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Modifying Factors in Pulmonary Arterial Hypertension

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ABSTRACT

Pulmonary arterial hypertension (PAH) is a debilitating disease of small pulmonary resistance arteries with vasoconstriction and vascular remodelling contributing to the disease pathology. A genetic basis for the disease was linked to heterozygous loss of function mutations in the bone morphogenetic protein receptor 2 (BMPR2) gene. The mutation is found in the majority of familial PAH cases and a significant number of apparently sporadic cases. The low penetrance of the disease in families carrying BMPR2 mutations and the absence of mutations in the majority of idiopathic patients indicates that BMPR2 deficiency alone is insufficient to induce PAH. It is generally accepted PAH has a multi-factorial pathology with endogenous and environmental factors acting in concert with genetic pre-disposition to create the disease phenotype. Enhancement of the serotonin (5-HT) system has been implicated in PAH with the 5-HT transporter (5-HTT) receiving the most attention as a modifying gene in the development of PAH and there is compelling animal and human data implicating a role for increased expression of the 5-HTT as a modulating factor.

The aim of this study was to investigate if genetic pre-disposition interacts with other additional modifying factors to create the symptoms of PAH. Transgenic mice overexpressing the 5-HTT (5-HTT+), deficient in BMPR2 ($BMPR2^{+/-}$) or a double transgenic (5-HTT+/$BMPR2^{+/-}$) were employed in addition to mice lacking tryptophan hydroxylase 1 (Tph1), the rate limiting enzyme for the synthesis of 5-HT, and therefore lacking peripheral 5-HT ($Tph1^{-/-}$). Additional known or suspected modifying factors assessed in these genetic models were hypoxia, dexfenfluramine (Dfen) and its major metabolite nordexfenfluramine (NDfen), 5-HT, bone morphogenetic protein-2 (BMP-2), KCNQ channels and the role of gender.

Mice were examined *in vivo* for evidence of a pulmonary hypertensive phenotype following exposure to hypoxia and Dfen. Female 5-HTT+ mice were the only group to

have a rise in two indices of PAH - namely right ventricular pressure (RVP) and vascular remodelling - in room air. Female 5-HTT+ mice also had an exaggerated pulmonary hypertensive phenotype in hypoxia. $BMPR2^{+/-}$ mice, were, unexpectedly least susceptible to hypoxic induced increases in RVP although female mice deficient in BMPR2 (both $BMPR2^{+/-}$ and 5-HTT+/$BMPR2^{+/-}$) had more extensive vascular remodelling under hypoxia compared with WT and 5-HTT+ mice. Male mice did not express the phenotypic changes just outlined. No synergistic effect was observed between 5-HTT+ and $BMPR2^{+/-}$ that resulted in a more pronounced pulmonary hypertensive phenotype.

WT and $BMPR2^{+/-}$ mice were chronically oral-dosed with Dfen. Female mice from both genotypes developed similar degrees of PAH. Male mice did not develop elevated RVP but $BMPR2^{+/-}$ males did have evidence of vascular remodelling, although at a lower level than the females. Female $Tph1^{-/-}$ mice did not develop PAH following Dfen indicating Dfen associated PAH is dependent on peripheral 5-HT synthesis. The presence of intact 5-HT synthesis was also associated with an increased vasoconstrictor response to 5-HT in isolated intralobar pulmonary arteries (IPAs), a situation not paralleled with the other serotonergic vasoconstrictors, Dfen and NDfen, indicating differing mechanisms of action underlying the respective vasoconstrictor responses.

