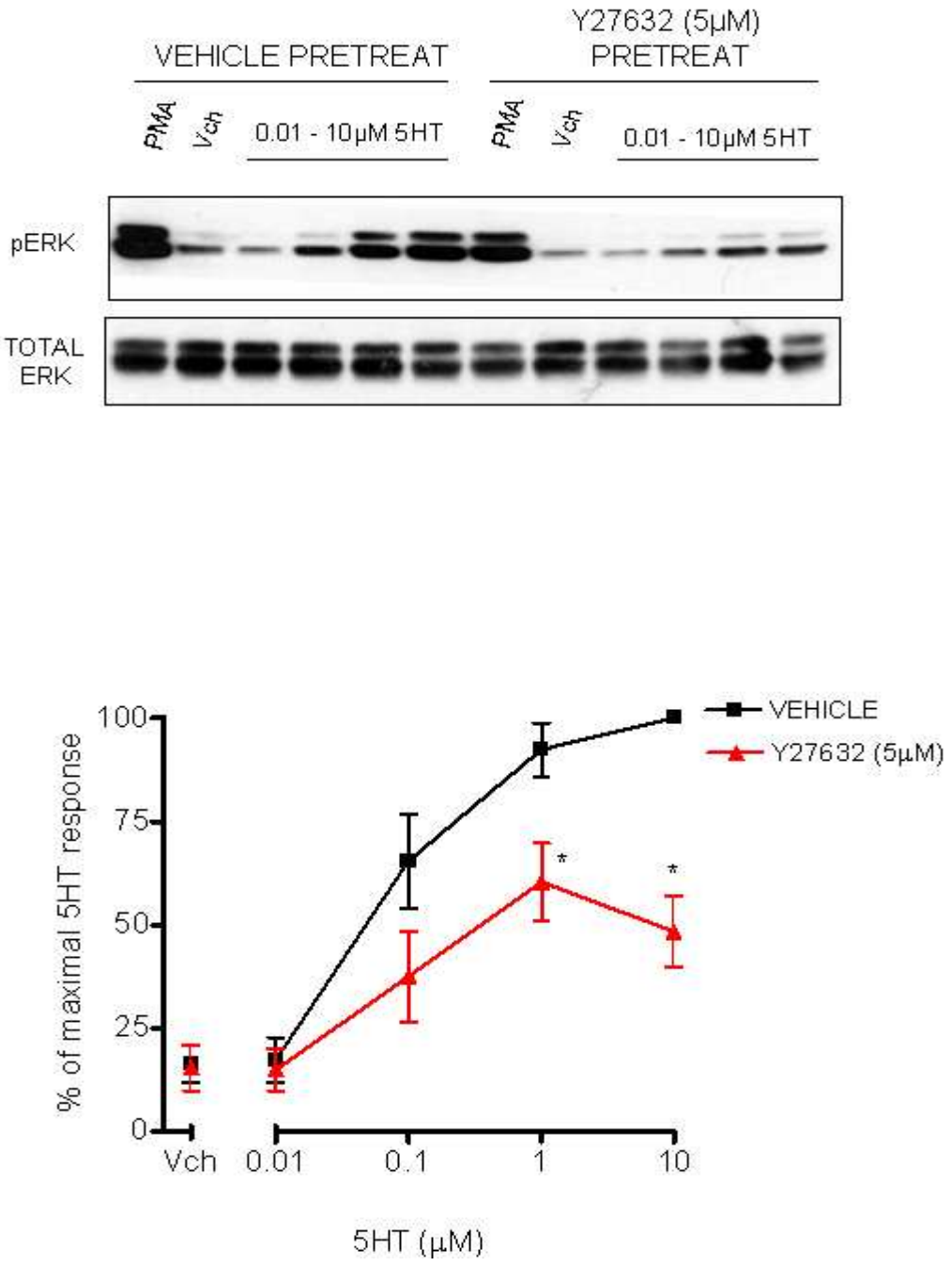

Figure 3.26 Effects of ROCK inhibition with Y27632 on 5HT-induced ERK activation.

After serum starvation for 16 hours CCL-39 cells were pre-treated for 30 minutes with Y27632 (5μM). Cells were then stimulated for 5 minutes with increasing concentrations of 5HT (0-10μM) or PMA (2μM). Cell lysates were prepared and equalised for protein concentration by BCA assay. Samples were then analysed by SDS-PAGE and immunoblotting using phospho-specific and total ERK antibodies. Blots shown are representative. Results are expressed as mean ± SEM (n=4). * $p<0.05$ *versus* vehicle pre-treated cells using an unpaired, two tailed t-test.

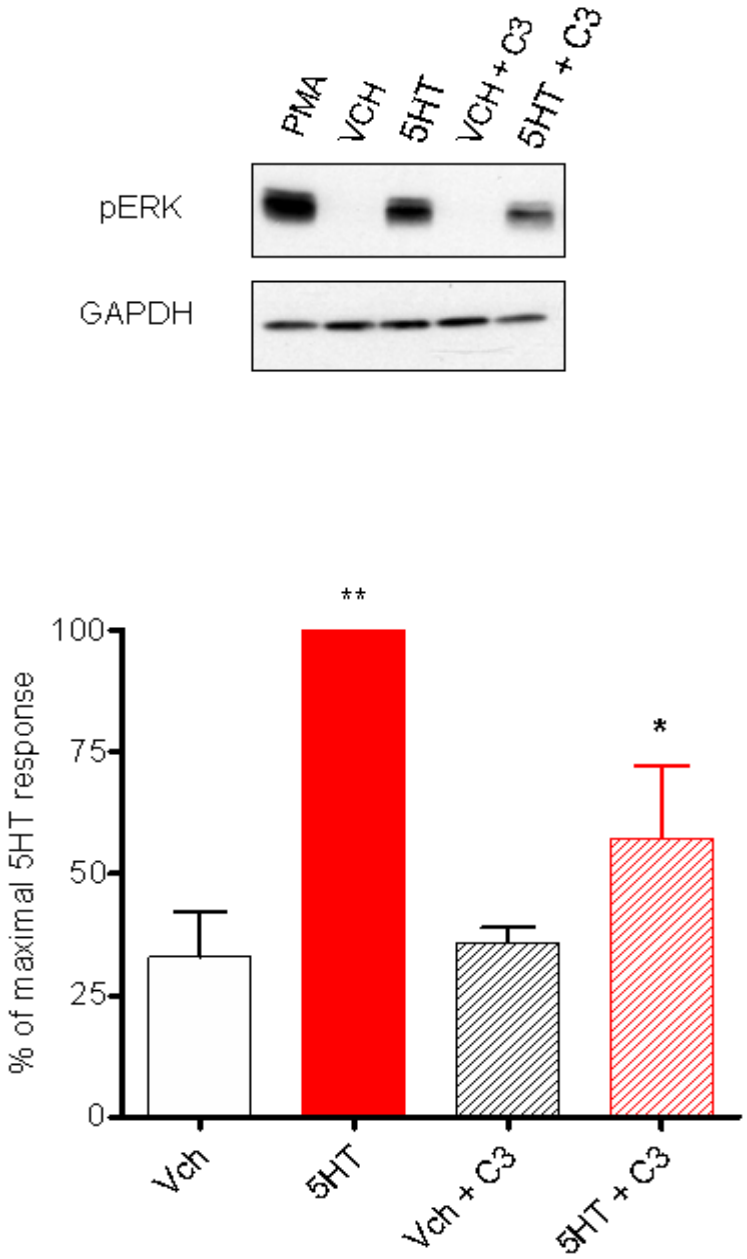

Figure 3.27 Effects of Rho-GTPase inhibition with C3 transferase on 5HT-induced ERK activation.

After serum starvation for 16 hours CCL-39 cells were pretreated for 6 hours with cell permeable C3 transferase (specific Rho-GTPase inhibitor). Cells were then stimulated for 5 minutes with 5HT (1μM) or vehicle. Treatment with PMA (2μM) for 5 minutes was used as a positive control for pERK. Cell lysates were prepared and equalised for protein concentration by BCA assay. Samples were then analysed by SDS-PAGE and immunoblotting using phospho-specific and total ERK antibodies. Blots shown are representative. Results are expressed as mean ± SEM (n=4). * $p<0.05$ *versus* 5HT**,** ** $p<0.01$ *versus* vehicle using Newman-Keuls multiple comparison test.

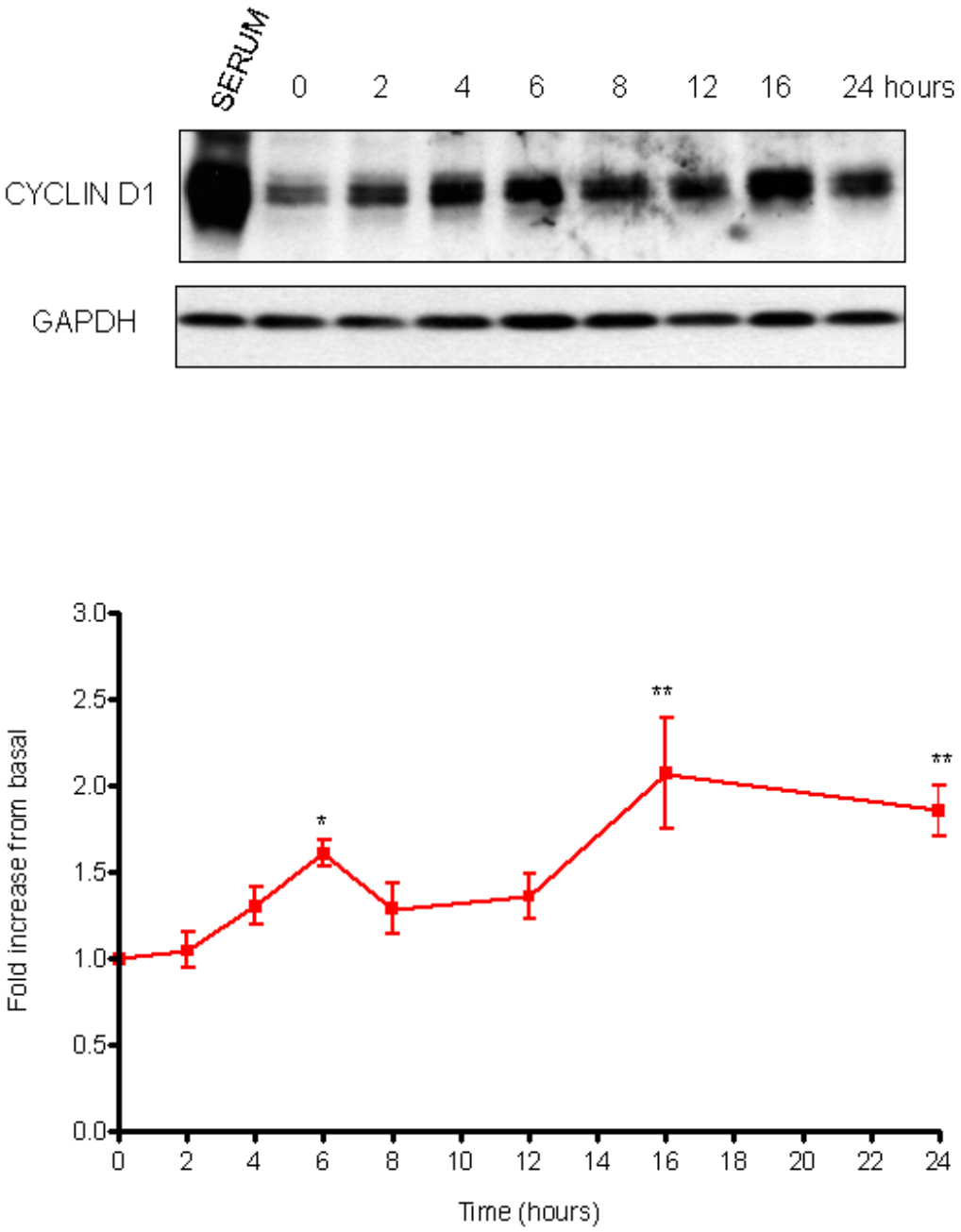

Figure 3.28 Timecourse of 5HT-induced cyclin D1 accumulation.

CCL-39 cells were serum starved for 16 hours and then stimulated with 5HT (1µM) for 0-24 hours. Cells grown in media containing 10% serum were used as a positive control for cyclin D1 accumulation. Cell lysates were prepared and equalised for protein concentration by BCA assay. Samples were then analysed by SDS-PAGE and immunoblotting using cyclin D1 and GAPDH antibodies. Blots shown are representative. Results are expressed as mean ± SEM (n=5). * $p<0.05$, ** $p<0.01$ *versus* vehicle using Newman-Keuls multiple comparison test.

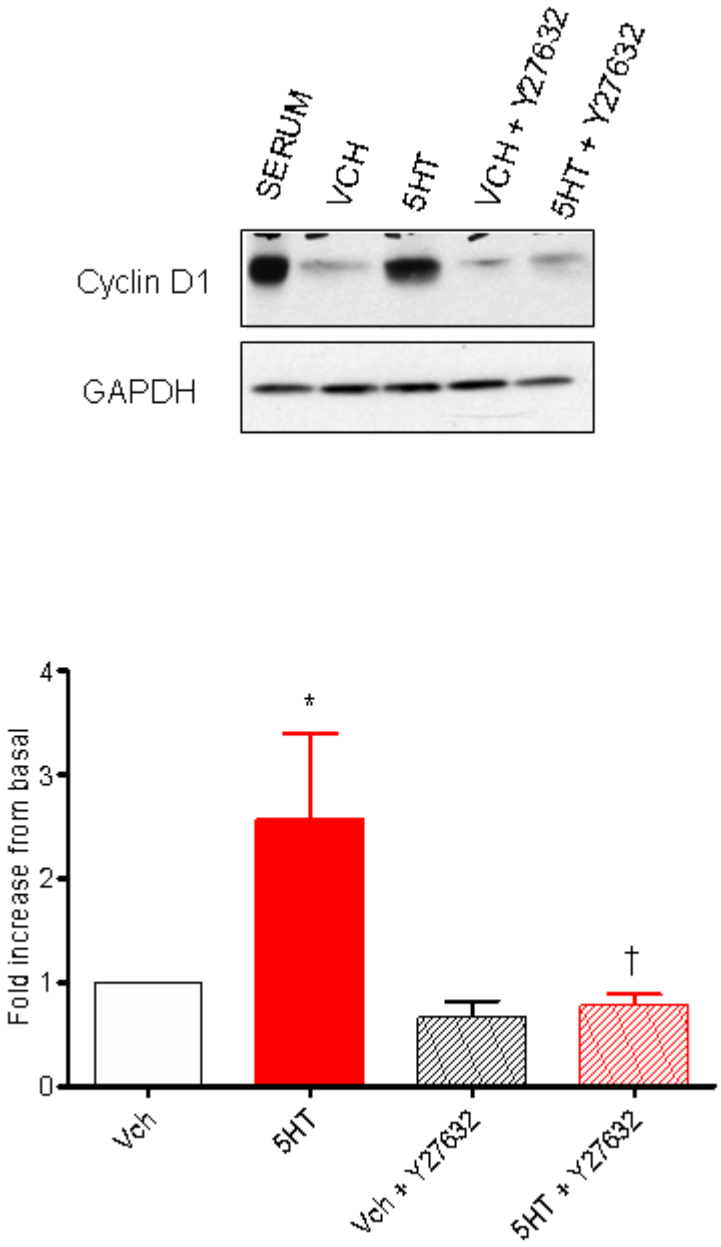

Figure 3.29 Effects of ROCK inhibition with Y27632 on 5HT-induced Cyclin D1.

After serum starvation for 16 hours CCL-39 cells were treated with Y27632 (5μM) and 5HT (1μM) for 6 hours. Cells grown in media containing 10% serum were used as a positive control for cyclin D1 accumulation. Cell lysates were prepared and equalised for protein concentration by BCA assay. Samples were then analysed by SDS-PAGE and immunoblotting using cyclin D1 and GAPDH antibodies. Blots shown are representative. Results are expressed as mean ± SEM (n=3). * $p<0.05$ *versus* vehicle and † $p<0.05$ *versus* 5HT using Newman-Keuls multiple comparison test.

In order to determine if the ROCK pathway functions selectively downstream of either 5HT receptors or 5HTT, various pharmacological agents were employed. As described previously, inhibition of ROCK significantly reduced 5HT-induced ERK1/2 activation (41±3% reduction, $p<0.05$, n=3). Stimulation with α-methyl-5HT (α-meth) (1μM), a $5HT_2$-selective agonist, resulted in ERK1/2 activation in CCL-39 cells (Figure 3.30). This activation was around 55±8% of that observed with 5HT. Co-administration of α-meth with Y27632 had no effect on the ability of agonist to induce ERK1/2 activation (α-meth induced 54.6±8% of the maximal response, while α-meth + Y27632 induced 56.3±13% of maximal 5HT response), suggesting that the ROCK pathway is not involved downstream of $5HT_2$ receptors. CP93129, a selective $5HT_{1B}$ receptor agonist was also used. CP93129 (1μM) was also found to induce ERK1/2 activation, with the maximal response produced reaching 51±10% of that elicited by 5HT (Figure 3.30). In contrast to α-meth, pre-treatment with Y27632 significantly attenuated the ability of the $5HT_{1B}$ agonist to activate ERK1/2, reducing the level of ERK1/2 activation by 76±3% ($p<0.05$, n=3). These findings suggest that ROCK is an important mediator specifically, downstream of the $5HT_{1B}$ receptor.

In light of the fact that there are no selective agonists for 5HTT, selective 5HT receptor antagonists were instead used in order to block 5HT receptors thus allowing 5HT to act preferentially on 5HTT (Figure 3.31). Inhibition of $5HT_{2A}$ and $5HT_{1B/1D}$ receptor significantly reduced 5HT-mediated ERK1/2 activation by 60±7% ($p<0.001$, n=3). However, pre-treatment with Y27632 had not effect on the attenuation induced by these antagonists. In addition to this, as shown previously, citalopram attenuated 5HT-mediated ERK1/2 activation (31±2% reduction, $p<0.01$, n=3) and this response also remained unaffected by ROCK inhibiton (32±9%, $p<0.01$, n=3).

To further elucidate the mechanism of action as to how ROCK modulates ERK phosphorylation, the effect of Y27632 on MEK, the upstream kinase responsible for phosphorylating ERK1/2, was studied. Immunoblotting using a phospho-specific MEK antibody revealed that Y27632-mediated inhibition of ROCK had no effect on 5HT-induced MEK activation (Figure 3.32).

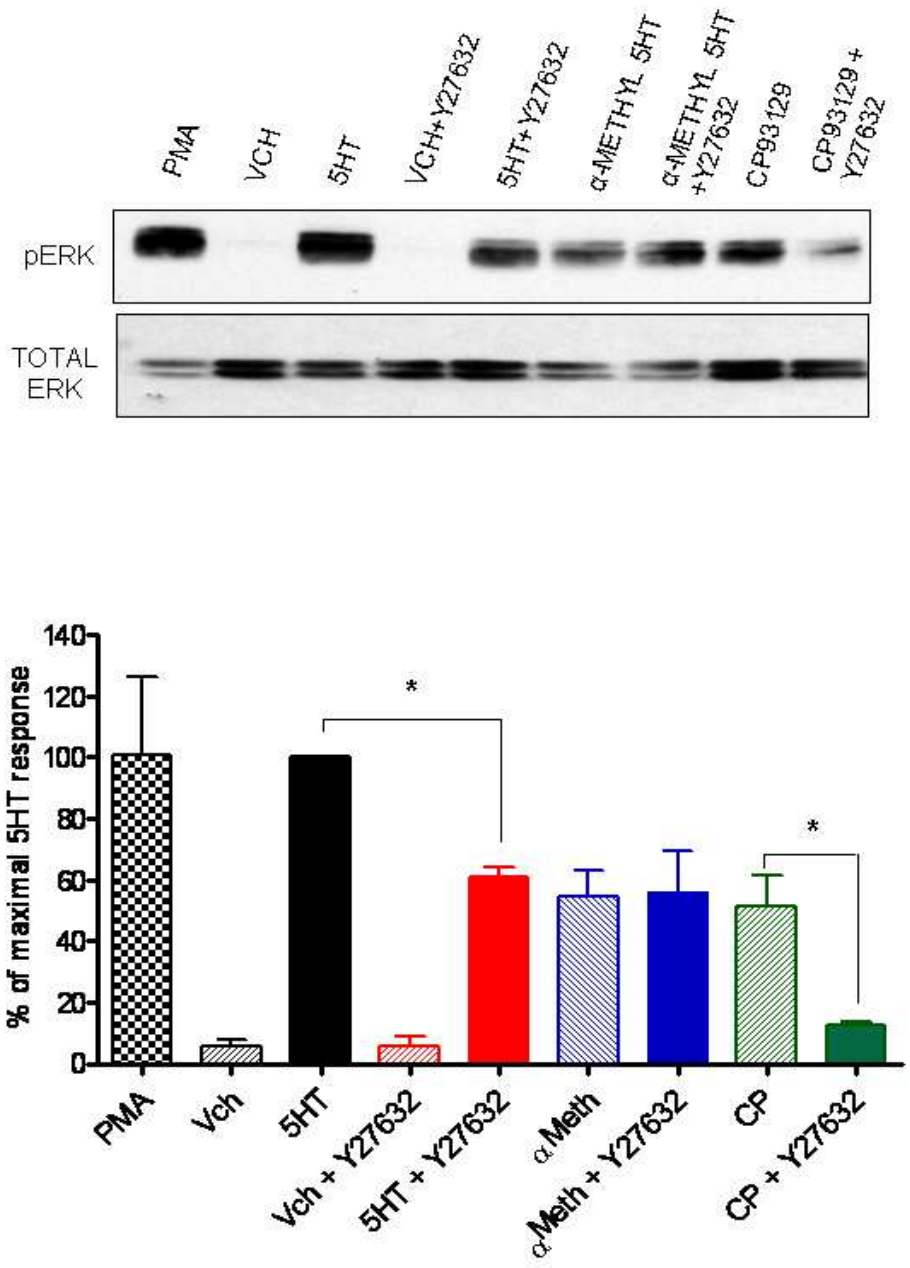

Figure 3.30 Effects of ROCK inhibition with Y27632 on $5HT_{2A}$ and $5HT_{1B}$ receptor-mediated ERK activation.