The vasoconstrictor action of 5-HT, Dfen, NDfen and the KCNQ potassium channel blocker linopirdine were all assessed in IPAs. Pulmonary arteries from $BMPR2^{+/-}$ mice showed enhanced vasoconstriction to 5-HT and NDfen. 5-HTT+ and 5-HTT+/$BMPR2^{+/-}$ mice showed enhanced vasoconstriction to NDfen but decreased vasoconstriction to 5-HT. Female 5-HTT+/$BMPR2^{+/-}$ mice were the only group tested to show significantly greater vasoconstriction to Dfen compared with WT. The vasoconstrictor response to linopirdine was significantly reduced in $BMPR2^{+/-}$ mice but neither linopirdine nor BMP-2 affected 5-HT induced vasoconstriction.

Female gender is an established risk factor for PAH. To investigate possible events that may underlie this risk, male (testosterone) and female (estradiol and 2-methoxyestradiol (2-ME)) sex hormones were assessed for their vasoactive properties in IPAs. All three hormones relaxed pre-constricted vessels but only at supraphysiological (>0.1 μM) concentrations. Each hormone also reduced the vasoconstriction exerted by 5-HT at 10^{-5} M in male mice but not in females. No such effect, however, was observed in either gender at a physiological (10^{-9} M) concentration. NDfen induced vasoconstriction was also unaffected by 10^{-9} M estradiol. Finally, male and female mouse lungs were assessed for protein expression of 5-HT and BMPR2 signalling compounds (p-Smad1/5/8, p-ERK1/2 and p-p38 MAPK). Female mouse lungs displayed higher expression of the mitogenic mediator p-ERK1/2 than male mouse lungs with the other proteins unchanged.

In conclusion, this study confirms overexpression of the 5-HTT as a trigger for elevated RVP and vascular remodelling in mice and a cause of more severe hypoxic PAH. $BMPR2^{+/-}$ mice are phenotypically normal in room air and show divergent pulmonary effects following hypoxia with loss of BMPR2 seemingly attenuating hypoxic induced increases in RVP but causing a simultaneous worsening of vascular remodelling, this latter effect consistent with the important role BMPR2 has in maintaining vascular integrity. Dfen induced PAH in mice was found to be dependent on peripheral 5-HT synthesis with BMPR2 mutation not acting as a risk factor. Loss of BMPR2 can enhance vasoconstriction to serotonergic agonists and when combined with overexpression of the 5-HTT, leads to a dramatic increase in sensitivity to Dfen induced vasoconstriction. Evidence was also found for altered KCNQ channel function in transgenic animals. Unexpectedly, female gender emerged as the most crucial risk factor for PAH in this thesis.

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DECLARATION

I declare that this thesis has been composed by myself and is a record of my work performed by myself (except where stated). It has not been previously submitted for a higher degree. Some of the results contained in this thesis have been published in peer-reviewed journals as follows:

1) DEMPSIE Y, MORECROFT I, WELSH DJ, MACRITCHIE NA, HEROLD N, LOUGHLIN L, NILSEN M, PEACOCK AJ, HARMAR A, BADER M, MACLEAN MR. (2008) Converging evidence in support of the serotonin hypothesis of dexfenfluramine-induced pulmonary hypertension with novel transgenic mice. *Circulation;* 117: 2928-37.

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ABBREVIATIONS

16α- OHE_1	16α-Hydroxyestrone
2-ME	2-Methoxyestradiol
5-HT	Serotonin
5-HTP	5-Hydroxytryptophan
5-HTT	Serotonin transporter
5-HTT+	Mice overexpressing human 5-HTT
5-LO	5-Lipooxygenase
ACE	Angiotensin-converting enzyme
Ach	Acetylcholine
AHPV	Acute hypoxic pulmonary vasoconstriction
ALK – 1	Activin-receptor–like kinase 1
ANOVA	Analysis of variance
ANP	Atrial or A-type natriuretic peptide
APAH	Associated pulmonary arterial hypertension
AR	Androgen receptor
ATP	Adenosine triphosphate
AVD	Apoptotic volume decrease
BAMBI	BMP and activin membrane bound inhibitor
BMPR2	Bone morphogenetic protein receptor 2
BMPs	Bone morphogenetic proteins
BNP	Brain or B-type natriuretic peptide
BSA	Bovine serum albumin
Ca^{2+}	Calcium
cAMP	Cyclic adenosine monophosphate
CCRC	Cumulative concentration-response curves

cGMP	Cyclic guanosine monophosphate
CHPV	Chronic hypoxic pulmonary vasoconstriction
Cl^-	Chloride ions
CNP	C-type natriuretic peptide
CNS	Central nervous system
CO	Cardiac output
CO_2	Carbon dioxide
COPD	Chronic obstructive pulmonary disease
CYP1B1	Cytochrome P450 1B1
Dfen	Dexfenfluramine
ECE	Endothelin-converting enzyme
ECM	Extracellular matrix
eNOS	Endothelial nitric oxide synthase
ER	Estrogen receptor
ERK	Extracellular regulated kinase
ET-1	Endothelin - 1
FBC	Final bath concentration
FGF	Fibroblast growth factor
FKN	Fractalkine
FPAH	Familial pulmonary arterial hypertension
GDF-5	Growth differentiation factor-5
GDF-6	Growth differentiation factor-6
H_2O_2	Hydrogen peroxide
HIF	Hypoxia inducible factor
HIF-1α	Hypoxia inducible factor-1α
HIF-2α	Hypoxia inducible factor-2α

HIF-3α	Hypoxia inducible factor-3α
HPV	Hypoxic pulmonary vasoconstriction
HR	Heart rate
HRE	Hypoxia inducible factor response element
Id1	Inhibitor of differentiation
IL-1	Interleukin-1
IL-6	Interleukin-6
iNOS	Inducible nitric oxide synthase
IP_3	Inositol triphosphate
IPA	Intralobar pulmonary artery
IPAH	Idiopathic pulmonary arterial hypertension
K^+	Potassium
KCl	Potassium chloride
Kv	Voltage-gated K^+ channels
LV	Left ventricle
MAO	Monoamine oxidase
MAPK	Mitogen-activated protein kinase
mCPP	*m*-Chlorophenylpiperazine
MCT	Monocrotaline
MDMA	3, 4-Methylenedioxymethamphetamine
MIS	Mullerian inhibiting substance
MMPs	Matrix metalloproteinases
mRVP	Mean right ventricular pressure
mSAP	Mean systemic arterial pressure
NANC	Nonadrenergic, non cholinergic
NDfen	Nordexfenfluramine

NFAT	Nuclear factor of activated T-cells
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NOS	Nitric oxide synthase
NP	Natriuretic peptide
O_2	Oxygen
PACAP	Pituitary adenylate cyclase-activating peptide
PAECs	Pulmonary arterial endothelial cells
PAFs	Pulmonary arterial fibroblasts
PAH	Pulmonary arterial hypertension
PAP	Pulmonary artery pressure
PASMCs	Pulmonary arterial smooth muscle cells
PDE5	Phosphodiesterase type 5
PDGF	Platelet-derived growth factor
PGI_2	Prostacyclin
PGI_2-S	Prostacyclin synthase
PKA	Protein kinase A
PKC	Protein kinase C
PKG	Protein kinase G
PLC	Phospholipase C
PO_2	Partial oxygen pressure
PPH	Primary pulmonary hypertension
PVDF	Polyvinylidene difluoride
PVR	Pulmonary vascular resistance
ROCK	Rho kinase
ROS	Reactive oxygen species