After serum starvation for 16 hours CCL-39 cells were pretreated for 30 minutes with Y27632 (5μM) or vehicle. Cells were then stimulated for 5 minutes with either 5HT (1μM), α-methyl5HT (1μM) ($5HT_{2A}$ agonist) or CP93129 (1μM) ($5HT_{1B}$ agonist). Treatment with PMA (2μM) for 5 minutes was used as a positive control for pERK. Cell lysates were prepared and equalised for protein concentration by BCA assay. Samples were then analysed by SDS-PAGE and immunoblotting using phospho-specific and total ERK antibodies. Blots shown are representative. Results are expressed as mean ± SEM (n=3). * $p<0.05$ *versus* CP93129 treated cells using an unpaired, two tailed t-test.

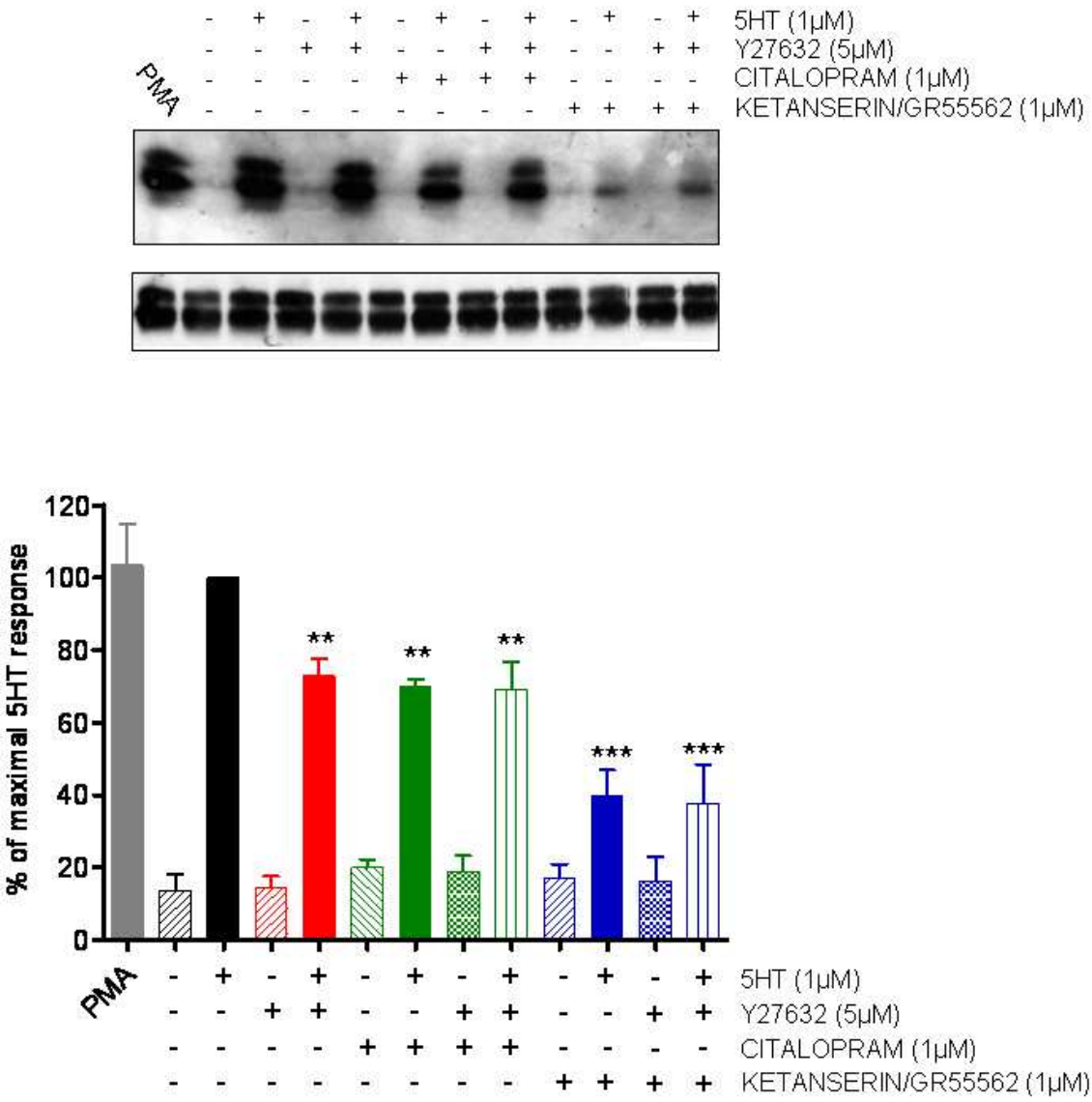

Figure 3.31 Effects of ROCK inhibition with Y27632 and selective antagonists on 5HT-induced ERK activation.

After serum starvation for 16 hours CCL-39 cells were pre-treated for 1 hour with Y27632 (5μM) and either citalopram (1μM), or a combination of ketanserin (1μM) and GR55562 (1μM) before stimulation with 5HT (1μM) for 5 minutes. Treatment with PMA (2μM) for 5 minutes was used as a positive control for pERK. Cell lysates were prepared and equalised for protein concentration by BCA assay. Samples were then analysed by SDS-PAGE and immunoblotting using phospho-specific and total ERK antibodies. Blots shown are representative. Results are expressed as mean ± SEM (n=3). ** $p<0.01$, *** $p<0.001$ *versus* 5HT using Newman-Keuls multiple comparison post test.

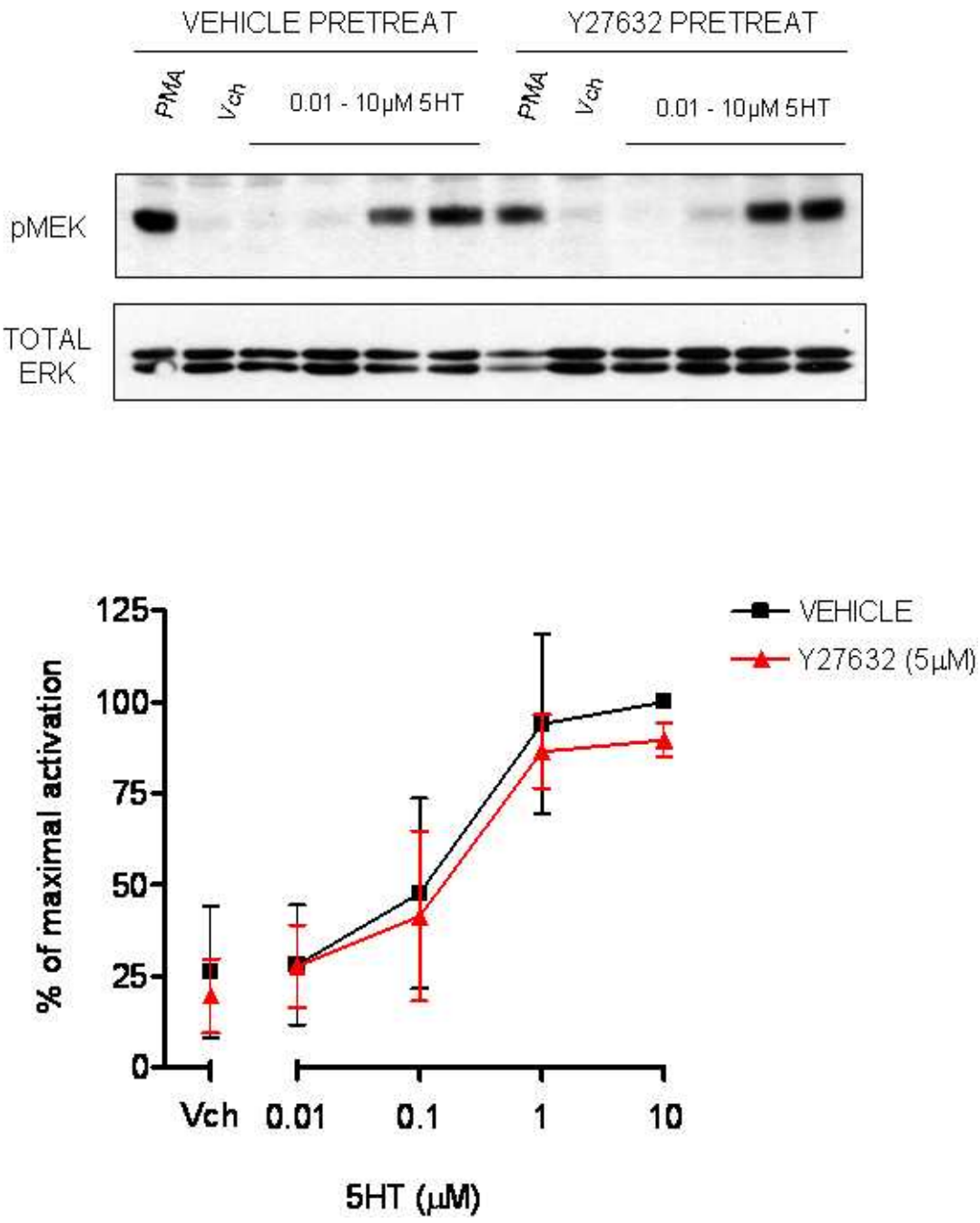

Figure 3.32 Effects of ROCK inhibition with Y27632 on 5HT-induced MEK activation.

After serum starvation for 16 hours CCL-39 cells were pre-treated for 30 minutes with Y27632 (5µM). Cells were then stimulated for 5 minutes with increasing concentrations of 5HT (0-10µM) or PMA (2µM). Cell lysates were prepared and equalised for protein conentration by BCA assay. Samples were then analysed by SDS-PAGE and immunoblotting using phospho-specific MEK and total ERK antibodies. Blots shown are representative. Results are expressed as mean ± SEM (n=3). Statistical analysis using an unpaired, two tailed t-test revealed no significant differences between vehicle and Y27632 treated cells.

ROCK has also been previously reported to be involved in facilitating the translocation of active ERK to the nucleus (Liu et al., 2004). Given the ability of Y27632 to attenuate the induction of cyclin D1 in CCL-39 cells (Figure 3.29), studies into the role of ROCK in the nuclear translocation of pERK in this cell type were also carried out. Stimulation with 5HT resulted in an increase in the level of pERK1/2 in both cytoplasmic and nuclear cell fractions (Figure 3.33). Inhibition of ROCK with 5μM Y27632 specifically attenuated ERK1/2 phosphorylation in the cytoplasm (33±8% reduction, $p<0.01$, $n=4$) but had no effect on the levels of pERK1/2 present in the nucleus in response to 5HT, suggesting inhibition of ROCK is unable to affect the ability of pERK1/2 to translocate into the nucleus.

Since inhibition of ROCK resulted in the attenuation of 5HT-induced ERK1/2 activation, yet had no effect on MEK activation, it may therefore regulate dual specificity phosphatases (DUSPs), responsible for de-phosphorylating ERK1/2. In order to investigate this hypothesis, the effects of the tyrosine phosphatase inhibitor, sodium-*ortho*-vanadate, on the ability of Y27632 to attenuate ERK1/2 activation was tested (Figure 3.34). Treatment with sodium-ortho-vanadate on its own significantly increased basal levels of ERK1/2 activation (51±5% versus vehicle, $p<0.001$, $n=3$). Stimulation with 5HT in the presence of sodium-ortho-vanadate resulted in an increase in ERK1/2 activation similar to that of 5HT alone. Moreover, the phosphatase inhibitor was unable to reverse the ability of the ROCK inhibitor to reduce 5HT-induced ERK1/2 activation, suggesting ROCK does not mediate its effects by regulating DUSPs responsible for de-phosphorylating ERK1/2.

In order to further elucidate the mechanisms by which 5HT utilises ROCK to mediate its mitogenic effects, the ability of 5HT to directly activate the ROCK pathway was studied. To do this, myosin phosphatase, a downstream substrate of ROCK was monitored. Activation of ROCK results in the phosphorylation of the myosin binding subunit of myosin phosphatase (MYPT1) (Amano et al., 2000). Immunoblotting using a phospho-specific antibody to MYPT1 (pMYPT1), revealed that over a period of 2 hours, stimulation with 5HT (1μM) had no effect on MYPT1 phosporylation, suggesting that 5HT does not directly activate this pathway (Figure 3.35).

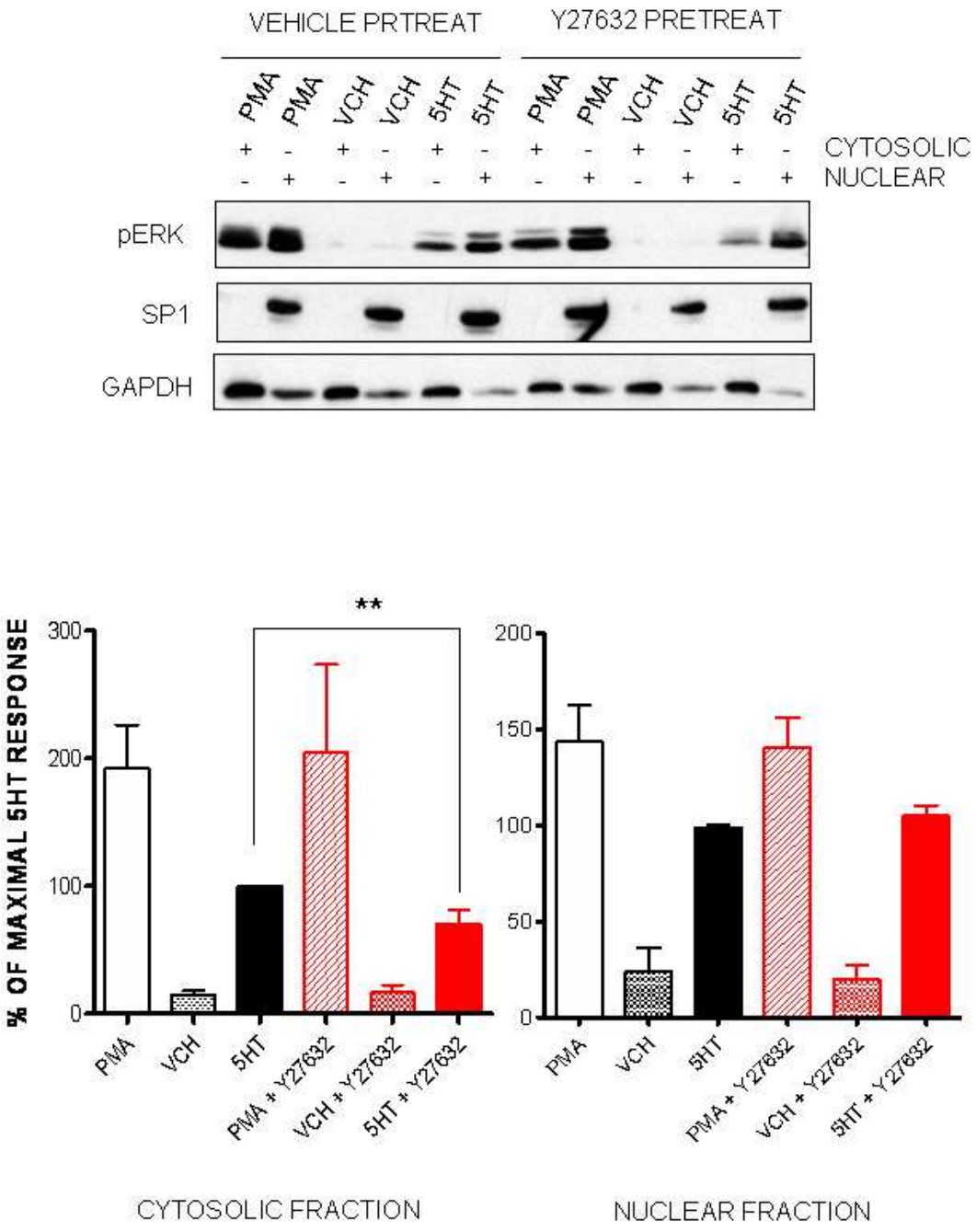

Figure 3.33 Effects of ROCK inhibition with Y27632 on the nuclear translocation of pERK.

CCL-39 cells were serum starved for 16 hours. The cell monolayer was then pre-treated for 30 minutes with Y27632 (5μM) or vehicle prior to stimulation with 5HT (1μM) or PMA (2μM) for 5 minutes. Cytosolic and nuclear fractions were prepared and equalised for protein concentration by Bradford's assay. Samples were then analysed by SDS-PAGE and immunoblotting using a phospho-specific ERK antibody. Immunoblotting using SP1 and GAPDH antibodies was also carried out to determine the integrity of nuclear and cytosolic fractions respectively. Blots shown are representative. Results are expressed as mean ± SEM (n=4). ** $p<0.01$ using an unpaired two-tailed t-test.

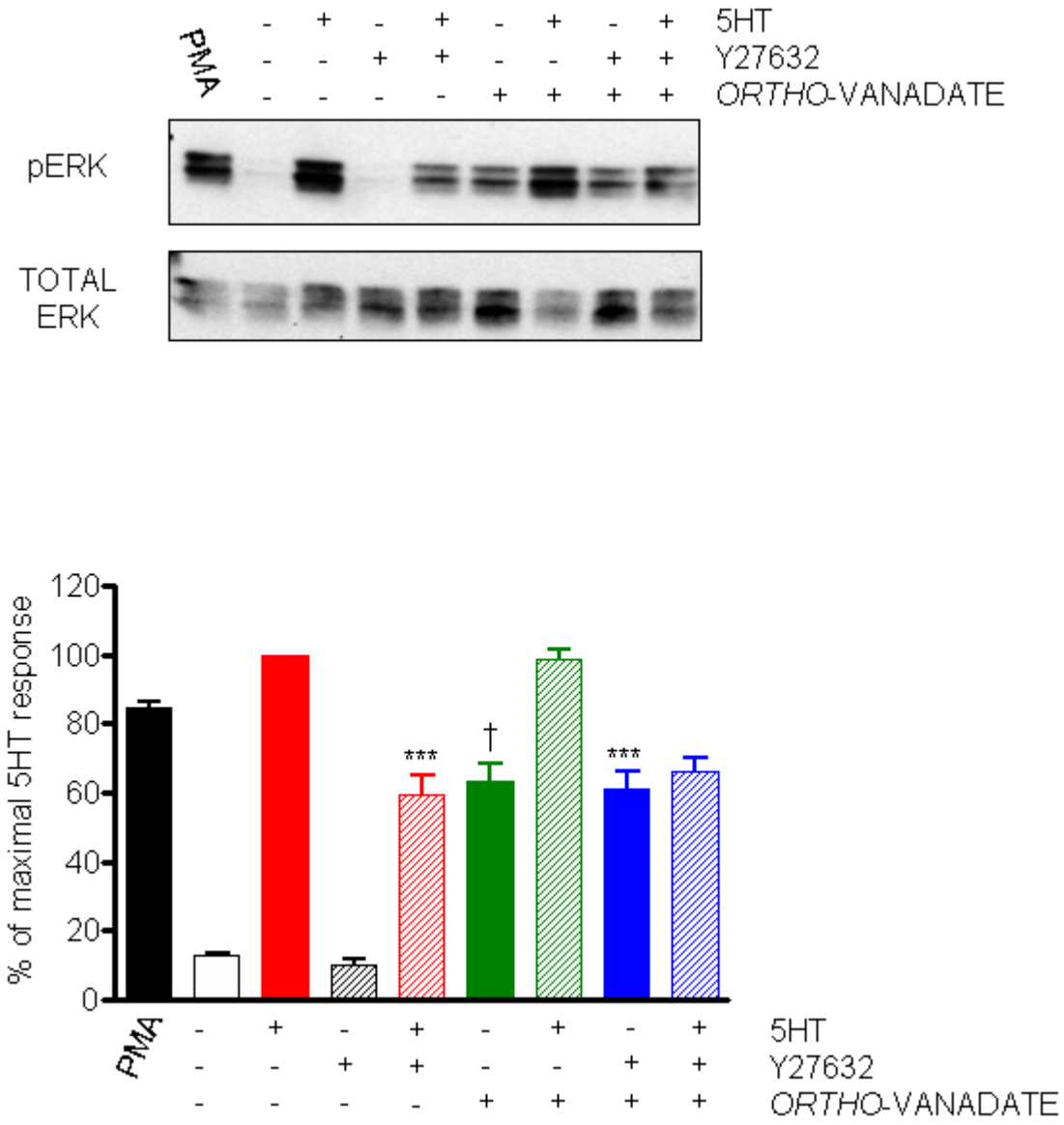

Figure 3.34 Effects of tyrosine phosphatase inhibition with vanadate and ROCK inhibition with Y27632 on 5HT-induced ERK activation.