RV	Right ventricle
RVH	Right ventricular hypertrophy
RVP	Right ventricular pressure
SAP	Systemic arterial pressure
SAPK	Stress-activated protein kinase
SDS	Sodium dodecyl sulphate
Smurf-1	Smad ubiquitination regulatory factor-1
SNPs	Single nucleotide polymorphisms
SOCE	Store operated Ca^{2+} entry
SPH	Secondary pulmonary hypertension
SR	Sarcoplasmic reticulumn
sRVP	Systolic right ventricular pressure
SSRIs	Selective serotonin reuptake inhibitors
ST	Staurosporine
SVR	Systemic vascular resistance
TBS	Tris buffered saline
TBST	Tween-TBS solution
TG	Transgenic
TGF-β	Transforming growth factor- β
TGF-βR2	Transforming growth factor- β receptor 2
TIE2	Endothelial-specific tyrosine kinase receptor
TPH	Tryptophan hydroxylase
TXA_2	Thromboxane A_2
VDCC	Voltage-dependent calcium channels
VEGF	Vascular endothelial growth factor
VIP	Vasoactive intestinal peptide

V_m	Cell membrane potential
$VMAT_2$	Vesicular monoamine transporter type 2
VNTR	Variable-number tandem repeat
WT	Wild-type

Chapter 1

Introduction

1.1 The pulmonary circulation

The human lung is the only organ in the body to receive the entire cardiac output (CO) at all times. In healthy adults the pulmonary circulation is a high-flow, low-resistance system that is composed of vessels that carry blood from the right side of the heart through the lungs and then returning it to the left side of the heart. Deoxygenated blood from the upper body including head, neck and upper limbs is returned to the right atrium via the superior vena cava. Deoxygenated blood from elsewhere in the body is returned to the right atrium via the inferior vena cava. When the right atrium is filled with blood, it contracts, forcing blood though the tricuspid valve into the right ventricle. Blood from the right ventricle is pumped through the pulmonary valve and into the main pulmonary artery for distribution to the lungs. The main pulmonary artery bifurcates with one branch entering the right lung and the other entering the left lung. Inside the lungs, pulmonary arteries run parallel to the airways and branches (thought to number 15 orders) run to the level of the terminal bronchioles before forming the vast network of capillaries in the alveolar walls. It is in these capillaries that the exchange of carbon dioxide (CO_2) and oxygen (O_2) take place. Oxygenated blood from the capillaries is returned to the heart via venules and veins before entering the left atrium via four pulmonary veins, two from each lung.

1.1.1 Structure of the pulmonary circulation

Fundamentally, pulmonary arteries, capillaries and veins are structurally similar to their systemic counterparts. Pulmonary arteries, like systemic arteries have an innermost layer called the tunica intima (figure 1.1). This is composed of a single layer of endothelial cells lying on a basement membrane. The endothelial cells are in direct contact with the blood flowing through the vessel lumen. Separating the tunica intima from the tunica media is a layer of elastic tissue called the internal elastic lamina. The tunica media is principally a layer of smooth muscle and is much thinner in healthy pulmonary arteries compared with

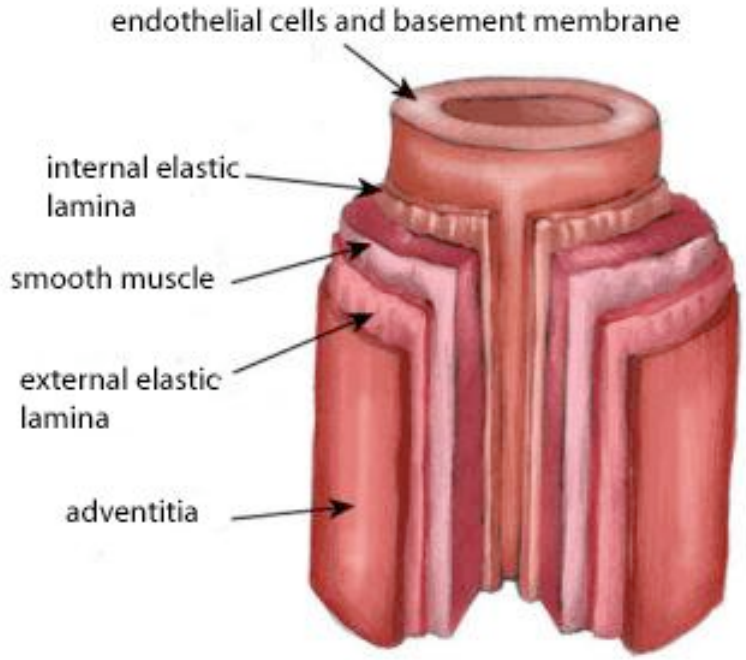


Figure 1.1 Structure of pulmonary artery. Illustration of the various layers that comprise the pulmonary arterial wall. Figure adapted from original image on teachPE.com.