After serum starvation for 16 hours CCL-39 cells were treated for 30 minutes with either sodium ortho-vanadate (100µM) or Y27632 (5µM) alone or in combination. Treatment with PMA (2µM) for 5 minutes was used as a positive control for pERK. Cells were then stimulated with 5HT (1µM) for 5 minutes before cells were harvested. Cell lysates were prepared and equalised for protein concentration by BCA assay. Samples were then analysed by SDS-PAGE and immunoblotting using phospho-specific and total ERK antibodies. Blots shown are representative. Results are expressed as mean ± SEM (n=3). † p<0.001 *versus* vehicle, *** p<0.001 *versus* 5HT using Newman-Keuls multiple comparison post test.

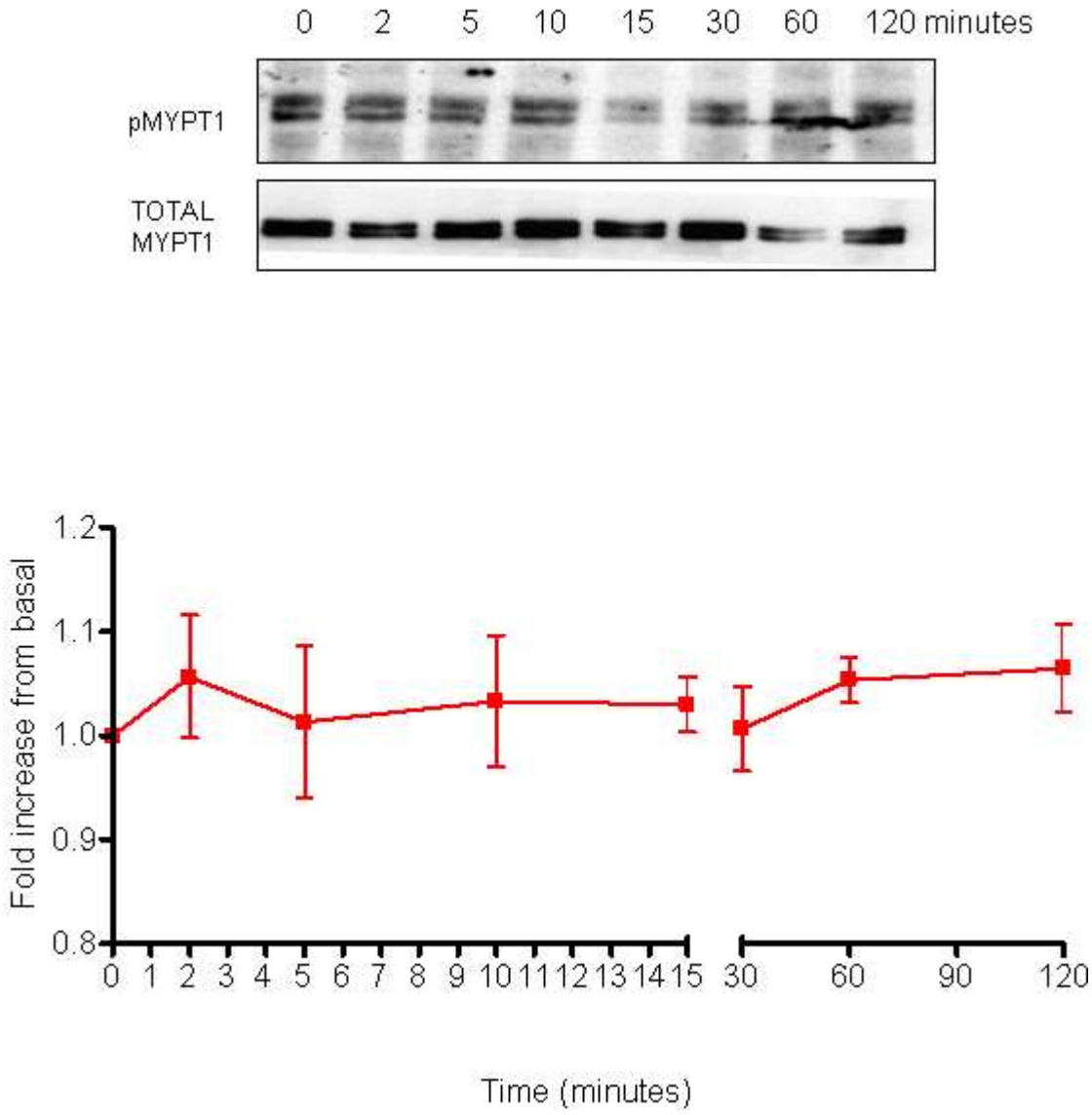

Figure 3.35 Timecourse of effects of 5HT on phosphorylation of myosin phosphatase.

Following serum starvation for 16 hours, CCL-39 cells were stimulated with 5HT (1µM) for 0-120 minutes. Cell lysates were prepared and equalised for protein concentration by BCA assay. Samples were then analysed by SDS-PAGE and immunoblotting using phospho-specific and total antibodies to the myosin binding subunit of myosin phosphatase. Blots shown are representative. Results are expressed as mean ± SEM (n=3). Statistical analysis using Newman-Keuls multiple comparison test revealed no significant differences.

In addition to this, cofilin, another downstream substrate of ROCK, was also studied. When active, ROCK phosphorylates LIMK, which in turn phosphorylates cofilin (Amano et al., 2000). Upon stimulation with 5HT, an initial rapid increase in the levels of phosphorylated cofilin (p-cofilin) was observed (51±12% increase from basal). However, due to large variability, this increase was not found to be statistically significant ($p>0.05$, $n=5$) (Figure 3.36). Furthermore, pre-treatment with the ROCK inhibitor Y27632 had no effect on the ability of 5HT to activate cofilin (Figure 3.37), suggesting cofilin is being phosphorylated independently of ROCK.

Finally, the effects of 5HT on actin stress fibres, the formation of which is a Rho mediated response, were studied. Under vehicle conditions, actin stress fibres were detected in CCL-39 cells by light microscopy (Figure 3.38). Stimulation with 5HT (1μM) over a period of 2 hours was unable to induce any change in the presence of actin stress fibres within the cells. However, inhibition of ROCK was able to disrupt the actin cytoskeleton and abolish the presence of actin stress fibres (Figure 3.39). In light of the findings that 5HT does not appear to directly activate the ROCK pathway, but ROCK inhibition is able to disrupt the formation of actin stress fibres, the role of the actin cytoskeleton in 5HT-induced ERK1/2 activation was investigated. Given that inhibition of ROCK attenuates 5HT-induced ERK1/2 activation and also disrupts the actin cytoskeleton, the effects of other inhibitors on the gross morphology of the actin cytoskeleton were determined. Citalopram, ketanserin, GR55562, NAC and U0126, which have all been previously show to attenuate the ability of 5HT to activate ERK1/2 had no effects on the actin cytoskeleton (Figure 3.40). The effects of agents such as cytochalasin D and latrunculin B which are known to disrupt actin cytoskeleton morphology were also analysed. Latrunculin B mediates its effects by associating with actin monomers, preventing them from repolymerising and forming filaments (Morton et al., 2000; Spector et al., 1983). Cytochalasin D has a different mode of action disrupting the actin cytoskeleton by binding both barbed and pointed ends of actin filaments causing both depolymerisation and inhibition of polymerisation (Cooper, 1987; Spector et al., 1983). Figure 3.41 demonstrates the ability of both cytochalasin D and latrunculin B to disrupt the actin cytoskeleton in CCL-39 cells. The effect of these agents on 5HT-induced ERK activation was also investigated. Cytochalasin D was found to significantly

attenuate 5HT-induced ERK1/2 activation, with a reduction of around 35±19% at the maximal 5HT response ($p<0.05$, $n=3$) (Figure 3.42). Similarly, latrunculin B also significantly reduced 5HT-induced ERK1/2 activation. The maximal 5HT response was reduced in this case by 62±8% ($p<0.01$, $n=3$) (Figure 3.43).

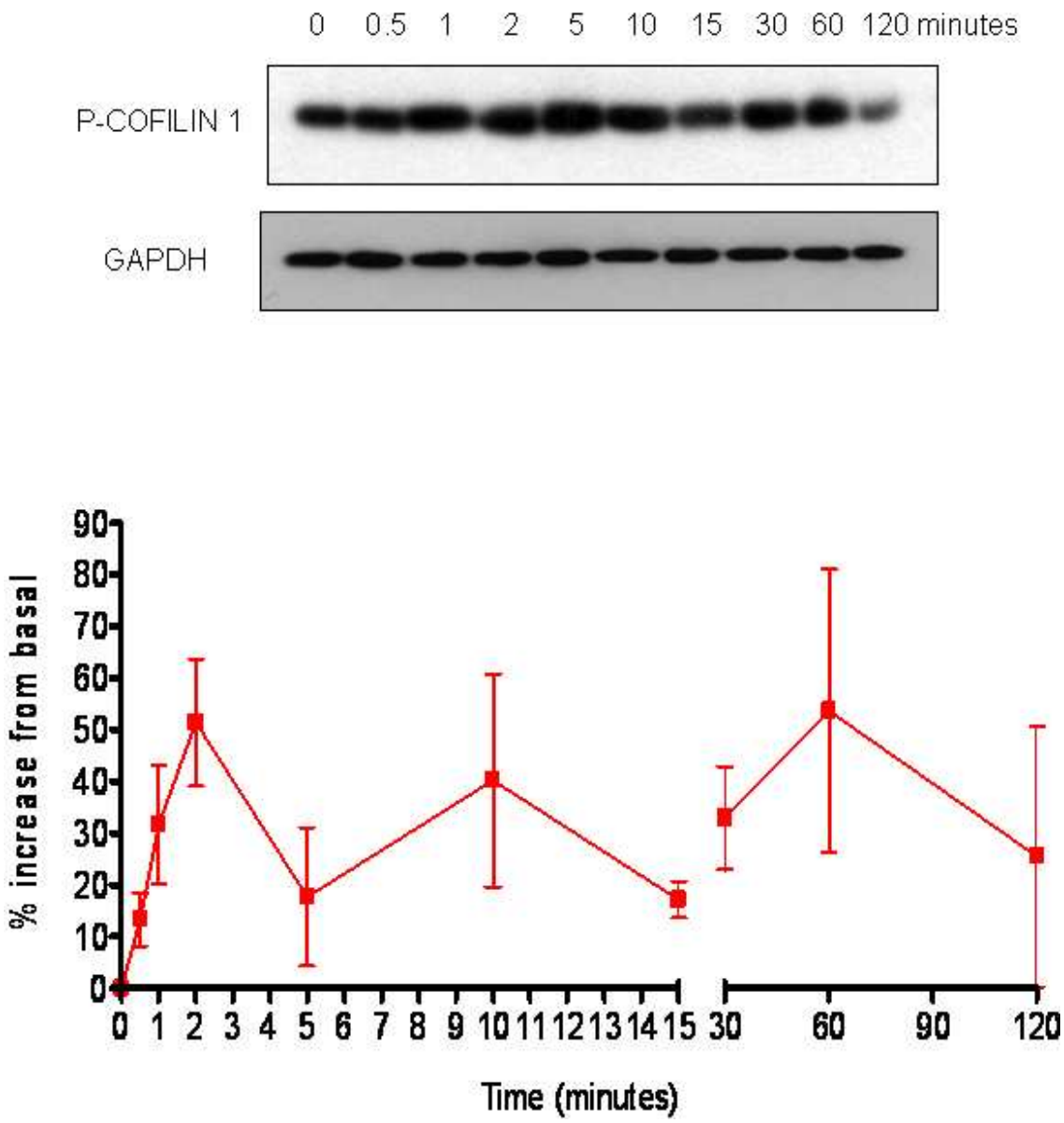

Figure 3.36 Timecourse of effects of 5HT on phosphorylation of cofilin 1.

Following serum starvation for 16 hours, CCL-39 cells were stimulated with 5HT (1µM) for 0-120 minutes. Cell lysates were prepared and equalised for protein concentration by BCA assay. Samples were then analysed by SDS-PAGE and immunoblotting using phospho-specific cofilin 1 and GAPDH antibodies. Blots shown are representative. Results are expressed as mean ± SEM (n=5). Statistical analysis using Newman-Keuls multiple comparison test revealed no significant differences.

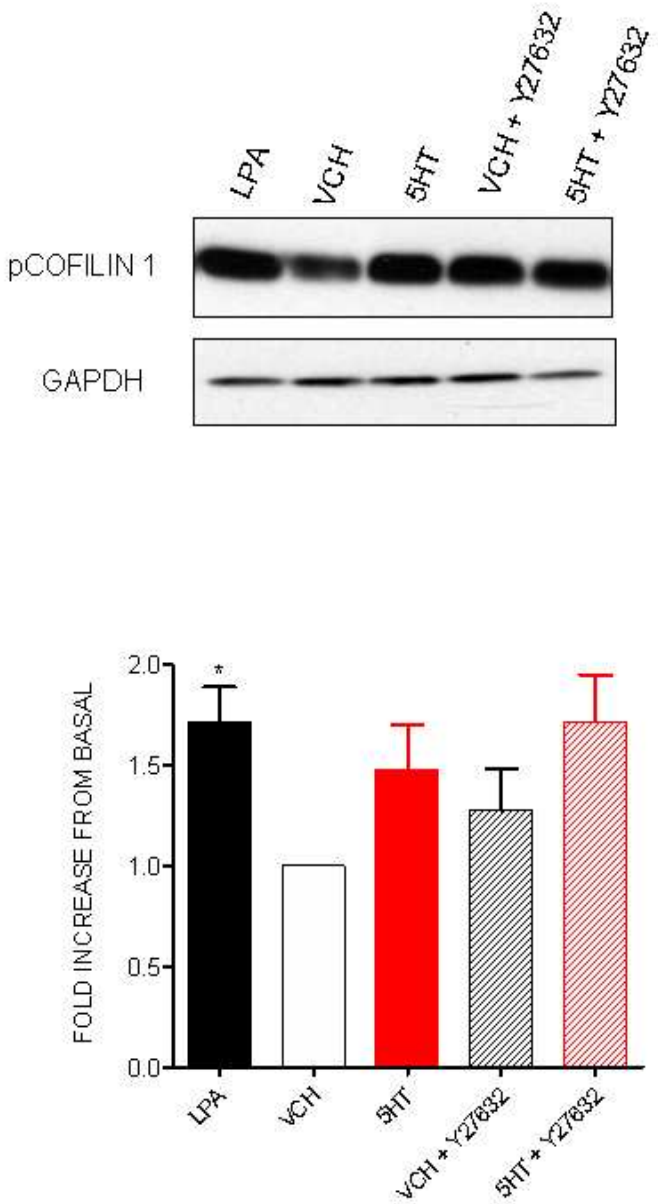

Figure 3.37 Effects of ROCK inhibition with Y27632 on cofilin 1 phosphorylation.

Following serum starvation for 16 hours cells were pre-treated for 30 minutes with either vehicle or Y27632 (5μM). Cells were then either treated with vehicle or 5HT (1μM) for 5 minutes. Treatment with LPA (100μM) for 15 minutes was used as a positive control. Cell lysates were prepared and equalised for protein concentration by BCA assay. Samples were then analysed by SDS-PAGE and immunoblotting using phospho-specific cofilin 1 and GAPDH antibodies. Blots shown are representative. Results are expressed as mean ± SEM (n=4). * $p < 0.05$ *versus* vehicle using Newman-Keuls multiple comparison test.

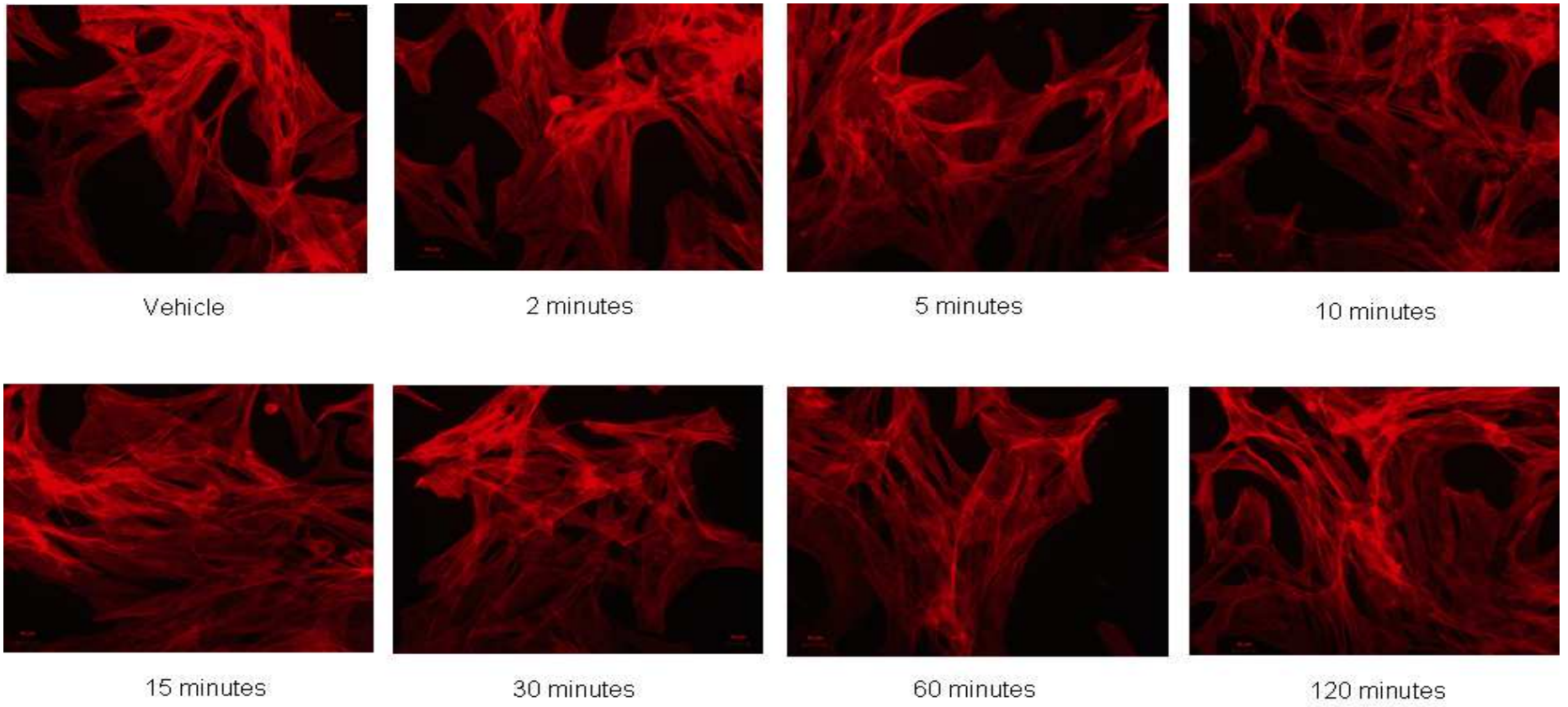

Figure 3.38 Timecourse of effects of 5HT on actin stress fibre formation in CCL-39 cells.

CCL-39 cells were serum starved for 16 hours then stimulated with 5HT (1µM) for 0-120 minutes. Cells were then fixed with 4% paraformaldehyde and the actin cytoskeleton detected by immunofluorescence using Alexa Fluor 594 conjugated-phalloidin. No changes in the actin cytoskeleton were detected following treatment with 5HT. Images were captured using a Ziess fluorescent microscope using x 40 objective lens. Pictures shown are representative of n=3 experiments. Scale bar represents 100µm.

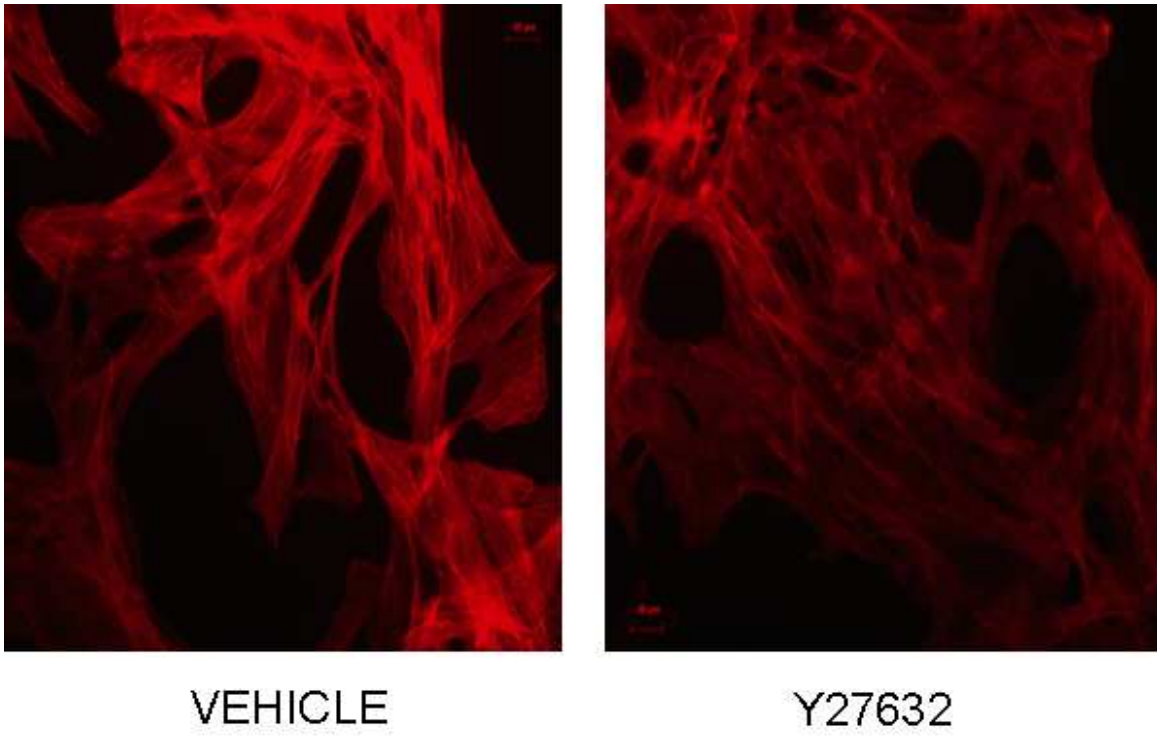

Figure 3.39 Effects of ROCK inhibition with Y27632 on actin stress fibres in CCL-39 cells.

CCL-39 cells serum starved for 16 hours then treated with either vehicle or Y27632 (5µM) for 30 minutes. Subsequently, cells were fixed with 4% paraformaldehyde and the actin cytoskeleton detected by immunofluorescence using Alexa Fluor 594-conjugated phalloidin. Treatment with Y27632 was observed to disrupt the actin cytoskeleton. Images were captured using a Ziess fluorescent microscope using x 40 objective lens. Pictures shown are representative of n=3 experiments. Scale bar represents 100µm.

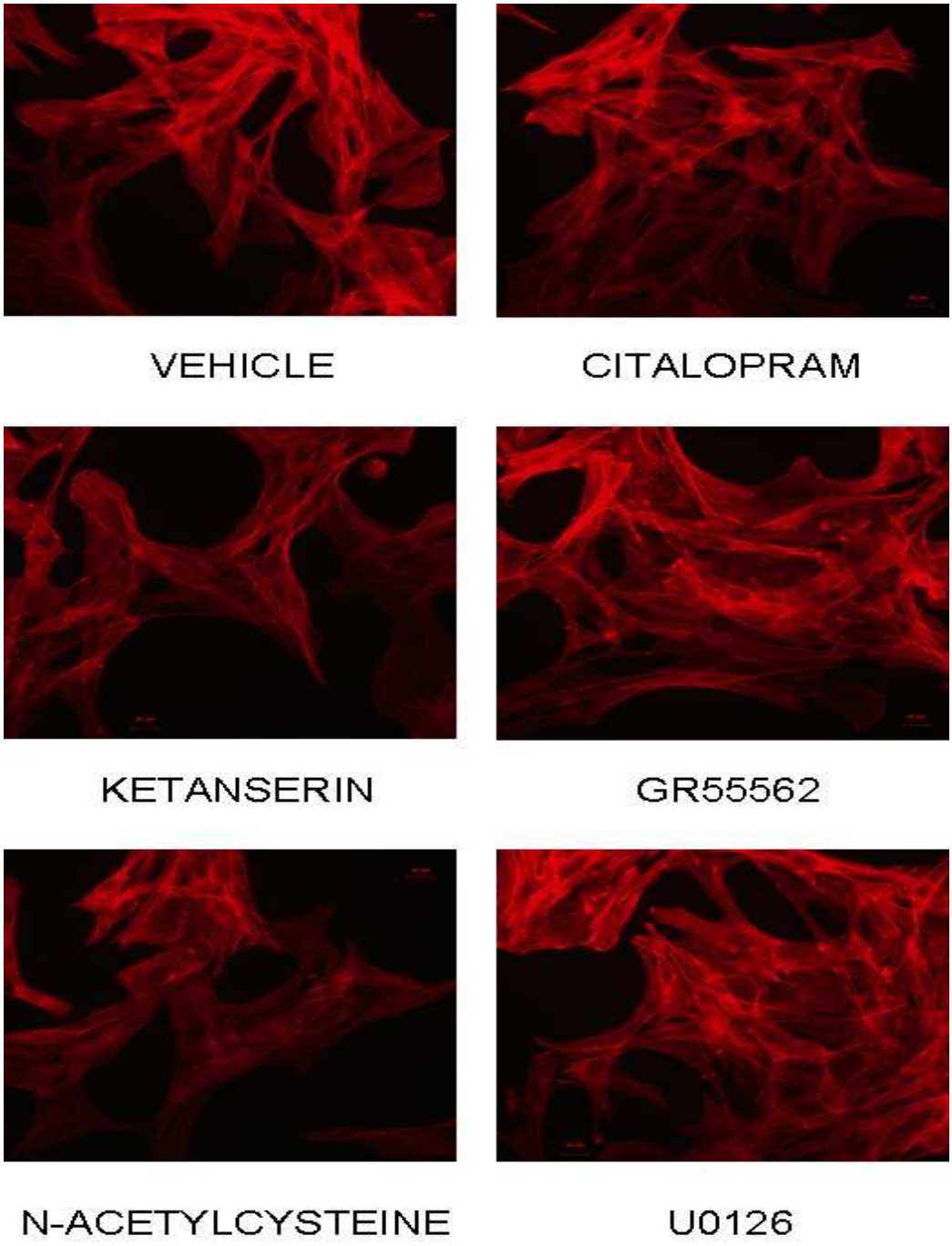

Figure 3.40 Effects of signal pathway inhibitors on actin stress fibres in CCL-39 cells.

CCL-39 cells were serum starved for 16 hours then treated with either citalopram (1μM), ketanserin (1μM), GR55562 (1μM) for 1 hour or N-acteylcysteine (0.1mM), U0126 (1μM) for 30 minutes. Subsequently, cells were fixed with 4% paraformaldehyde and the actin cytoskeleton detected by immunofluorescence using Alexa Fluor 594-conjugated phalloidin. No changes in the actin cytoskeleton were detected following treatment with inhibitors. Images were captured using a Ziess fluorescent microscope using x 40 objective lens. Pictures shown are representative of n=3 experiments. Scale bar represents 100μm.

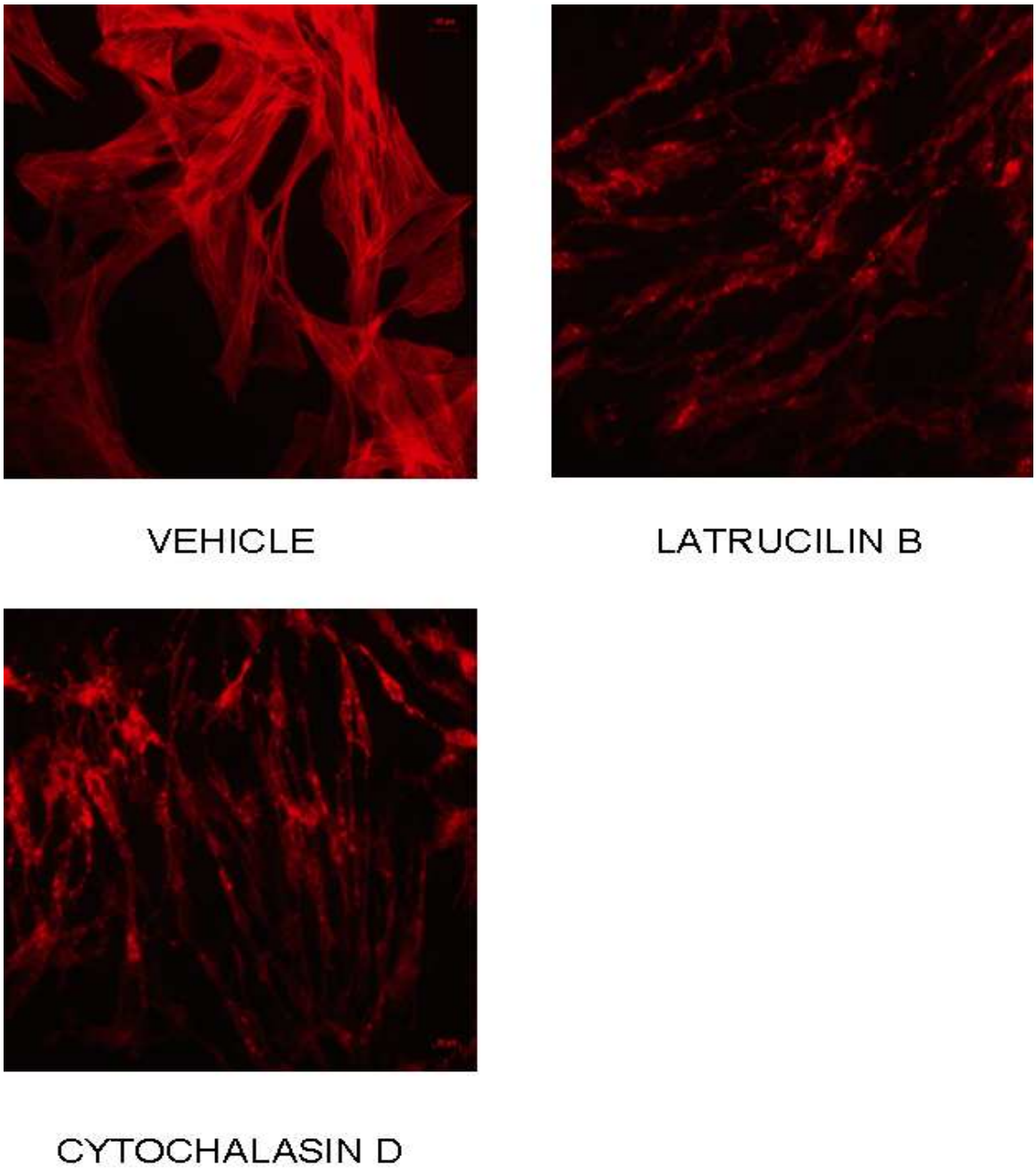

Figure 3.41 Effects of Cytochalasin D and Latrunculin B on the actin cytoskeleton of CCL-39 cells.

After serum starvation for 16 hours CCL-39 cells were pre-treated for 30 minutes with either latrunculin B (10µM) or cytochalasin D (10µM). Subsequently, cells were fixed with 4% paraformaldehyde and the actin cytoskeleton detected by immunofluorescence using Alexa Fluor 594-conjugated phalloidin. Images were captured using a Ziess fluorescent microscope using x 40 objective lens. Treatment with latrunculin B or cytochalasin D was observed to disrupt the actin cytoskeleton. Pictures shown are representative of n=3 experiments. Scale bar represents 100 µm.

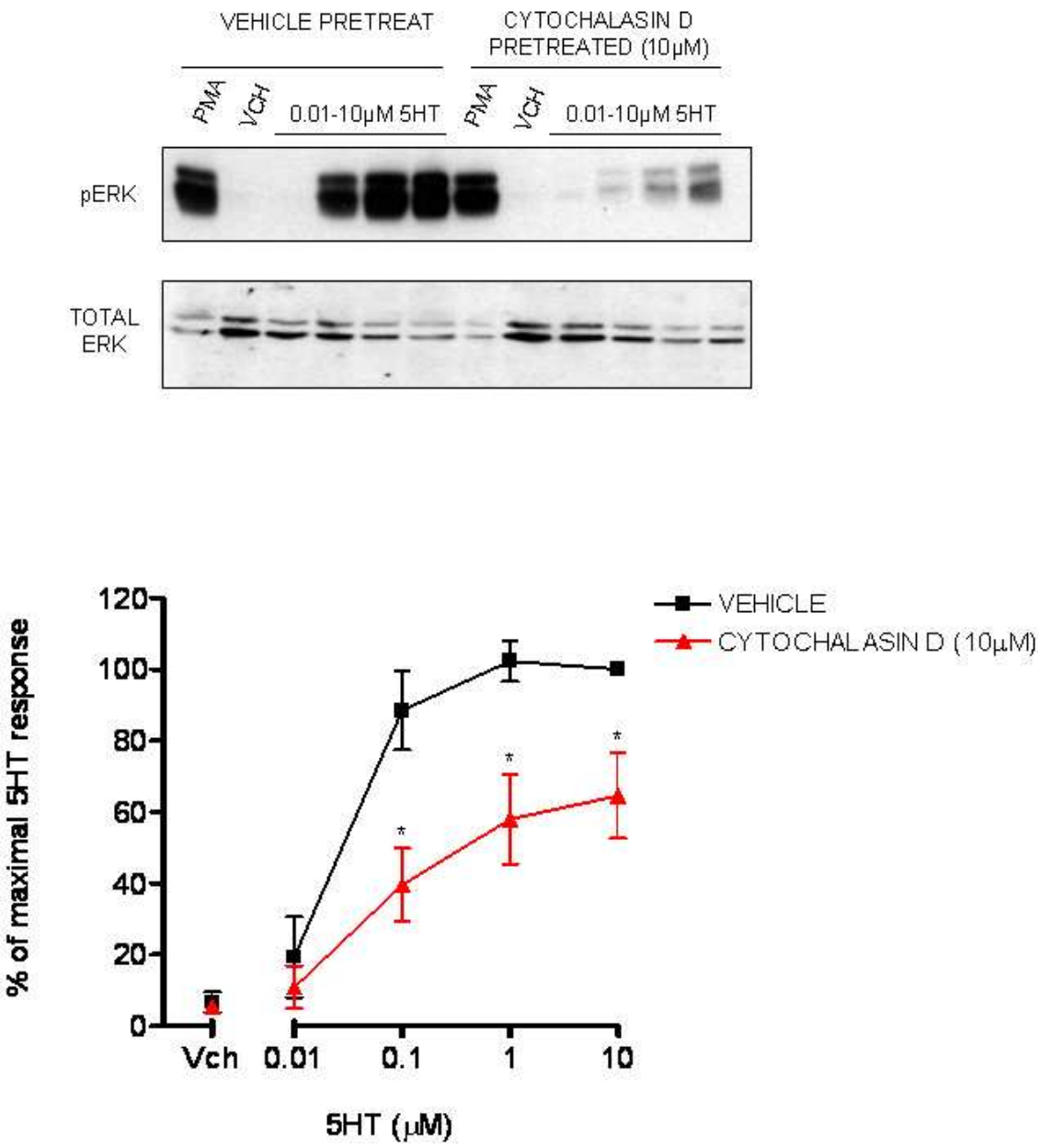

Figure 3.42 Effects of Cytochalasin D on 5HT-induced ERK activation.

After serum starvation for 16 hours CCL-39 cells were pre-treated for 30 minutes with cytochalasin D (10μM). Cells were then stimulated for 5 minutes with increasing concentrations of 5HT (0-10μM) or PMA (2μM). Cell lysates were prepared and equalised for protein concentration by BCA assay. Samples were then analysed by SDS-PAGE and immunoblotting using phospho-specific and total ERK antibody. Blots shown are representative. Results are expressed as mean ± SEM (n=3). * $p < 0.05$ *versus* vehicle pre-treated cells using an unpaired, two tailed t-test.

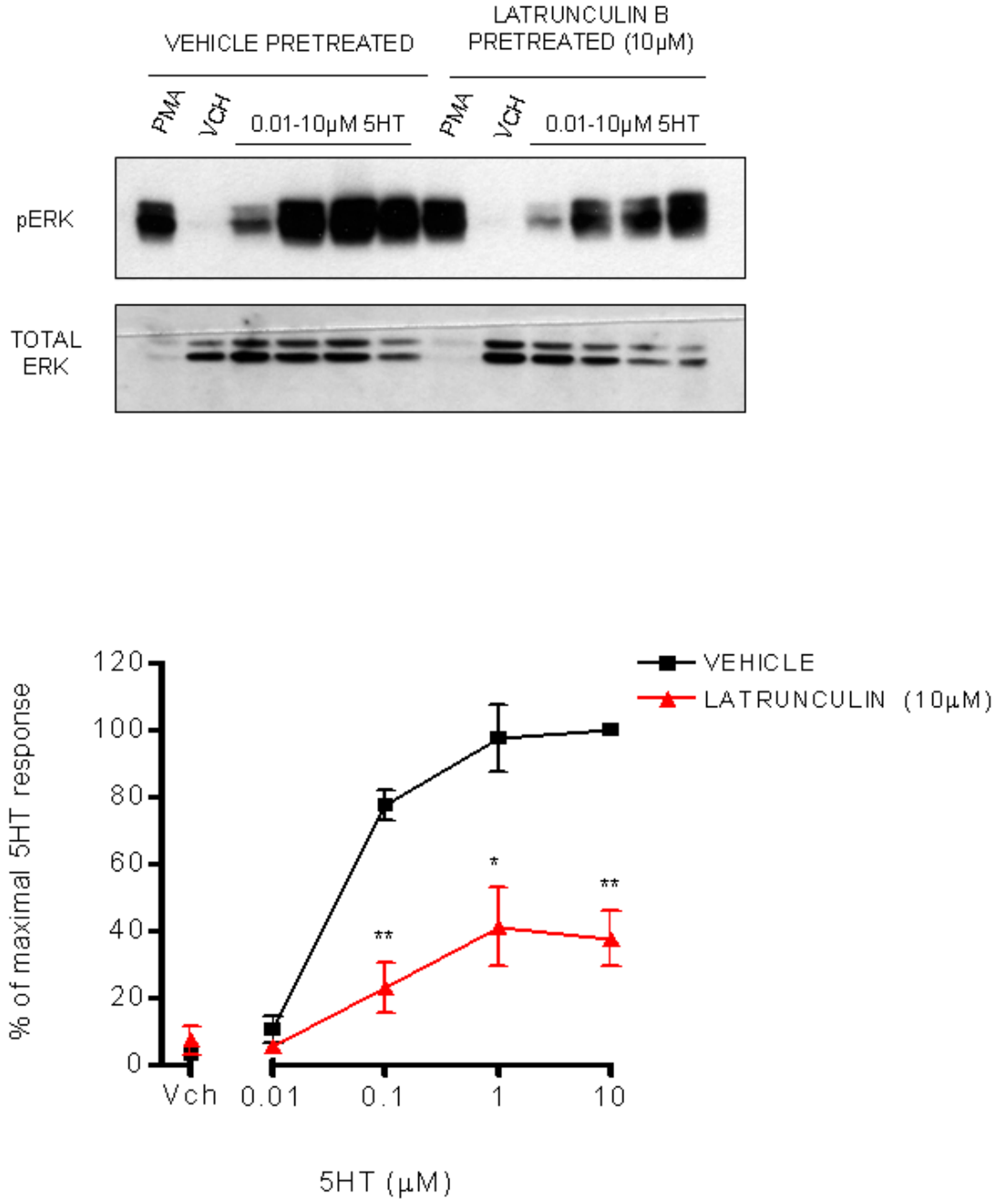

Figure 3.43 Effects of Latrunculin B on 5HT-induced ERK activation.

After serum starvation for 16 hours CCL-39 cells were pre-treated for 30 minutes with latrunculin B (10μM). Cells were then stimulated for 5 minutes with increasing concentrations of 5HT (0-10μM) or PMA (2μM). Cell lysates were prepared and equalised for protein concentration by BCA assay. Samples were then analysed by SDS-PAGE and immunoblotting using phospho-specific and total ERK antibodies. Blots shown are representative. Results are expressed as mean ± SEM (n=3). * $p < 0.05$, ** $p < 0.01$ *versus* vehicle pre-treated cells using an unpaired, two tailed t-test.

3.3 DISCUSSION

As stated in the introduction, the ability of 5HT to activate ERK MAP kinase plays a critical role in its mitogenic effects and as such may contribute to the remodelling process that occurs during PAH. Therefore, investigation into the signalling mechanisms utilised by 5HT may give rise to potential therapeutic targets for new drugs in the treatment of PAH. To further elucidate these signalling pathways, this study investigated potential mediators of the 5HT induced response and how these components contributed to 5HT-mediated ERK activation and proliferation.

Previous studies have characterised the rapid activation of ERK1/2 in PASMCs with maximal activation occurring around 5 minutes (Lee et al., 1999). This is consistent with the results shown in Figure 3.1, where 5HT is able to induce a rapid and transient activation of ERK1/2. Furthermore, in CCL-39 cells the mitogenic effects of 5HT appear to be dependent on its ability to activate ERK1/2 (Figure 3.2). Other studies have also confirmed the requirement for ERK1/2 activation in 5HT-induced proliferation (Bianchi et al., 2005; Lawrie et al., 2005). Interestingly, 5HT only resulted in an initial rapid and transient activation of ERK, with no further activation witnessed over the 24 hour period. Previous studies have indicated that in this cell type sustained MAP kinase activation is required for cyclin D1 induction and DNA synthesis (Balmanno and Cook, 1999). The study suggested agonists such as LPA and TRP-7, which caused only transient ERK activation, were poor inducers of proliferation. However, stimulation with thrombin, which induced a sustained activation of ERK, resulted in a proliferative response. Work carried out by other groups has also shown 5HT was unable to induce late phase ERK activation (Meloche et al., 1992) but still induced proliferation in CCL-39 cells (Pouyssegur et al., 1988; Lee et al., 1999).

The role of p38 in 5HT-mediated signalling remains unclear. It has previously been shown that 5HT is able to activate p38 *via* the $5HT_{2A}$ receptor under hypoxic conditions (Welsh et al., 2004). Furthermore, in NIH3T3 cells overexpressing the $5HT_{2A}$ receptor, 5HT was shown to activate both ERK1/2 and p38 (Kurrasch-Orbaugh et al., 2003). In human astrocytoma cells, 5HT has also been reported to activate p38 *via* the $5HT_7$ receptor (Lieb et al., 2005). In

contrast to these findings, 5HT was unable to induce activation of p38 in CCL-39 cells (Figure 3.4). This is consistent with findings in both bovine PASMCs and in rat vascular SMCs where 5HT had no effect on the activation status of p38 (Banes et al., 2001; Lee et al., 2001). Despite being unable to activate p38, inhibition of p38 resulted in a marked reduction in 5HT induced proliferation in CCL-39s (Figure 3.5). This phenonenom has also been witnessed in PASMCs suggesting p38 may act as a negative regulator of 5HT-induced proliferation (Lee et al., 2001). However, in the present study, the concentration of SB203580 that was effective in abolishing proliferation also significantly attenuated the activation of ERK by PMA (Figure 3.7). This suggests the ERK cascade in general may be affected, thus explaining the effects of SB203580 on 5HT-induced proliferation. In addition to this, inhibition of p38 has been reported to decrease 5HTT activity and 5HT uptake (Samuvel et al., 2005). The effects of p38 inhibition on 5HTT described in this study may contribute to reduction in proliferation observed in Figure 3.5.