corresponding systemic arteries. The role of the smooth muscle is to provide support for the vessel wall and to alter vessel diameter to regulate blood flow and pressure. A second layer of elastic tissue, the external elastic lamina, separates the tunica media from the tunica adventitia, a layer composed of connective tissue, elastic and collagen fibres in addition to other components such as dispersed fibroblasts and adipose cells. The connective tissue of the adventitia is dense in the region closest to the tunica media but becomes thinner towards the outer edges of the vessel wall where it disperses into the surrounding area.

Although pulmonary arteries are similar to systemic arteries, there are important differences between them. The main pulmonary artery is significantly thinner walled than the aorta and there is less smooth muscle in the wall of intra-lobar pulmonary arteries than in corresponding systemic vessels. Pre-capillary arteries in the lung are only partially muscularised and some lack smooth muscle altogether (Levitzky, 2002).

1.1.2 Pulmonary vascular resistance

One consequence resulting from the above differences in anatomy between the pulmonary and systemic circulations is that the pulmonary circulation has a low resistance to blood flow. Pulmonary vascular resistance (PVR) is about $1/10^{th}$ that of systemic vascular resistance (SVR) (Levitzky, 2002). Pulmonary artery pressure (PAP) is normally low with a mean pressure of ~15 mm Hg. This differs from a mean of ~100 mm Hg in systemic arteries (Hickey, 1996). PAP does, however, vary with age but does not usually exceed a mean pressure of 20 mm Hg in people aged below 60. In people aged beyond 60, PAP can vary depending on physiological and disease conditions (Mandegar et al., 2004). In addition, PAP in healthy people can be affected by factors such as altitude, sea diving or exercise (Mandegar et al., 2004). PAP is the product of CO and PVR (equation 1).

Equation 1 $$PAP = CO \times PVR$$

Equation 1) Relationship of pulmonary artery pressure, cardiac output and pulmonary vascular resistance.
PAP = pulmonary artery pressure, CO = cardiac output, PVR = pulmonary vascular resistance

The pulmonary and systemic circulations receive approximately the same CO; therefore, the lower PAP in the pulmonary circulation is entirely due to lower PVR. Even during periods of increased CO, PAP remains relatively stable. There are two mechanisms responsible for lowering PVR when intravascular pressures are increased. These are:

1) Recruitment. Vessels that were previously unperfused now start to conduct blood.
2) Distension. Vessels expand. A large radius equates with lower resistance (equation 2).

Equation 2, the Poiseuille equation, is a physical law that describes three factors that determine the resistance of a fluid (in this case blood) to flow in a cylindrical tube (such as a blood vessel). These factors are vessel length, fluid (blood) viscosity and inner vessel radius. The most important of these both quantitatively and physiologically is the vessel radius. Vessel length does not significantly alter and blood viscosity is usually maintained within a small range, although there are occasions during which blood viscosity may change by a greater degree. These are associated with changes in hematocrit levels or body temperature. Vessel radius can be changed by contraction/relaxation of the smooth muscle in the tunica media and as equation 2 shows, PVR is inversely proportional to the fourth power of the radius of the vessel (r^4), thus PVR is extremely sensitive to changes in vessel radius. This is illustrated in a graph of relative radius of a single vessel plotted against relative flow (figure 1.2). From this graph, it is clear that even very small changes in vessel radius can have dramatic effects on blood flow. Both equation 2 and figure 1.2 assume constant laminar flow, constant viscosity and a steady pressure flow. They also

assume the length of the vessel to be held constant and the vessel to be rigid. Although blood flowing through a vessel does not behave in such an ideal manner i.e. does not behave as a Newtonian fluid, the Poiseuille equation is sufficient for conveying the importance that vessel radius has on determining PVR.