CCL-39 cells have been shown to uptake 5HT, a process which can be blocked by various inhibitors of 5HTT (Lee et al., 1999), confirming the presence of functional 5HTTs in this cell type. By using pharmacological approaches, this study and that of others also suggested the presence of $5HT_{1B/1D}$ and $5HT_{2A}$ receptors (Lee et al., 1999). In the present investigation the role of 5HT receptors and transporter in 5HT-induced ERK activation were assessed. Inhibition of 5HTT (Figure 3.8), $5HT_{2A}$ (Figure 3.12) or $5HT_{1B/1D}$ receptors (Figure 3.14) all significantly reduced 5HT-mediated ERK activation. In addition to this, inhibition of anyone of these components resulted in almost complete abolition of the proliferative response. Furthermore, simultaneous inhibition of $5HT_{1/2}$ receptors and 5HTT did not have an additive effect, resulting in a 50% reduction in ERK1/2 activation, similar to that witnessed by inhibition of one component alone (Figure 3.16). These findings suggest 5HT receptors and 5HTT may co-operatively interact to mediate the effects of 5HT. Several examples of cross- talk between GPCRs have been documented. G_i-coupled receptors have been shown to amplify the effects of those coupled to G_q. For example, stimulation of $5HT_{1B}$ receptors augments SMC contraction induced by histamine (H_1) receptors or thromboxane A_2 in rat femoral arteries and iliac arteries (MacLennan et al., 1993; Yildiz and Tuncer, 1995). Furthermore, G_q-

coupled receptors have also been reported to augment G_s-coupled receptor-stimulated adenylyl cyclase activity (Selbie and Hill, 1998). In support of the hypothesis of cross-talk, functional interactions between $5HT_{1B}$ receptors and 5HTT have been reported to mediate vasoconstriction in small pulmonary arteries (Morecroft et al 2005). Interactions between 5HTT and 5HT receptors have also been demonstrated by the finding that chronic inhibition of 5HTT affects $5HT_{2A}$ receptor signalling (Damjanoska et al., 2003). Inhibition of 5HTT has also been shown to dampen 5HT receptor signalling in HEK293 cells (Johnson et al., 2003). Furthermore, stimulation of $5HT_{2A}$ receptors results in activation of PKC and increases levels of intracellular calcium (Rahimian and Hrdina, 1995), both of which are involved in the regulation of 5HTT (Ramamoorthy et al., 1998). In addition, inhibition of 5HTT has also been reported to upregulate $5HT_{2B}$ receptors in mouse astrocytes (Kong et al., 2002). The contribution of 5HTT and 5HT receptors in mediating mitogenic effects has been most extensively characterised in bovine PASMCs, where interactions between signalling pathways induced by receptors and transporter are required to produce cell proliferation. In this cell type, while 5HTT appears to be responsible for initiating signalling that results in ERK activation. $5HT_2$ and $5HT_{1B/1D}$ receptors are also required to perpetuate the proliferative response (Liu and Fanburg, 2006). However, in this case inhibition of 5HT receptors does not decrease ERK activation but modulates processes required for ERK to induce its mitogenic effects. In human PASMCs stimulation with 5HT appears to result in ERK activation *via* $5HT_{2A}$ receptor and not 5HTT (Lawrie et al., 2005). In these PASMCs, 5HTT also plays a role in mediating the effects of 5HT but is not involved in the activation of ERK. Taken together these findings suggest functional interactions between 5HT receptors and 5HTT and cross-talk between the signalling pathways induced in executing cellular mitogenesis. $5HT_{2A}$ receptors have previously been found to interact with caveolin-1 in different cell types, a process which modulates 5HT signalling (Bhatnagar et al., 2004; Cogolludo et al., 2006). Caveolin-1 forms an important component of caveolae, specialised plasma membrane microdomains that have been found to compartmentalize and integrate numerous signalling events including the MAP kinase cascade (Shaul and Anderson, 1998). 5HTT has also been reported to be partitioned into lipid microdomains in the plasma membrane. The

compartmentalisation of 5HT receptors and 5HTT into specialised areas of the membrane may facilitate their ability to cross-talk.

The role of internalisation in 5HT-induced ERK activation was also assessed. In certain circumstances clathrin-mediated endocytosis and caveolin have been implicated in the regulation of signalling from 5HT receptors and 5HTT (Bhatnagar et al., 2004; Della Rocca et al., 1999; Jayanthi et al., 2005). In this instance inhibition of internalisation using the structurally unrelated compounds ConA and MDC had little effect on the 5HT-induced ERK activation (Figures 3.17 and 3.18). ConA significantly increased ERK activity under basal conditions and a similar increase was also witnessed in response to 5HT, suggesting conA may activate ERK MAP kinase pathway in this cell type. The increased activity of ERK observed may be due to the binding of ConA to cell surface glycoproteins. For instance ConA has previously been shown to crosslink PZR glycoproteins, an immunoglobulin superfamily of cell surface proteins, resulting in their tyrosine phosphorylation (Zhao et al., 2002). In CCL-39 cells ConA may crosslink receptor tyrosine kinases, resulting in their activation and thus contributing to the elevated basal levels of ERK activation. No effects of 5HT-induced ERK activation were witnessed in response to MDC treatment suggesting internalisation from the plasma membrane is not a requirement for 5HT-induced ERK activation. In addition to this, MDC reversed the inhibitor effects of 5HT receptor antagonists (Figure 3.20) and the 5HTT blocker fluoxetine (Figure 3.19). This may be due to the ability of MDC to inhibit receptor desensitisation and down regulation of 5HTT by preventing receptor and 5HTT internalisation from the plasma membrane, thus potentiating the effects of 5HT. These findings suggest endocytosis is not a requirement for 5HT-induced ERK activation and proliferation. However, it would be beneficial to monitor receptor endocytosis directly in this model before drawing a final conclusion. Attempts were made to generate CCL39 cell lines stably overexpressing a green fluorescent protein (GFP)-tagged rat $5HT_{2A}$ receptor in order to monitor receptor internalisation. Cells transfected with $5HT_{2A}$-GFP receptor also confer resistance to the neomycin derivative G418, allowing selection of $5HT_{2A}$-GFP positive cells. Despite isolation of multiple G418-resistant clones over several separate transfections that were resistant to treatment with 1 mg/ml G418, only 5-10% of the cells expressed the GFP-

tagged receptor construct, as determined by fluorescence microscopy. Moreover, the $5HT_{2A}$-GFP cells were also refractory to further enrichment by fluorescence-activated cell sorting (FACS) (data not shown).

ROS have also been implicated in mediating the effects of 5HT. As stated previously, ROS appear to be a downstream mediator of 5HTT, required to induce ERK1/2 activation in PASMCs. In this study, N-acetyl-cysteine, an antioxidant, significantly reduced 5HT-induced ERK1/2 activation, suggesting ROS are also required in CCL-39 cells to form part of the mitogenic signalling response (Figure 3.21). This is consistent with previous studies that have reported the generation of superoxide in this cell type in response to stimulation of 5HT (Lee et al., 1999). The ability of ROS such as O^{2-} and H_2O_2 to activate ERK has been well documented. A number of growth factors have been shown to generate intracellular ROS production, a process that appears to be essential for their mitogenic signalling. For instance, PDGF stimulated increases in intracellular ROS are required for ERK activation, DNA synthesis and regulation of gene expression (Sundaresan et al., 1995). Furthermore, in mesangial cells, ROS generation *via* the $5HT_{2A}$ receptor is though to be responsible for ERK activation (Grewal et al., 1999). The mechanisms by which ROS regulate ERK activation remain unclear, although it has been proposed they are able to regulate the activity of phosphatases responsible for modulating ERK activity (Kim et al., 2003).

ROS have also been shown to play a critical role in cardiac hypertrophy (Bianchi et al., 2005) and a variety of other vascular diseases (Yung et al., 2006). In cardiomyocytes, entry of 5HT into the cell *via* 5HTT is thought to result in the formation of H_2O_2 due to its breakdown by MAO. The reactive oxygen species formed are then responsible for ERK activation and subsequent hypertrophy (Bianchi et al., 2005). In the present study, inhibition of MAO had no effect on 5HT-mediated ERK activation, suggesting production of ROS by MAO has no role in the proliferative response in CCL-39 cells. This is similar to findings in PASMCs (Lee et al., 1999). Another study has suggested that ROS generation by MAO is not required for ERK activation, but does play a role in mediating the nuclear translocation of pERK (Lawrie et al., 2005). However, given that inhibition of MAO had no effect on 5HT-induced proliferation this mechanism is unlikely to occur in CCL-39 cells.

The RhoA/ROCK pathway has also been implicated in the actions of 5HT. 5HT has been shown to activate RhoA in rat aortic rings (Sakurada et al., 2001). Furthermore, RhoA/ROCK appears to be involved in 5HT-mediated contractions in bovine cerebral arteries (Nishikawa et al., 2003). In addition to its role in contraction, ROCK may also mediate mitogenic effects and has been suggested to play a role in PDGF BB-induced proliferation in systemic vascular SMCs (Chapados et al., 2006). Based on data presented here, ROCK also appears to be involved in mediating 5HT-induced proliferation in CCL-39 cells (Figure 3.25). This effect may be due to the ability of the Rho/ROCK pathway to modulate 5HT-induced ERK activation as inhibition of both Rho (Figure 3.27) and ROCK (Figure 3.26) significantly reduce 5HT-induced ERK activation. This effect appears to be specific to 5HT as inhibition of ROCK has no effect on PMA-induced ERK activation. Studies in human aortic SMCs have also shown the inhibition of ROCK attenuates 5HT-induced ERK activation (Matsusaka and Wakabayashi, 2005b). Conversly, sustained activation of ROCK has been found to promote ERK activation in NIH-3T3 fibroblasts (Croft and Olson, 2006). ROCK also plays a role in ERK activation induced by angiotensin II in mesenteric resistance arteries (Matrougui et al., 2001). However, in these studies the mechanisms by which inhibition of ROCK modulates ERK activation were not addressed.

The requirement of ROCK for 5HT-induced proliferation has been shown previously in PASMCs (Liu et al., 2004). However in that study inhibition of ROCK had no effect on ERK phosphorylation, but was required to mediate the translocation of active ERK to the nucleus to stimulate the transcription factor phosphorylation required for cell cycle progression and cellular proliferation. Thus, the role of ROCK in nuclear translocation was also investigated in CCL-39 cells. These experiments suggested that ROCK attenuates the phosphorylation of ERK in the cytoplasm but has no effect on the ability of pERK to translocate to the nucleus (Figure 3.33). In addition to this, inhibition of ROCK was also able to completely abolish 5HT-induced cyclin D1 expression (Figure 3.29), consistent with findings by Liu et al 2004. Rho has previously been associated with normal cell cycle progression and expression of cyclin D1 (Welsh et al., 2001b; Jaffe and Hall, 2005). Moreover, ROCK is also involved in the regulation of the cyclin D1-cdk4 complex *via* ERK-dependent cyclin D1

expression (Roovers and Assoian, 2003). The current study would suggest that cytoplasmic effectors of ERK are responsible for mediating 5HT-induced proliferation. It has previously been reported that in PC12 cells proliferative signals cause a transient activation of ERK which remains mainly in the cytoplasm (Marshall, 1995).

The RhoA/ROCK pathway may interact with components of the ERK MAPK cascade upstream of ERK thus explaining the effects of RhoA/ROCK inhibition on 5HT-induced ERK activation. However in CCL-39 cells inhibition of ROCK had no effect on 5HT-mediated MEK activation (Figure 3.32). This suggests the Rho/ROCK pathway regulates the phosphorylation status of ERK independently of MEK its upstream kinase. Various studies have highlighted interactions between the RhoA/ROCK pathway and members of the Ras/Raf/MEK/ERK signalling pathway. Rho-GTPases have been shown play a role in Ras mediated Raf activation (Li et al., 2001). Furthermore Raf-1 appears to operate as a regulator of Rho downstream signalling associating with ROCK II *via* its amino-terminus in primary mouse keratinocytes (Ehrenreiter et al., 2005). This interaction controls the subcellular distribution of ROCK II and limits its activation. In mouse embryonic fibroblasts where the BRAF gene has been knocked out, levels of ROCK II and pERK where decreased (Pritchard et al., 2004). In other cell types active Rho has been shown to bind to MEKK1 and stimulate its kinase activity (Gallagher et al., 2004). MEKK1, an upstream kinase in the JNK and p38 pathways is known to bind and function as a scaffold for ERK (Morrison and Davis, 2003). Mechanisms as to how interactions between the Rho/ROCK pathway and ERK MAP kinase pathway mediate 5HT-induced proliferation remain unclear. It is possible that scaffolding proteins which interact with the MEK/ERK pathway may mediate cross talk between the pathways. It is well documented that scaffolding proteins target MEK/ERK complexes to specific cellular location and affect the activity of specific components of the signalling cascade (Kolch, 2005).

Phosphatases play an important role in the regulation of ERK MAP kinases. It is possible that in CCL-39 cells the Rho/ROCK pathway may modulate phosphatases involved in the regulation of the ERK phosphorylation and as such, affect its activation in response to 5HT. Using vanadate as a non-specific tyrosine phosphatase inhibitor had no effect on ability of the ROCK inhibitor to

attenuate 5HT-induced ERK activation, suggesting phosphatases are not involved in mediating the effects of ROCK (Figure 3.34). Treatment with vanadate alone elevates basal activity of ERK presumably by inhibiting DUSPs, which are responsible for maintaining ERK activation status under basal conditions. This effect may mask any alterations in ERK phosphorylation induced by vanadate in the presence of the ROCK inhibitor. Therefore the ability of ROCK to modulate phophatases involved in 5HT-induced ERK activation cannot be ruled out. It may be beneficial to investigate the role of phosphatase by other means such as using inhibitors to specific phosphatases or monitoring ERK-specific phosphatase activity within the cells.

It has previously been reported that 5HT modulates the Rho/ROCK pathway *via* the $5HT_{1B/1D}$ receptor (Liu et al., 2004). Using selective agonists, this was also shown to be the case in CCL-39 cells (Figure 3.30), with $5HT_{1B/1D}$ receptors involved in mediating the effects of ROCK but not $5HT_2$ receptors. Interestingly, using 5HTT blockers to ensure the effects of 5HT were mediated *via* 5HT receptors, ROCK inhibition had no further effect in reducing 5HT-receptor mediated ERK activation (Figure 3.31). This implies ROCK is not involved downstream of 5HT receptors present in CCL-39 cells. Furthermore, using 5HT receptor antagonists to ensure 5HT acts preferentially *via* 5HTT, inhibition of ROCK had no further effect on the reduction in ERK activation, suggesting ROCK does not function downstream of 5HTT.

To try and address this issue further, the ability of 5HT to activate the Rho/ROCK pathway was assessed. Prior studies have shown the ability of 5HT to activate this pathway in a variety of tissues and cell types including PASMCs (Liu et al., 2004), and aortic SMCs (Matsusaka and Wakabayashi, 2005b).

In PASMCs 5HT has been shown to activate Rho and its downstream effector MYPT1. However, in CCL-39 cells, following stimulation with 5HT no activation of MYPT1 (Figure 3.35) was observed. This may be due to the high basal level of MYPT1 phosphorylation present. 5HT did however elevate levels of p-cofilin, another downstream mediator of ROCK (Figure 3.36). Although, this response was consistently observed, due to high basal levels and variability it was not found to be statistically significant. Furthermore, this variable phosphorylation could not be attenuated by inhibition of ROCK (Figure 3.37). LIMK is the direct upstream mediator of cofilin and can be activated *via* the Rac/PAK pathway.

This may explain why inhibition of ROCK is unable to prevent the phosphorylation of cofilin. In addition to this, under quiescent conditions CCL-39 cell displayed a high degree of actin stress fibres. Stimulation with 5HT resulted in no notable change in the presence of these fibres (Figure 3.38). These findings are in contrast to a study in PASMCs, where application of 5HT was shown to cause disruption of the actin cytoskeleton (Day et al., 2006), highlighting the differences in 5HT signalling pathways between different cell types. Furthermore the high levels of pMYPT1, p-cofilin and presence of actin stress fibres observed in unstimulated CCL-39 cells suggests a high degree of basal Rho activity, making any potential increase in the activity of the Rho/ROCK pathway induced by 5HT difficult to quantify. In the future it may be valuable to use other methods, such as a GST-fusion proteins containing domains derived from Rho target proteins (e.g. GST-tagged rhotekin-RBD protein) in order to detect the effects of 5HT on the Rho/ROCK pathway.

Figure 3.39 shows the ability of the ROCK inhibitor to disrupt the presence of actin stress fibres in CCL-39 cells. Given the effects of ROCK inhibition on actin stress fibre formation it was hypothesised that the actin cytoskeleton may play a crucial role in the regulation of 5HT-induced ERK activation. Others have reported the role of the cytoskeleton in mediating ERK activity. For example, disruption of the actin cytoskeleton by cytochalasin D has been shown to prevent Raf activation by inhibiting its translocation to the plasma membrane in response to GTP loading of Ras (Krepinsky et al., 2005). The cytoskeleton also plays a role in mediating the translocation of active ERK to the nucleus. For instance, disruption of the actin cytoskeleton has been shown to prevent stretch-induced nuclear localisation of ERK in ventricular myocytes, a process that can be restored by agents such as jasplakinolide, which cause actin polymerisation (Kawamura et al., 2003). ROCK and the actin cytoskeleton are also involved in mediating pressure-induced ERK activation and phosphorylation in mesenteric arteries (Matrougui et al., 2001). In order to address whether inhibition of 5HT receptors or 5HTT resulted in any change in the cytoskeleton and thus resulted in a reduction in ERK activation, actin stress fibres were assessed. None of the antagonists or inhibitors used previously had any notable effect on the presence or distribution of actin stress fibres within the cells, suggesting the ability of these inhibitors to attenuate 5HT-induced ERK

activation is not *via* modulation of the actin cytoskeleton (Figure 3.40). However, treatment with cytochalasin D and latrunculin B, agents that disrupt the actin cytoskeleton by distinct mechanisms, resulted in a significant reduction in 5HT-mediated ERK activation but had no effect on ERK activation induced by treatment with PMA (Figure 3.42 and 3.43) suggesting these findings are specific to 5HT. Disruption of the actin cytoskeleton using agents such as cytochalasin D has previously been suggested to inhibit internalisation from the plasma membrane. However, neither MDC nor con A, agents that also inhibit internalisation were able to attenuate ERK activation, suggesting that the effects of cytochalasin D are due to its ability to disrupt the actin cytoskeleton alone. These results imply that in this cell type the actin cytoskeleton, and ROCK in particular, play a crucial role in mediating 5HT-induced ERK activation. The role of the actin cytoskeleton in meditating the distribution of signalling components within the cell may be key in the ability of 5HT to activate ERK. As mentioned previously ROCK and the actin cytoskeleton have been reported to mediate the translocation of various components of the ERK cascade and thus regulate its activity (Krepinsky et al., 2005; Kawamura et al., 2003, Liu et al., 2004). Interestingly, stress fibre formation can also directly regulate gene expression of a subset of serum response factor-dependent genes (Copeland and Treisman, 2002; Geneste et al., 2002; Gineitis and Treisman, 2001).

5HT has previously been shown to induce reorganisation of vimentin filaments in tracheal SMCs (Tang et al., 2005). It may therefore be beneficial to determine the role of other components of the actin cytoskeleton, such as microtubules and intermediate fibres in mediating the effects of 5HT. The PAK/LIMK pathway is also involved in regulation of the cytoskeleton. Given the apparent importance of the actin cytoskeleton in mediating the effects of 5HT, it may prove useful to investigate the potential role of this pathway in the process. LIMK has already been implicated in the expression of cyclin D1 (Roovers et al., 2003) and PAK is thought to be involved in the 5HT-mediated reorganisation of vimentin (Tang et al., 2005).

In CCL-39 cells, entry of 5HT into the cell *via* 5HTT appears to be required to mediate its proliferative effects. What is unclear however, is where 5HT acts once inside the cell. As stated previously, studies suggest intracellular 5HT may be broken down by MAO to produce ROS, which are involved in ERK

activation and thus contribute to cellular proliferation. This appears not to be the case in this cell type as inhibition of MAO is unable to modulate 5HT-induced ERK activation. One other possibility is that once inside the cell 5HT acts as a "second messenger" modulating proliferative signalling pathways. One group has suggested that once inside the cell 5HT is transamidated to Rho rendering it constitutively GTP-bound and active (Walther et al., 2003; Guilluy et al., 2007). Other molecules such as spingosine-1- phosphate (S1P) have been shown to regulate ERK1/2 activity *via* both its extracellular and intracellular actions (Goodemote et al., 1995). Thus, it is possible that inside the cell 5HT may activate intracellular receptors which form part of a 5HT signal transduction pathway. Indeed, several GPCRs have been detected intracellularly and are thought to function in mediating intracellular signalling. For instance the LPA_1 receptor has been reported to be constitutively expressed in the nucleus of several mammalian cell types where it participates in the intracellular signalling of LPA and regulates protein phosphorylation (Waters et al., 2006). If this is the case for 5HT, our data would suggest that ROCK may be important in maintaining the intracellular localisation of these receptors.

It may also prove fruitful to further investigate the role of ROS in this system. ROS have previously been shown to be involved in the activation of the Rho/ROCK pathway (Jin et al., 2004). Rho is also thought to contribute to the formation of superoxide in certain cells (Kim et al., 2004). Given the requirement of ROS in 5HT-mediated ERK activation, investigations into the effects of ROCK inhibition on ROS production may shed some light on the mechanisms by which ROCK regulates the 5HT response. It has previously been shown that H_2O_2 inhibits phosphatases PP1 and PP2A resulting in increased ERK phosphorylation (Kim et al., 2003). Therefore, if the Rho/ROCK pathway *via* stimulation with 5HT is responsible for ROS generation in CCL-39 cells, its inhibition may affect the activity of phosphatases responsible for activating ERK thus attenuating 5HT-incuded ERK activation.

To summarise, $5HT_{1B/1D}$, $5HT_{2A}$ receptors and 5HTT all appear to play a role in mediating 5HT-induced ERK activation and proliferation, suggesting cross-talk between receptor and transporter signalling pathways. ROCK also plays a role in mediating the mitogenic effects of 5HT *via* the $5HT_{1B/1D}$ receptor. However, 5HT does not appear to directly activate the Rho/ROCK pathway, at least not

within the means of detection used in this study. The ability of ROCK to modulate cytoskeletal dynamics appears to be pivotal in its ability to modulate 5HT-induced ERK activation.

Chapter 4

Characterisation of the Effects of ROCK Inhibition on an *In Vivo* Model of Pulmonary Hypertension.

4.1 INTRODUCTION

As described previously, ROCK is a major downstream effector of the small GTP-ase Rho. Its functions include: the dynamic regulation of cytoskeletal proteins, cell migration, proliferation and smooth muscle contraction. Dysregulation of the Rho/ROCK pathway has also been implicated in numerous pathophysiological processes in the cardiovascular system such as atherosclerosis (Mallat et al., 2003), angina (Masumoto et al., 2002; Mohri et al., 2003), myocardial infarction (Satoh et al., 2003), restenosis (Sawada et al., 2000) and hypertension (Kobayashi et al., 2002).

RhoA has been found to play a particularly important role in the hypertensive process in several *in vivo* models. For instance, increased expression and activity of RhoA has been observed in vascular SMCs (Seko et al., 2003), in addition to the implication of Rho-dependent signaling pathways, in resistance artery remodeling occurring during hypertensive process (Mukai et al., 2001; Wesselman et al., 2004). Moreover, ROCK is thought to mediate calcium sensitization in VSMCs, a process involved in the pathobiology of hypertension (Uehata et al., 1997). Furthermore, in spontaneously hypertensive rats, upregulation of ROCK was found to precede the development of hypertension (Mukai et al., 2001). ROCK has also been shown to be involved in hypertension in humans (Masumoto et al., 2001).

A growing body of evidence also exists highlighting the role of the Rho/ROCK pathway in the pulmonary circulation. Pulmonary artery endothelial cells from pulmonary hypertensive piglets have been shown to display increased RhoA activity (Wojciak-Stothard et al., 2006). In addition to this, RhoA activity and ROCK 1 protein expression are each elevated in mildly hypoxic fawn hooded rats compared to those maintained at sea level (Nagaoka et al., 2005), with similar findings also reported in chronically hypoxic rats (Hyvelin et al., 2005). The activities of RhoA and ROCK have also been reported to be elevated by high flow-induced PAH (Li et al., 2007). Moreover, exposure of rat PASMCs to hypoxia has also been shown to significantly increase ROCK activity (Wang et al., 2001).

ROCK appears to play a dual role in the pathophysiology of pulmonary hypertension contributing to both vasoconstriction and vascular remodeling. Several laboratories have reported the vasodilatory properties of ROCK inhibition on hypoxia-induced vasoconstriction in pulmonary vessels. In intrapulmonary vascular rings maintained in hypoxic conditions, slow sustained contraction was markedly reduced by treatment with Y27632, a ROCK inhibitor (Wang et al., 2001; Robertson et al., 2000a). Furthermore, studies carried out in rats suggest that ROCK-mediated calcium sensitization plays a pivitol role in the increased basal pulmonary artery tone and sustained vasoconstriction observed in pulmonary vessels during chronic hypoxia (Nagaoka et al., 2004). ROCK-mediated vasoconstriction is also thought to be an important component of severe occlusive PAH (Oka et al., 2007).

In addition to this, chronic treatment with ROCK inhibitors has also been shown to reduce elevated pulmonary artery pressure in models of pulmonary hypertension. Administration of fusadil reduced pulmonary artery pressures in chronic hypoxic (Nagaoka et al., 2005), high flow-induced (Li et al., 2007) and monocrotaline-induced animal models of pulmonary hypertension (Abe et al., 2004). Moreover, Y27632 had similar effects, significantly reducing pulmonary artery pressures in the chronic hypoxic rat (Hyvelin et al., 2005). ROCK may also couple vasoconstriction with vascular remodeling in pulmonary arteries by contributing to matrix synthesis in vascular smooth muscle cells (Chapados et al., 2006). Indeed, the beneficial effects of ROCK inhibition on RVH and pulmonary vascular remodeling have been described. For example, administration of fusadil was found to decrease RVH and medial pulmonary artery wall thickness in *in vivo* models (Nagaoka et al., 2005; Abe et al., 2004; Li et al., 2007). In addition to this, the potentially beneficial effects of sildenifil (Sauzeau et al., 2003; Guilluy et al., 2005) and statins (Girgis et al., 2007; Xing et al., 2006) in the treatment of PAH may be partially owing to the ability of these drugs to inhibit the RhoA/ROCK pathway.

The results discussed previously in chapter 3 suggest ROCK as a possible downstream mediator of 5HT in its proliferative response. Given the pivotal role of 5HT and 5HTT in the development of pulmonary hypertension (Eddahibi et al., 2001) and the apparent importance of ROCK, any cross-talk between these systems *in vivo* were investigated. Specifically, in this chapter, the effects of

ROCK inhibition on chronic hypoxia-induced pulmonary hypertension in WT and $5HTT^{+}$ mice were assessed.

4.2 RESULTS

Initially, relative expression levels of ROCK 1 and ROCK 2 transcripts in whole lung tissue from WT and $5HTT^+$ under both normoxic and hypoxic conditions were assessed. In the case of ROCK 1, under normoxic conditions WT and $5HTT^+$ mice display similar levels of expression (Figure 4.1). Hypoxia resulted in the elevation of ROCK 1 transcript levels in WT and $5HTT^+$ mice (WT: 359±21% increase, $p<0.05$, n=5. $5HTT^+$: 1041±27%, $p<0.01$, n=5), with the increase more pronounced in $5HTT^+$ mice. Furthermore, under normoxic conditions relative levels of ROCK 2 are similar in both WT and $5HTT^+$ mice (Figure 4.2). Exposure to chronic hypoxia on the other hand caused a significant increase in ROCK 2 levels in both WT (600±28% increase from normoxic, $p<0.01$, n=6) and $5HTT^+$ mice (863±37% increase from normoxic, $p<0.05$, n=6) resulting in similar levels of transcript expression. This suggests that overexpression of 5HTT enhances the hypoxia-induced transcription of ROCK 1.

The effects of ROCK inhibition on RVP in WT and $5HTT^+$ mice maintained in both normoxic and hypoxic conditions were assessed. Hypoxia was found to cause a significant elevation in mRVP in WT and $5HTT^+$ mice (WT: $p<0.01$, n=5 $5HTT^+$: $p<0.05$, n=5) (Figure 4.3). Under normoxic conditions $5HTT^+$ mice exhibited higher mRVP than WT mice (WT: 12.5±1.5mmHg, $5HTT^+$: 16.08±3mmHg). Furthermore, inhibition of ROCK had no effect on mRVP in normoxic WT mice (n=6); it did however reduce the elevated mRVP in normoxic $5HTT^+$ mice ($p<0.05$, n=7). In addition to this, under hypoxic conditions, administration of Y27632 attenuated the elevated mRVP seen in WT mice ($p<0.05$, n=8). Moreover, when administered to $5HTT^+$ mice in hypoxic conditions, Y27632 reduced mRVP ($p<0.001$, n=8) resulting in pressures similar to than seen in normoxic vehicle-dosed WT mice ($5HTT^+$ hypoxic Y27632: 12.9±3mmHg, n=8, WT normoxic vehicle: 12.5±1.5mmHg, n=8).

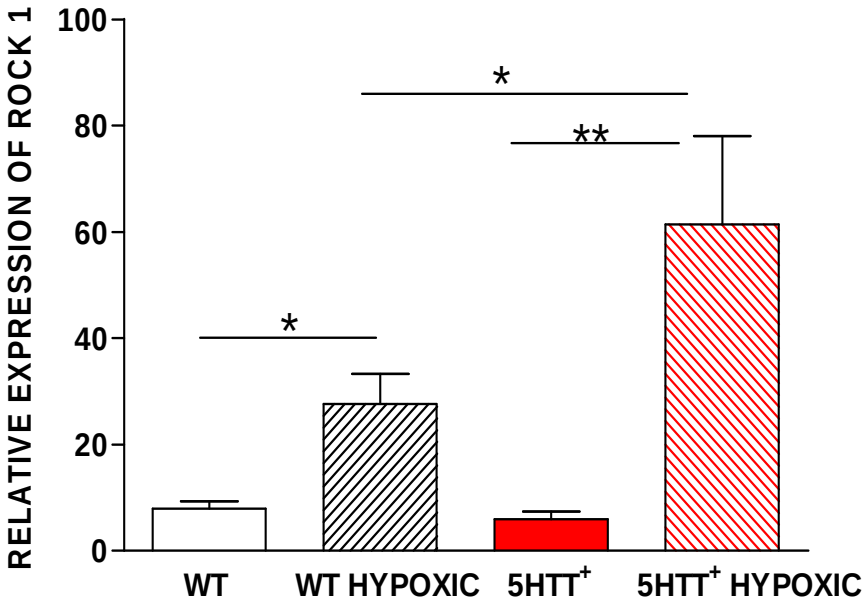

Figure 4.1 Relative expression levels of ROCK 1 transcript in whole lung tissue from WT and 5HTT$^+$ under both normoxic and hypoxic conditions.

Whole lungs were removed from euthanised animals for RNA isolation. After reverse transcription, relative transcript levels of ROCK1 were determined by TaqMan RT-PCR. Results expressed as mean ± SEM relative to levels of 18s ribosomal RNA, WT n=5, WT hypoxic n=5, 5HTT$^+$ n=5, 5HTT$^+$ hypoxic n=5. * $p < 0.05$, ** $p < 0.01$ using Newman-Keuls multiple comparison test.

Figure 4.2 Relative expression levels of ROCK 2 transcript in whole lung tissue from WT and 5HTT$^+$ under both normoxic and hypoxic conditions.

Whole lungs were removed from euthanised animals for isolation of RNA. After reverse transcription, relative transcript levels of ROCK 2 were determined by TaqMan RT-PCR. Results expressed as mean ± SEM relative to levels of 18s ribosomal RNA, WT n=5, WT hypoxic n=6, 5HTT$^+$ n=5, 5HTT$^+$ hypoxic n=6. * $p < 0.05$, ** $p < 0.01$ using Newman-Keuls multiple comparison test.

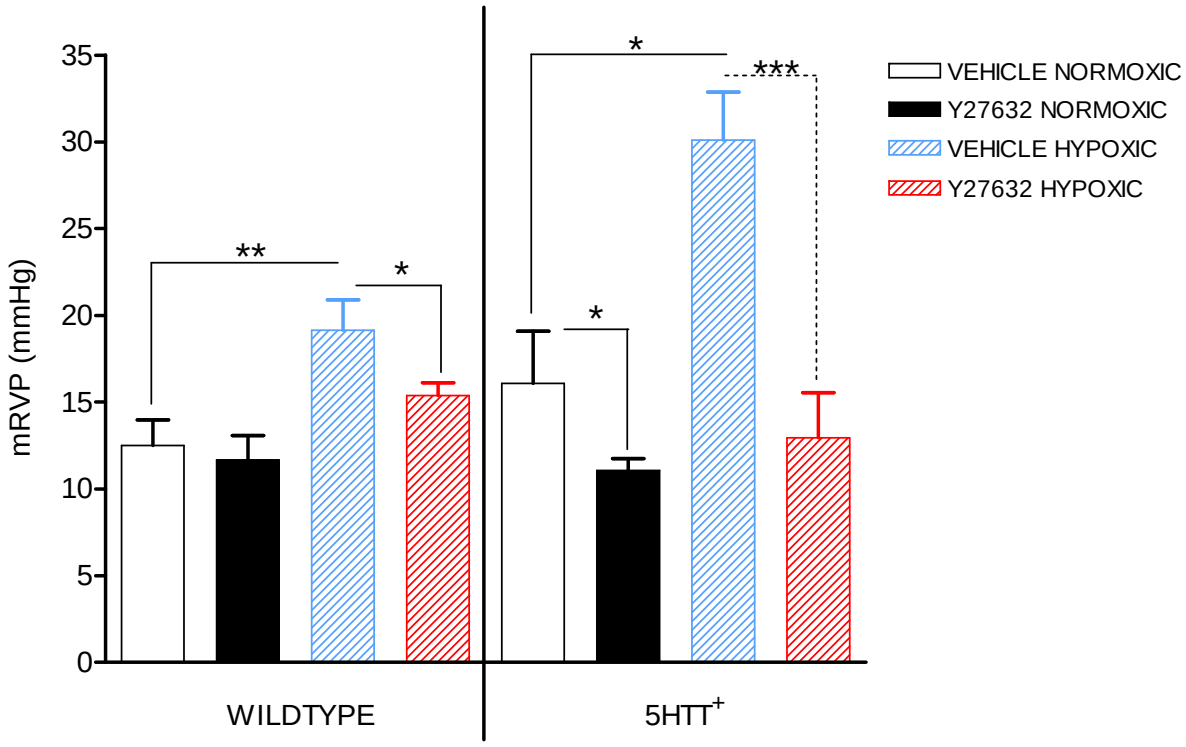

Figure 4.3 Effects of Y27632 administration on mean right ventricular pressure (mRVP).
WT and 5HTT^{+} mice were maintained in either normoxic or hypoxic conditions for 14 days during which period they were administered with either vehicle or Y27632 (30mgkg^{-1}) daily by oral gavage. Subsequently mRVP was measured by a transdiaphramatic approach. Results expressed as mean ± SEM, WT vehicle n=8, WT Y27632 n=6, WT vehicle hypoxic n=5, WT Y27632 hypoxic n=8, 5HTT^{+} vehicle n=7, 5HTT^{+} Y27632 n=7, 5HTT^{+} vehicle hypoxic n=5, 5HTT^{+} Y27632 hypoxic n=8. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ using Newman-Keuls multiple comparison test.

Similar effects were also observed on sRVP. Under normoxic conditions $5HTT^+$ mice displayed a pulmonary hypertensive phenotype, exhibiting elevated sRVP compared to WT mice (WT: 18.7±1.2mmHg, n=8, $5HTT^+$: 27.6±3.4mmHg, n=7, $p<0.05$) (Figure 4.4). Again, hypoxia significantly elevated sRVP in both WT and $5HTT^+$ mice (WT: $p<0.05$, $5HTT^+$: $p<0.01$). Y27632 significantly attenuated this response (WT: $p<0.05$, $5HTT^+$: $p<0.01$) having a more pronounced effect in the $5HTT^+$ group.

Comparable trends in dRVP were also noted, with hypoxia elevating pressures in both WT ($p<0.05$) and $5HTT^+$ ($p<0.01$) mice (Figure 4.5). Under normoxic conditions dRVP in $5HTT^+$ animals appears elevated compared to WT (WT: 5.7±1.5mmHg, n=8, $5HTT^+$: 8.9±3.4mmHg, n=7, $p>0.05$). Administration of Y27632 had no effect on dRVP observed under normoxic conditions in WT mice; it did however marginally reduce the elevated pressure seen in $5HTT^+$ mice. Notably, the most marked effects of ROCK inhibition occurred in $5HTT^+$ mice under hypoxic conditions, where Y27632 significantly reduced the elevation in dRVP ($p<0.01$) and returned pressures near to levels witnessed in normoxic WT animals (WT normoxic vehicle: 5.7±1.5mmHg, n=8, $5HTT^+$ hypoxic Y27632: 5.5±2.4mmHg, n=8). These findings show that ROCK inhibition, attenuates hypoxia-induced increases in RVP and that this effect is most notable on sRVP. Given that sRVP is significantly higher in $5HTT^+$ mice, the benefical effects of ROCK inhibition are more pronounced in these mice. The effects of Y27632 observed were specific to the pulmonary circulation as mean systemic arterial pressures and heart rates were found to be unaltered by administration of Y27632 (Table 4.1). Furthermore, neither overexpression of the 5HTT gene nor chronic hypoxia has any effect on systemic haemodynamics (Table 4.1).

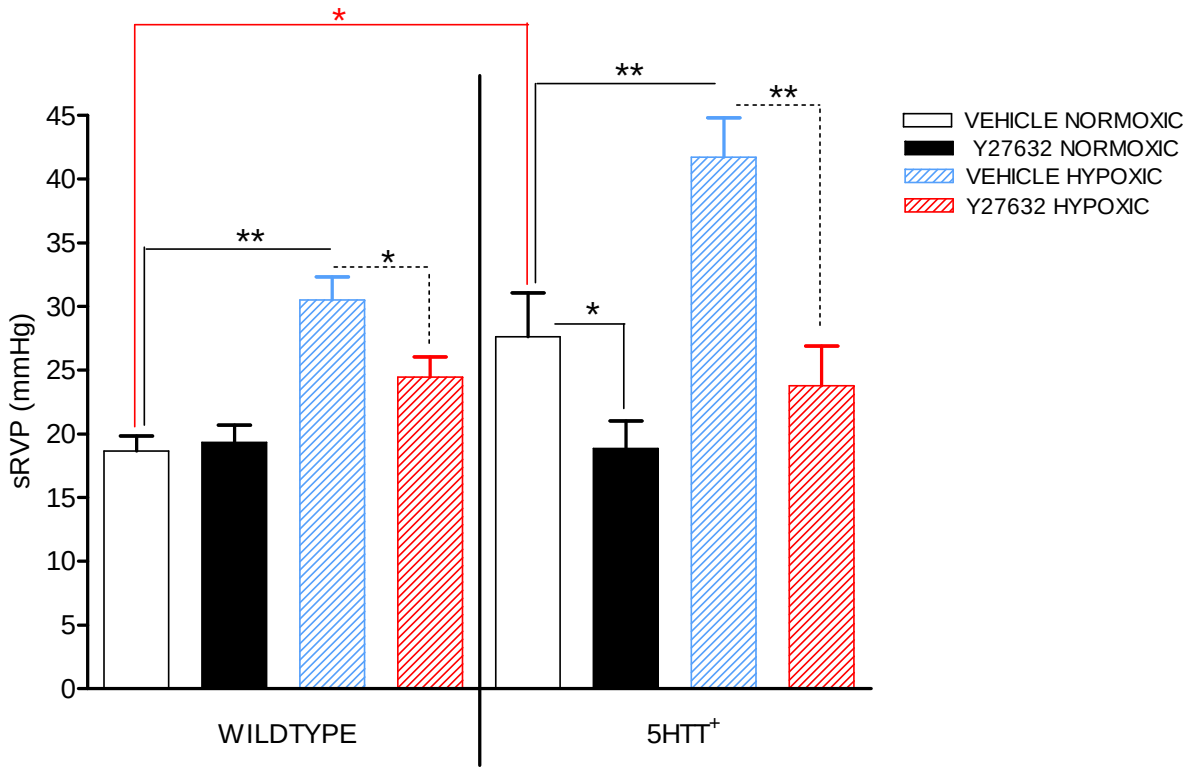

Figure 4.4 Effects of Y27632 administration on systolic right ventricular pressure (sRVP).
WT and $5HTT^+$ mice were maintained in either normoxic or hypoxic conditions for 14 days during which period they were administered with either vehicle or Y27632 ($30mgkg^{-1}$) daily by oral gavage. Subsequently sRVP was measured by a transdiaphramatic approach. Results expressed as mean ± SEM, WT vehicle n=8, WT Y27632 n=6, WT vehicle hypoxic n=5, WT Y27632 hypoxic n=8, $5HTT^+$ vehicle n=7, $5HTT^+$ Y27632 n=7, $5HTT^+$ vehicle hypoxic n=5, $5HTT^+$ Y27632 hypoxic n=8. * $p < 0.05$, ** $p < 0.01$ using Newman-Keuls multiple comparison test.