The relationship governing the radius of a single vessel to the total resistance of the lung is determined by the structure of the pulmonary arterial tree. In the pulmonary arterial tree vessels are arranged either in series or in parallel. A vessel is in series with its daughter branches and each of these branches is in parallel to each other (Richardson et al., 1998). The total resistance of vessels connected in series is the sum of each individual vessels resistance (equation 3). Conversely, when vessels are connected in parallel, the reciprocal of the total resistance is equal to the sum of the reciprocals of each individual vessels resistance (equation 4). The total resistance of vessels connected in parallel will, therefore, always be less than the resistance of the single vessel with the lowest individual resistance (equation 4). From the resistance equations: 3 and 4, it is deducible that the greater the number of vessels that exist in parallel networks, the lower the overall resistance of the system will be, while the greater the number of vessels there are in series, the greater the overall resistance. This explains why, as the radius of pulmonary arteries become exponentially smaller from order 1 to 15, total resistance does not increase. The radius might decrease exponentially but the numbers of branches increase exponentially creating a greater number of arteries connected in parallel and thus maintaining a low PVR.

Equation 2 $$PVR = \frac{8L\eta}{\pi r^4}$$

Equation 2) The Poiseuille equation for determining the relationship of pulmonary vascular resistance to blood flow
PVR = pulmonary vascular resistance, L = length of vessel, ŋ = viscosity of blood, r = inner radius of vessel

Equation 3 $$R_T = R_1 + R_2 + R_3 \ldots + \mathbf{R}_n$$

Equation 3) Relationship of individual vessel resistance to total resistance for a series network

Equation 4 $$\frac{1}{R_T} = \frac{1}{R_1} + \frac{1}{R_2} + \frac{1}{R_3} + \ldots + \frac{1}{R_n}$$

Equation 4) Relationship of individual vessel resistance to total resistance for a parallel network

1.1.2.1 Regulation of pulmonary vascular resistance

The regulation of vascular resistance in the pulmonary circulation is different to the systemic circulation due to the unique properties of the pulmonary vasculature. In the systemic circulation arterioles are the major site of vascular resistance whereas in the lung, vessels of all sizes contribute to PVR. Unlike the systemic circulation, pulmonary vessels have a low basal tone, display little or no autoregulation and their response to autonomic neuronal control is not well understood (Richardson et al., 1998). It is generally accepted that PVR is regulated by mechanisms that fall into two categories: 1) passive control and 2) active control.

1.1.2.1.1 Passive control

Passive control involves changes in vessel radius/diameter independent of a change in vascular tone. Distension and recruitment of vessels is able to occur due to the low basal tone of pulmonary vessels. Small pulmonary vessels also have a larger radius than their systemic counterparts and these properties allow the lung to accommodate greater CO; for