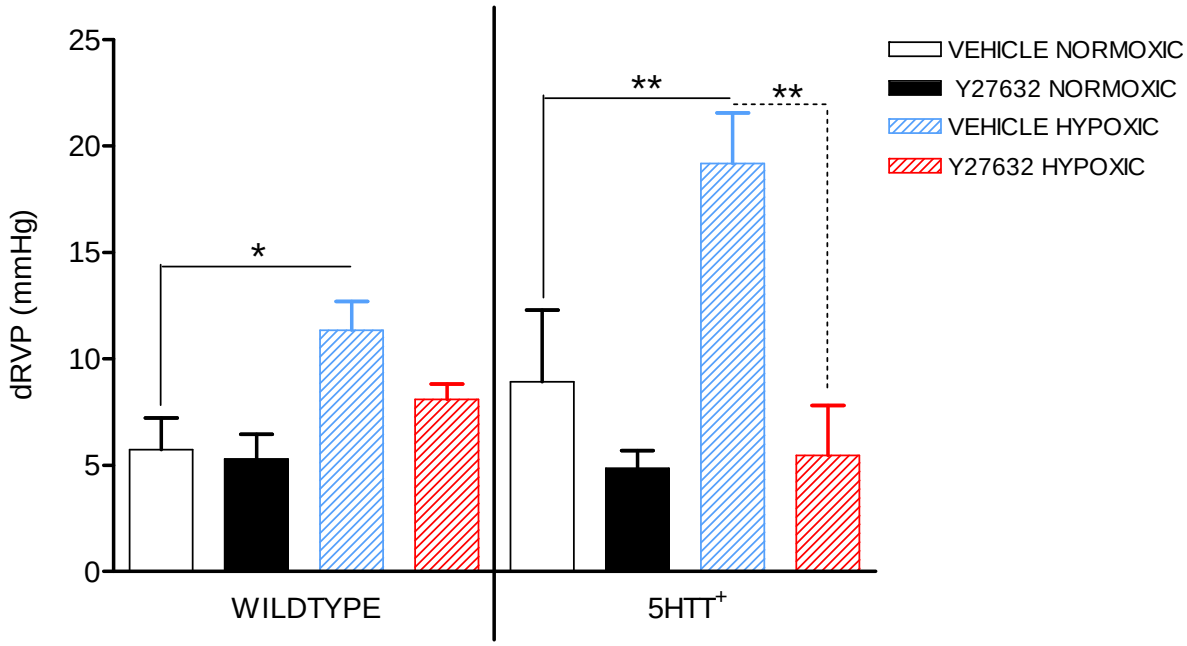

Figure 4.5 Effects of Y27632 administration on diastolic right ventricular pressure (dRVP).
WT and 5HTT^{+} mice were maintained in either normoxic or hypoxic conditions for 14 days during which period they were administered with either vehicle or Y27632 (30mgkg^{-1}) daily by oral gavage. Subsequently dRVP was measured by a transdiaphramatic approach. Results expressed as mean ± SEM, WT vehicle n=8, WT Y27632 n=6, WT vehicle hypoxic n=5, WT Y27632 hypoxic n=8, 5HTT^{+} vehicle n=7, 5HTT^{+} Y27632 n=7, 5HTT^{+} vehicle hypoxic n=5, 5HTT^{+} Y27632 hypoxic n=8. * $p < 0.05$, ** $p < 0.01$ using Newam-Keuls multiple comparison test.

GROUP	mean SAP (mmHg)	Heart Rate (mmHg)	n
Wildtype			
Vehicle	92±4	438±19	8
Y27632	85±7	461±22	6
Hypoxic Vehicle	85±3	470±16	5
Hypoxic Y27632	88±3	497±21	8
5HTT$^+$			
Vehicle	78±10	470±19	7
Y27632	86±4	505±18	7
Hypoxic Vehicle	97±4	476±21	5
Hypoxic Y27632	77±10	500±32	8

Table 4.1 Effects of Y27632 administration on systemic arterial pressure (SAP) and heart rate (HR).
WT and 5HTT$^+$ mice were maintained in either normoxic or hypoxic conditions for 14 days during which period they were administered with either vehicle or Y27632 (30mgkg^{-1}) daily by oral gavage. Subsequently SAP and HR were measured by cannulation of the right carotid artery. Results expressed as mean ± SEM. No significant differences between groups were found using Newman-Keuls multiple comparison test.

The effects of ROCK inhibition on RVH were also assessed (Figure 4.6). In WT animals under normoxic conditions, administration of Y27632 had no effect on the RV/LV+S ratio (n=7). Exposure to chronic hypoxia induced a marked elevation in this ratio ($p<0.001$, n=14) and treatment with Y27632 had no effect on this hypertrophic response (n=9). Similarly, in $5HTT^+$ mice, Y27632 had no effect on the RV/LV+S ratio under normoxic conditions (n=10) and exposure to chronic hypoxia markedly increased RVH ($p<0.001$, n=14). However, in contrast to WT mice, when administered with Y27632, $5HTT^+$ mice exhibited significantly reduced RVH under hypoxic conditions ($p<0.001$, n=9).

Pulmonary vascular remodeling was also assessed (Figure 4.7). Hypoxia significantly elevates the percentage of remodeled arteries in WT and $5HTT^+$, with the remodelling observed in $5HTT^+$ mice more marked (16.8±1% versus 23.3±2%, $p < 0.001$, n=6). In addition, administration of Y27632 under hypoxic conditions significantly attenuates the percentage of remodeled pulmonary arteries in WT (12.6±0.8%, $p<0.01$, n=4) and $5HTT^+$ mice (11.7±0.8%, $p<0.001$, n=7).

Taken together, these observations suggest that inhibition of ROCK not only reduces elevated pulmonary artery pressure but also markedly attenuates pulmonary vascular remodeling. These inhibitory effects are more prominent in $5HTT^+$ mice, highlighted by the fact that ROCK inhibition selectively reduces RVH in these mice.

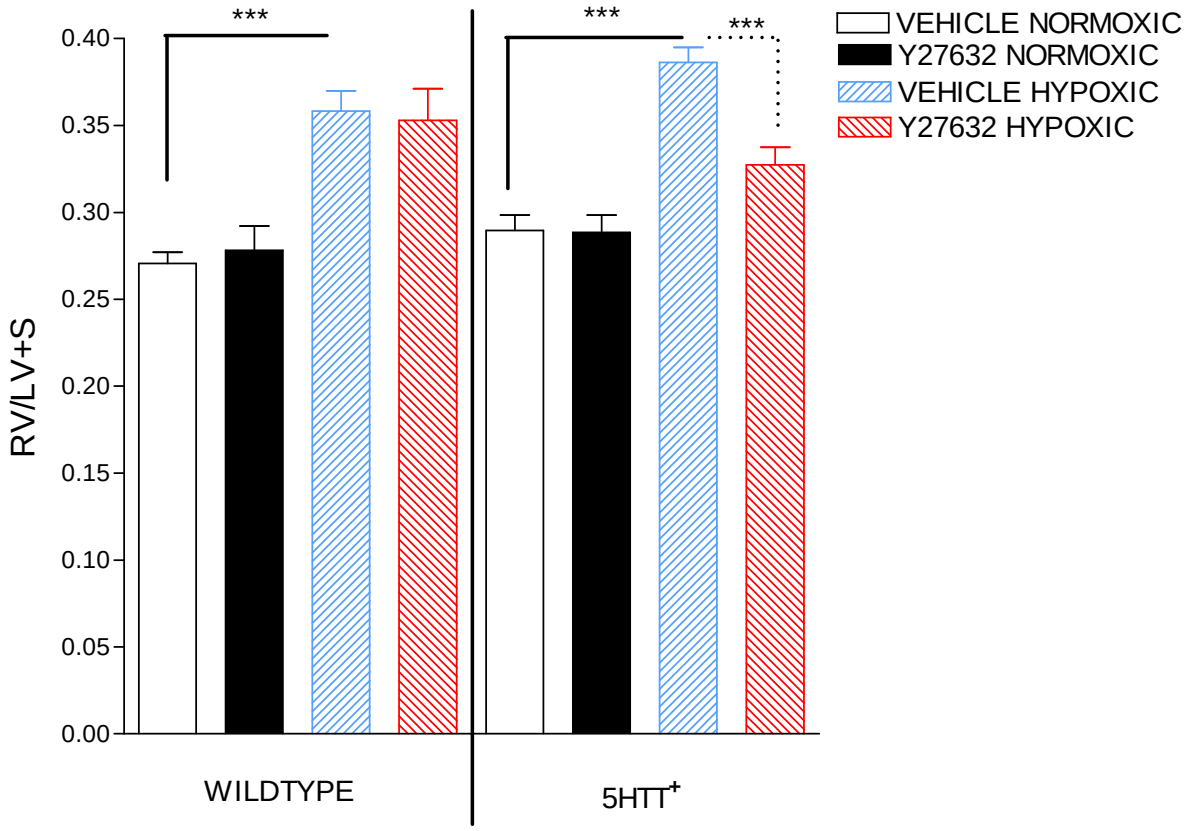

Figure 4.6 Effects of Y27632 administration on right ventricular hypertrophy.
WT and 5HTT^{+} mice were maintained in either normoxic or hypoxic conditions for 14 days during which period they were administered with either vehicle or Y27632 (30 mgkg^{-1}) daily by oral gavage. Following haemodynamic measurement hearts were removed and right ventricular hypertrophy assessed by calculating the ratio of the right ventricle to left ventricle plus septum (RV/LV+S). Results expressed as mean ± SEM, WT vehicle n=12, WT Y27632 n=7, WT vehicle hypoxic n=14, WT Y27632 hypoxic n=9, 5HTT^{+} vehicle n=14, 5HTT^{+} Y27632 n=10, 5HTT^{+} vehicle hypoxic n=14, 5HTT^{+} Y27632 hypoxic n=9. *** $p < 0.001$ using Newman-Keuls multiple comparison test.

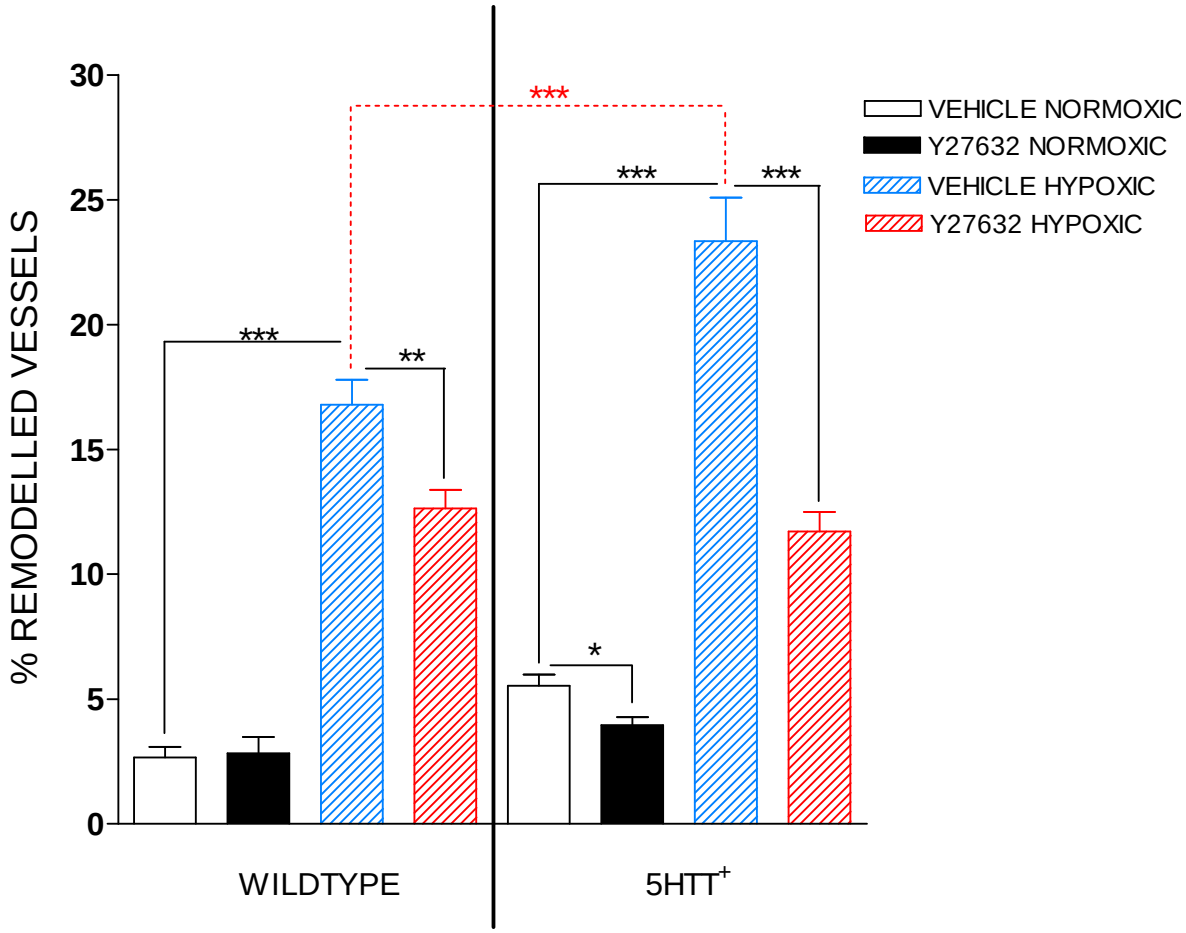

Figure 4.7 Effects of Y27632 administration on the pulmonary vascular remodelling.
WT and 5HTT^{+} mice were maintained in either normoxic or hypoxic conditions for 14 days during which period they were administered with either vehicle or Y27632 (30 mgkg^{-1}) daily by oral gavage. Following haemodynamic measurement lung tissue was removed for analysis. Results expressed as mean ± SEM, WT vehicle n=6, WT Y27632 n=4, WT vehicle hypoxic n=6, WT Y27632 hypoxic n=4, 5HTT^{+} vehicle n=7, 5HTT^{+} Y27632 n=5, 5HTT^{+} vehicle hypoxic n=6, 5HTT^{+} Y27632 hypoxic n=7. **p<0.05, ** p< 0.01, *** p < 0.001 using Newman-Keuls multiple comparison test.

4.3 DISCUSSION

ROCK is thought to play a role in the pathophysiology of several vascular diseases including PAH. 5HT and 5HTT are also implicated in the development of the condition. Given the results discussed in Chapter 3 of this thesis and studies carried out by others (Liu et al., 2004), potential cross-talk between 5HT signaling pathways and ROCK were investigated *in vivo*.

Chronic hypoxia is a commonly used model of pulmonary hypertension (Marcos et al., 2003; Long et al., 2006; Eddahibi et al., 1998), and in this study, exposure of WT mice to hypoxia resulted in elevated RVP and RVH, hallmarks of experimental PAH. As mentioned previously 5HTT is thought to be involved in the development of PAH and consistent with previously published results (MacLean et al., 2004; Guignabert et al., 2006), $5HTT^{+}$ mice appeared to display a spontaneously pulmonary hypertensive phenotype, with significantly elevated sRVP under normoxic conditions. Polymorphisms in the 5HTT gene promoter associated with overexpression of 5HTT, have also been observed in a large percentage of patients with PAH (Eddahibi et al., 2001).

ROCK is an important mediator of vascular tone and has previously been implicated in hypoxic pulmonary vasoconstriction, with its inhibition attenuating vasoconstriction in isolated rat lung and pulmonary arteries (Robertson et al., 2000a; Fagan et al., 2004). This may explain the ability of ROCK inhibition to significantly attenuate sRVP in both WT and $5HTT^{+}$ mice (Figure 4.4). The effects of ROCK inhibition appears specific to the pulmonary circulation, as Y27632 had no effect on mSAP or HR (Table 4.1). The beneficial effects of ROCK inhibition on RVP observed in this study are consistent with findings by others in chronically hypoxic, fawnhooded and monocrotaline treated rats (Hyvelin et al., 2005; Nagaoka et al., 2006; Abe et al., 2004; Guilluy et al., 2005).

The beneficial effects of ROCK inhibition on RVP appear more pronounced in $5HTT^{+}$ mice, with all parameters of RVP significantly attenuated (Figures 4.3 to 4.5). The more marked effect of ROCK inhibition in these mice is also highlighted by the fact that Y27632 appears to specifically attenuate hypoxia induced RVH in $5HTT^{+}$ mice but not WT mice (Figure 4.6). Others have also suggested the beneficial effects of ROCK inhibition on RVH in chronically

hypoxic rats (Abe et al., 2004; Guilluy et al., 2005; Nagaoka et al., 2006). Furthermore, inhibition of ROCK has previously been shown to attenuate pulmonary vascular remodelling and vessel wall thickness in a variety of models of pulmonary hypertension (Abe et al., 2004; Guilluy et al., 2005; Nagaoka et al., 2006). These finding agree with the present study in which administration of the ROCK inhibitor Y27632 significantly attenuated hypoxia-induced pulmonary vascular remodelling (Figure 4.7).

Moreover, the effects of chronic hypoxia on relative levels of ROCK 1 and ROCK 2 transcript were assessed. Hypoxia resulted in elevation of ROCK 2 in both WT and $5HTT^{+}$ to a similar extent. In the case of ROCK 1 however, a more marked increase was observed in $5HTT^{+}$ mice (Figure 4.1). This observation may also be related to the augmented effects of ROCK inhibition on RVP, vascular remodelling and RVH in 5HTT mice. These findings suggest a potential link between 5HTT and ROCK 1 expression.

A number of studies have previously shown the ability of hypoxia to upregulate RhoA in a variety of cell types and tissues, including PASMCs (Bailly et al., 2004), PAECs (Wojciak-Stothard et al., 2006) and rat pulmonary arteries (Guilluy et al., 2005). Increased protein levels of ROCK 1 have also been reported in the pulmonary arteries of fawnhooded rats kept at high altitude (Nagaoka et al., 2006). Furthermore, consistent with findings in the present study, elevated ROCK 1 and 2 mRNA levels have been observed in lungs from chronically hypoxic rats (Hyvelin et al., 2005). However, the mechanisms resulting in increased ROCK expression during hypoxia have not been addressed.

Hypoxia is known to modulate the expression of a wide variety of genes. One major class of transcription regulators activated in response to hypoxia are hypoxia inducible factors (HIF) (Wang et al., 1995). HIF-1α, HIF-2α (Ema et al., 1997; Tian et al., 1997; Flamme et al., 1997), HIF-3α (Gu et al., 1998) subtypes have been identified, with HIF-1α most ubiquitously expressed and extensively characterised (Semenza, 2004). HIF comprises of heterodimeric transcription factors HIFα and HIFβ, which bind to consensus DNA binding motifs within the regulatory promoter region of hypoxia responsive genes, known as the hypoxia response elements (HRE), resulting in transcriptional regulation of the target gene (Wang et al., 1995).

A large number of genes with a wide range of functions are regulated by HIF-1 including VEGF (Levy et al., 1995), inducible NOS (iNOS) (Melillo et al., 1995), cyclo-oxygenase 2 (COX-2) (Kaidi et al., 2006) erythropoietin (EPO) (Semenza and Wang; 1992, Semenza et al., 1991) and GLUT1 (Chen et al., 2001). It is possible that ROCK genes may also be upregulated by HIF-1 under hypoxic conditions, thus explaining the increased mRNA levels observed in this study in chronically hypoxic mice. Indeed, data analysis of the promoter region of murine ROCK 1 and ROCK 2 genes identified potential HIF-1 consensus sequences, suggesting HIF-1 may be involved in hypoxia-mediated ROCK upregulation (Figure 4.8).

Other transcription factors, such as nuclear factor kappa B (NFκB), cAMP response element binding protein (CREB), p53, Egr-1 Sp-1 and AP-1 have all been found to be activated either directly or indirectly by hypoxia (Cummins and Taylor, 2005), contributing to altered gene transcription. Hypoxic activation of the afore mentioned transcription regulators may also contribute to the increased transcript levels of ROCK observed under chronically hypoxic conditions in the present study. Indeed, data analysis of the promoter region of the human ROCK gene suggested several possible cis DNA elements including AP-1 and Sp-1 (Shimokawa and Takeshita, 2005). However, the function of these elements has not been evaluated. Similarly, data anlaysis of murine ROCK 1 and ROCK 2 identified a potential Sp-1 and AP-1 binding sites in each promoter region. This analysis also detected potential NFκB and CREB transcription factor binding sites.

In addition to this, inflammatory stimuli may be involved in the regulation of ROCK expression during hypoxic conditions. Hypoxic activation of NFκB has been found to increase the level of inflammatory mediators such as COX-2, tumour necrosis factor α (TNFα) and interleukin-6 (IL-6). The upregulation of inflammatory mediators by this mechanism may influence ROCK expression, as angiotensin II and interleukin 1β (IL-1β) have previously been shown to increase the expression and function of ROCK in vascular SMCs. This process was dependent of NFκB (Hiroki et al., 2004).

Hypoxia also activates various kinases, which may result in altered gene transcription. The MAP kinases ERK (Stenmark et al., 2002; Minet et al., 2000), p38 (Stenmark et al., 2002; Welsh et al., 2001a) and JNK (Stenmark et al.,

2002) have been found to be activated under hypoxic condition, as have other protein kinases such as PKC (Yuan et al., 2005), PKA (Beitner-Johnson et al., 1998), CaMK (Beitner-Johnson et al., 1998; Yuan et al., 2005) and Src (Thobe et al., 2006). Activation of such kinases during hypoxia may result in the modulation of signalling pathways and alterations in gene transcription, accounting for the increased levels of ROCK transcript. For instance, there is evidence to suggest that in various circumstances activation of p38 results in the expression of various inflammatory mediators including TNFα and iNOS (Wang et al., 2004). Given the effect of inflammatory stimuli on ROCK, this may in turn result in its upregulation. Furthermore the increased entry of 5HT into cells in $5HTT^+$ mice may potentiate signal transduction resulting in the increased transcription of ROCK 1.

Paracrine factors released during hypoxia may also contribute to the regulation of ROCK activity. Mediators such as adenosine are released during hypoxic conditions. The accumulation of extracellular adenosine has previously been found to affect the transcription of genes such as VEGF and eNOS (Ramanathan et al., 2007; Min et al., 2006). Therefore, accumulation of adenosine could potentially effect ROCK expression by activating signal transduction pathways that mediate its transcription. Furthermore, given both adenosine and p38 both play important roles in the regulation of 5HTT activity (Zhu et al., 2004; Zhu et al., 2005), the increased presence of these mediators during hypoxic conditions may contribute to the augmented hypoxic-upregulation of ROCK 1 in $5HTT^+$ mice.

To conclude, the more pronounced beneficial effects of ROCK inhibition on RVP and vascular remodelling in $5HTT^+$ mice and the ability of Y27632 to reduce right ventricular hypertrophy in 5HTT+ mice but not WT mice, suggests cross-talk between 5HT and ROCK signaling systems. This hypothesis is consistent with the marked elevation in the expression of ROCK1 observed in the lungs of 5HTT+ mice in response to hypoxia. Whilst, both ROCK 1 and ROCK 2 promoter regions contain potential HIF-1 binding sites explaining their upregulation during hypoxia, the mechanisms as to how overexpression of 5HTT results in increased transcript levels of ROCK 1 in the lung requires further investigation.

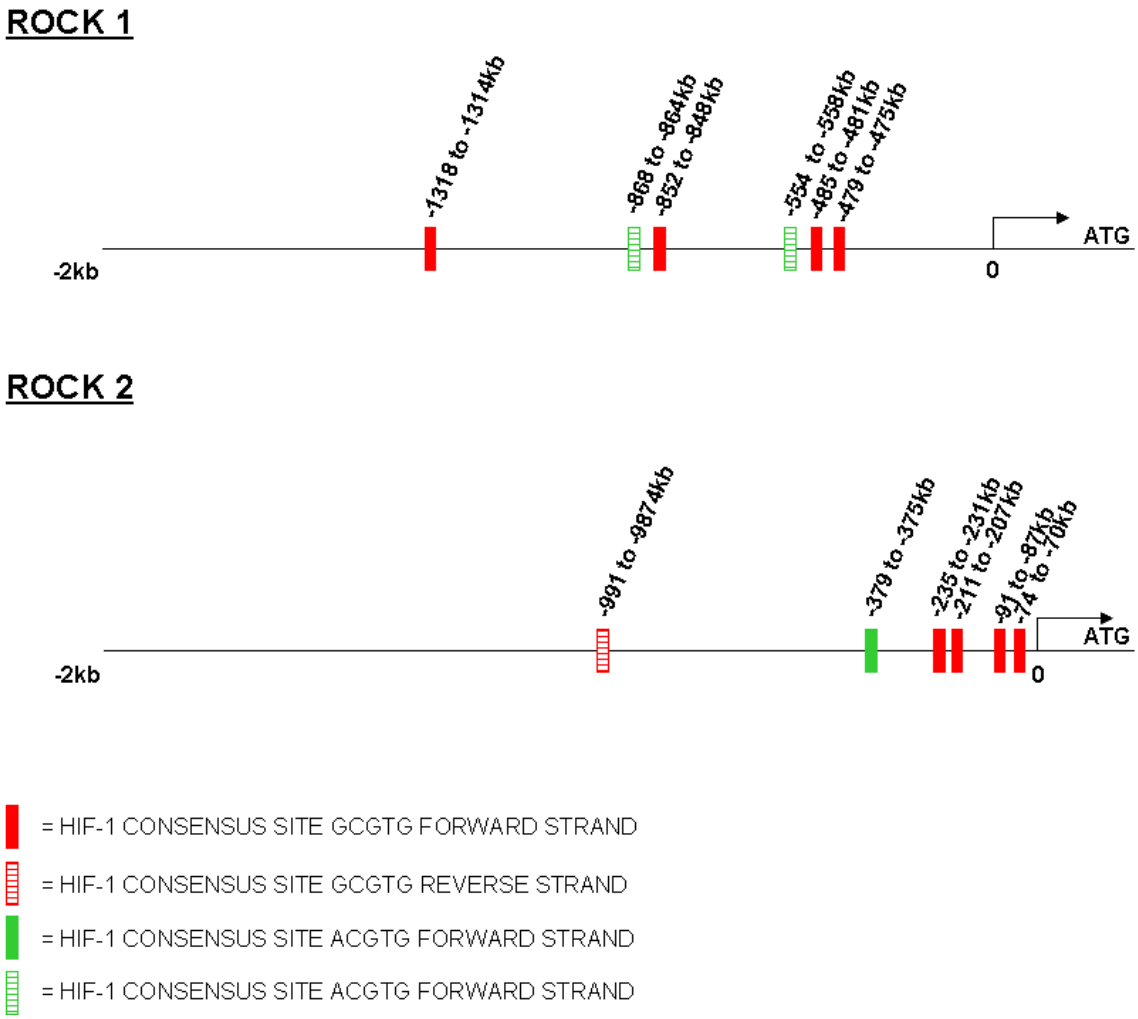

Figure 4.8 Schematic diagram showing the potential position of HIF-1 consensus sequences in the promoter region of murine ROCK 1 and ROCK 2 genes.

Data analysis of ROCK 1 and ROCK 2 genes revealed several potential HIF-1 binding sites in their promoter regions. For each gene, a 2kb region upstream of the transcription initiation site was analysed for HIF-1 consensus sequences 5'-GCTGT-3' and 5'-ACTGT-3'.

Chapter 5
FINAL DISCUSSION

SUMMARY

5HT is a monoamine, synthesised by the body from dietary tryptophan and plays a major role in wide variety of physiological and pathological processes, including pulmonary hypertension (Levy, 2006, MacLean et al., 2000, Hoyer et al., 2002). Evidence exists implicating 5HT in the vasoconstriction and vascular remodelling that occurs during PAH (MacLean et al., 2000). 5HT has been shown to act as a mitogen in a variety of cell types (Lee et al., 1999, Liu and Fanburg, 2006, Lawrie et al., 2005, Bianchi et al., 2005) and it is this effect that may contribute to the vascular remodelling that occurs in patients with PAH. This project has characterised signalling pathways utilised by 5HT to induce fibroblast proliferation, in particular highlighting the role of the Rho/ROCK in this process. Furthermore, using an *in vivo* model where the 5HTT protein is overexpressed, inhibition of the Rho/ROCK pathway was found to have marked beneficial effects on the symptoms of PAH.

In Chapter 3, examination of 5HT mitogenic signalling using CCL-39 hamster lung fibroblasts revealed that proliferation was ERK-dependent and that $5HT_{1B/1D}$ receptors, $5HT_{2A}$ receptors and 5HTT were all required for optimal ERK activation and proliferation, suggesting cross-talk between 5HT receptors and the transporter. Furthermore, pharmacological inhibition of Rho/ROCK pathway significantly inhibited 5HT induced ERK activation, cyclin D1 accumulation and proliferation. However, no effect on 5HT-induced MEK activation or on the ability of active ERK to translocate to the nucleus was observed. Instead, inhibition of the ROCK was found to specifically inhibit the activation of a cytoplasmic pool of ERK. These findings suggest that ROCK inhibition may reduce the ability of MEK to promote ERK phosphorylation. Furthermore, ROCK functions downstream of the $5HT_{1B}$ receptor in mediating 5HT-induced ERK activation. In the cell type used in this study, the direct activation of Rho/ROCK pathway by 5HT was not detected by means of immunoblotting for the activation of downstream substrates of ERK. However, actin stress fibres were detected in the cells prior to stimulation with 5HT, suggesting a high basal activity of Rho which may mask the effects of 5HT on the Rho/ROCK pathway. The function of ROCK in maintaining cytoskeletal

intergrity is central to its role in 5HT-induced ERK activation in this cell type as disruption of the actin cytoskeleton by other agents such as latrunculin B and cytochalasin D also markedly attenuate the ability of 5HT to activate ERK. The role of the ROCK and the actin cytoskeleton in mediating 5HT-induced ERK activation are specific to 5HT pathways as neither inhibition of ROCK nor disruption of the actin cytoskeleton had any effect on the ability of PMA to activate ERK.

In Chapter 3, this study characterised the effects of ROCK inhibition on 5HT-induced ERK activation and proliferation, however it may also be useful to determine the effects of ROCK activation on these processes, as this would be expected to promote proliferation. One possible way in which to address the effects of ROCK activation would be to use a conditionally active ROCK construct. Indeed, a study carried out by Croft *et al* 2006, using a conditionally activated ROCK-estrogen receptor (ROCK-ER) fusion construct, reported that activation of ROCK-ER by an estrogen analogue was sufficient to stimulate cell cycle progression in NIH 3T3 mouse fibroblasts. Based on the findings in Chapter 3, estrogen-mediated ROCK-ER activation in CCL-39 cells would be expected to potentiate 5HT-stimulated proliferation and ERK activation.

Chapter 3 also suggesst that the ROCK pathway, *via* $5HT_{1B}$ stimulation controls MEK activation of a cytoplasmic pool of ERK. One possible mechanism by which many GPCRs are able to activate specific pools of ERK is *via* the action of scaffolding proteins such as β-arrestin, which can mediate the recruitment of Src or Raf/MEK/ERK signalling complexes following agonist stimulated receptor phosphorylation (DeFea et al., 2000, Wang et al., 2006b, Shenoy et al., 2006). Much work has been carried out on the regulation of $5HT_{2A}$ receptor phosphorylation and interactions with β-arrestin (Bhatnagar et al., 2001, Gray et al., 2003, Gray and Roth, 2001, Gray et al., 2001). However little evidence on $5HT_{1B}$ receptor regulation exists. Interestingly, data analysis of the protein sequence of the human $5HT_{1B}$ receptor revealed a cluster of serine and threonine residues within its third intracellular loop. This is characteristic of G-protein-coupled receptor kinase (GRK) phosphorylation sites, which have a high affinity of β-arrestins. Similar sites have previously been described for other GPCRs, including the V2 vasopressin receptor and the α_{2A} adrenoreceptor (Pao and Benovic, 2005, Innamorati et al., 1997). Therefore, future experiments to

characterise $5HT_{1B}$ receptor regulation may be useful in determining the mechanisms by which 5HT, *via* the $5HT_{1B}$ receptor and the Rho/ROCK pathway specifically controls a cytoplasmic pool of ERK.

The results presented in Chapter 3 also identify a role for the actin cytoskeleton in 5HT-mediated mitogenesis. It appears that ROCK maintains the integrity of the cytoskeleton in order to facilitate 5HT-mediated ERK activation and proliferation. These findings may be of physiological relevance as other components involved in the control of actin cytoskeletal dynamics have been implicated in the development of PAH. LIMK, a key regulator of actin dynamics, has been found to interact with the tail region of the BMPR2 receptor, a process which inhibits LIMK activity (Foletta et al., 2003). A BMPR2 mutant with a truncation in the COOH-terminus that has also been described in patients with PAH was reported to be unable to bind or inactivate LIMK (Foletta et al., 2003). Given the role of BMPR2 in the development of PAH these findings suggest that deregulation of actin dynamics may contribute to the pathobiology of PAH. Furthermore, the upregulation cofilin-2 and LIMK 2 have been reported in cultured PASMCs and in pulmonary arteries from monocrotaline-treated rats (Dai et al., 2006). Increased levels of these proteins were suggested to promote cell motility, a process that may contribute to vascular remodelling and occlusion of pulmonary arteries. The cytoskeleton also plays a vital role in the regulation of pulmonary vascular permeability. For example, actin filaments are of importance to endothelial cell permeability, with disruption of these filaments resulting in decreased endothelial barrier integrity and infiltration of inflammatory mediators, a process that occurs in disease states such as acute lung injury and acute respiratory distress syndrome (Dudek and Garcia, 2001). Therefore, cytoskeletal integrity may have a widespread role in PAH affecting both BMPR2 and 5HT signalling.

Give the apparent role of ROCK in mitogenic 5HT-activated signalling pathways, Chapter 4 investigated the role of ROCK in a chronic hypoxic mouse model of PAH, using transgenic mice that ubiquitously overexpress 5HTT in order to determine any potential interactions between 5HT and ROCK pathways *in vivo*.

Using quantitative RT-PCR, chronic hypoxia was found to upregulate both ROCK 1 and ROCK 2 transcripts, with the upregulation of ROCK 1 potentiated

in $5HTT^+$ mice. Furthermore, ROCK inhibition resulted in a marked reduction in hypoxia-induced sRVP and vascular remodelling, effects that were significantly greater in $5HTT^+$ mice compared to WT. In addition, the hypoxia-induced RVH that occurred in both WT and $5HTT^+$ groups was only sensitive to ROCK in $5HTT^+$ mice. Taken together, Chapters 3 and 4 highlight a role for Rho/ROCK pathway in the development of PAH and suggest interactions between 5HT and Rho/ROCK systems in mediating the mitogenic effects of 5HT.

In Chapter 4, inhibition of ROCK was found to have more significant effects in $5HTT^+$ mice, an effect which may be due to the potentiated hypoxia-induced upregulation of ROCK 1. The increased expression of ROCK transcript detected in the chronic hypoxic mouse model, suggested increased ROCK activity, which may contribute to the remodelling process and cell proliferation. Analysis of mouse lung tissue by immunoblotting is required to determine the levels of ROCK protein, as well as the activation status of downstream ROCK substrates, in order to confirm that ROCK activity is increased during hypoxia.

Recently it has been demonstrated that entry of 5HT into the cell *via* 5HTT mediates the prolonged activation of Rho by the transglutaminase-mediated serotinylation of RhoA (Guilluy et al., 2007, Walther et al., 2003). This may contribute to the potentiated effects of hypoxia in $5HTT^+$ mice, given the more marked beneficial effects of ROCK inhibition in these animals. If this mechanism is functional in fibroblasts, using the data presented in Chapters 3 and 4, a potential model to explain how the enhanced expression of ROCK 1 observed in $5HTT^+$ mice promotes remodelling by enhancing the mitogenic effects of 5HT in fibroblasts may be proposed (Figure 5.1). In this model, entry of 5HT into the cell *via* 5HTT facilitates the transglutaminase (TG)-catalysed serotonylation of Rho, rendering it constitutively active. Serotonylated Rho proteins can then bind and activate ROCK1 and ROCK2. As ROCK1 transcript levels are markedly elevated in response to hypoxia and 5HTT overexpression, this may be the predominant isoform affected. In pulmonary fibroblasts, enhanced ROCK expression may facilitate $5HT_{1B}$ receptor activation of the ERK pathway by assisting MEK-mediated ERK phosphorylation. To confirm this hypothesis the phenomenon of serotonylation must be confirmed in the fibroblasts studied. For instance, detection of 5HT binding to RhoA may be determined by immunoprecipitating RhoA from 5HT stimulated fibroblasts and

subsequently immunoblotting using an anti-5HT antibody. However, in disagreement with the hypothesis of serotonylation, pretreatment of CCL-39 cells with MDC had no effect on 5HT stimulated ERK activation (Figure 3.18). MDC inhibits the transglutaminase enzyme required for serotonylation to occur and thus if serotonylation occurs it CCL-39 cells it may have been expected that MDC would inhibit 5HT-induced ERK activation. MDC has previously been found to inhibit binding of 5HT to RhoA in mouse aortic SMCs (Guilluy et al., 2007). However, the concentration of MDC (200µM) used in this study was significantly higher than that used in Chapter 3 (100µM) and this may explain why MDC pre-treatment was unable to inhibit 5HT-stimulated ERK activation. Therefore, the process of serotonylation cannot be ruled out in CCL-39 cells.

Furthermore, given the hypoxia-induced upregulation of ROCK in mice, future experiments may include the use of a hypoxic cellular model to determine if this phenomenon also occurs *in vitro* and to ascertain any changes that may arise in the ROCK-dependent 5HT mitogenic signalling during hypoxia. Studying signalling pathways under hypoxic conditions and also the use of a primary cell line derived from mouse pulmonary arteries would allow a more direct comparision and intregration between the cellular signal transduction pathways investigated and the chronic hypoxic mouse model of PAH.

This study highlights the role of ROCK in the development of pulmonary hypertension and indicates cross-talk between 5HT and Rho/ROCK signalling pathways. The results are of clinical relevance, given ROCK inhibitors such as fusadil are currently being investigated as potential therapies for PAH (Ishikura et al., 2006). Furthermore, inhibition of ROCK appears to have selective effects on the pulmonary circulation, as shown in this study (Table 4.1) and by others where fusadil selectively induces vasodilation in pulmonary arteries but not aorta in an animal model of PAH (Jiang et al., 2007). In addition to this, other agents such as sildenafil and statins appear to mediate their beneficial effects on the symptoms of PAH *via* inhibition of the ROCK pathway (Xing et al., 2006, Guilluy et al., 2005). To conclude, ROCK appears to be a promising therapeutic target in the treatment of PAH, with drugs targeting ROCK having beneficial effects on pulmonary vasoconstriction and pulmonary vascular remodelling. The findings in this study confirm ROCK's importance in the development PAH

and suggest potential mechanisms of action for the therapeutic effects of ROCK inhibitors.

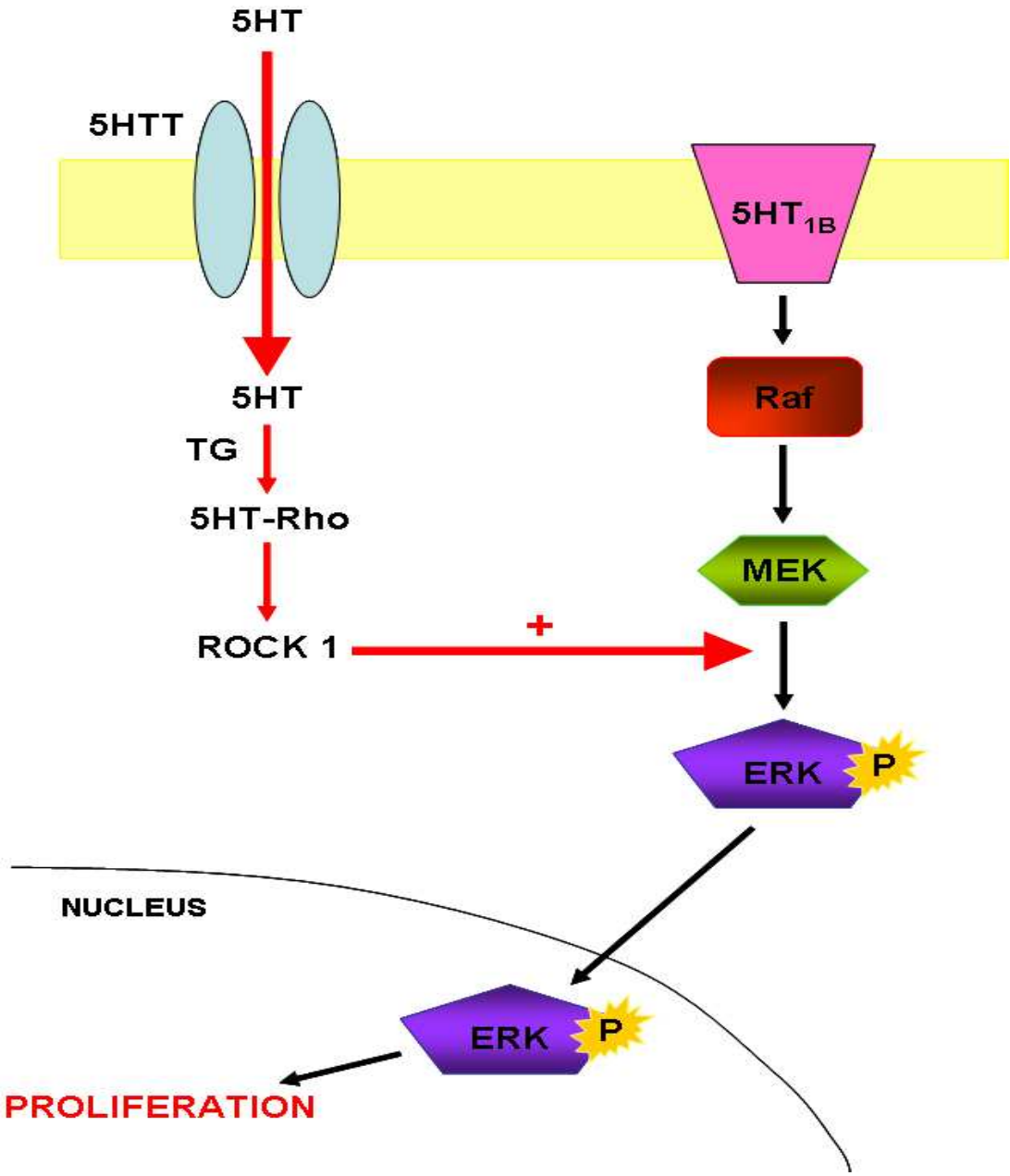

Figure 5.1 Proposed model of how chronic hypoxia and increased 5HTT activity control 5HT-mediated proliferation *via* ROCK.

Overexpression of 5HTT results in elevated levels of 5HT entering the cell. Entry of 5HT facilitates the transglutaminase (TG)-catalysed serotinylation of Rho, resulting in the constitutive activation of Rho. Active Rho then binds and activates ROCK, predominantly ROCK 1. Active ROCK is required for $5HT_{1B}$ receptor-mediated phosphorylation and activation of ERK by MEK. Once active ERK mediates cellular proliferation and contributes to vascular remodelling.

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