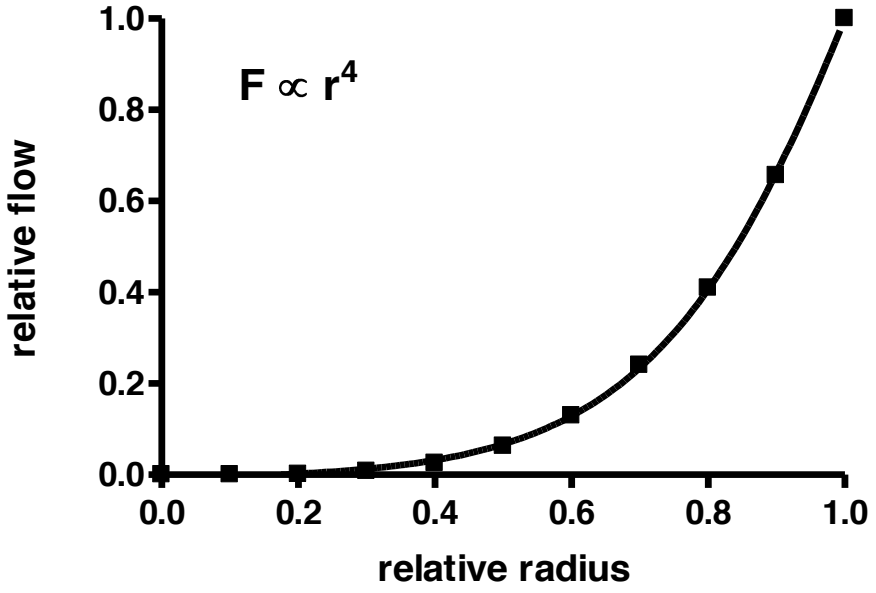


Figure 1.2 Relationship of relative radius to relative flow in a single vessel.
Graph illustrating the mathematical relationship between radius of vessel to flow within that vessel. PVR is inversely proportional to the fourth power of the radius of the vessel (r^4), F = blood flow, r = vessel radius. Reproduced from Klabunde, 2004.

example, during times of exercise, while maintaining a low PVR.

Gravity has a significant effect on the distribution of blood within the lung. In an upright person of average height the lowest point of the lungs is approximately 30 cm lower than the highest point. The consequence of gravity on the lung is a pressure difference between the top part and the bottom part of the lungs. The top part will have a PAP 15 mmHg lower than the mean PAP and in the lower part of the lung, PAP will be 8 mmHg higher than mean PAP (Guyton, 1981). These pressure differences have important effects on blood flow within the lung. In an upright person, there is low blood flow in the top section of the lung. Conversely, in the lower lung, blood flow may be 5-10 times greater than at the top (Guyton, 1981). As a consequence of hydrostatic pressure, the lung can be divided into three zones to represent the different dynamics of pulmonary blood flow:

Zone 1: The apical regions of the lung which are predominately above the level of the heart. At this level, pulmonary vascular pressure tends to be lower than alveolar pressure (mean of ~ 0 mmHg) at both arterial and venular ends of the pulmonary capillaries. This results in collapse of a large number of capillaries shutting off blood flow. Zone 1 therefore is an area of no blood flow.

Zone 2: Vessels in this region lie just above heart level. Arterial pressure is positive (higher than alveolar pressure) and higher than venous pressure which remains negative (lower than alveolar pressure). Therefore the venous ends of capillaries remain collapsed. In Zone 2 blood flows intermittently, with capillaries open during systole and collapsed during diastole.

Zone 3: In the lowest region of the lung, pulmonary venous pressure is generally higher than alveolar pressure and consequently capillaries are open at all times and blood flows continuously from the arteries to the veins.

1.1.2.1.2 Active control

Active control involves a wide range of biological factors capable of altering smooth muscle contraction/relaxation and consequently vascular tone. The mechanisms by which vascular tone is actively regulated are:

1) Neuronal control
2) Humoral and endothelial derived factors
3) Oxygen tension

1.1.2.1.2.1 Neuronal control

The autonomic nervous system is composed of sympathetic (adrenergic) and parasympathetic (cholinergic) nerves and both are found in the pulmonary vasculature; however, their relative contribution to the regulation of vascular tone has proved difficult to ascertain (Downing and Lee, 1980). Using fluorescent imaging, adrenergic nerves are easily indentified in the adventitia of human pulmonary arteries and veins. In large elastic pulmonary arteries, nerve fibres penetrate the outer third of the media but in smaller muscular arteries, there is no such innervation of the smooth muscle layer. No adrenergic fibres are found in human pulmonary arteries smaller than 30 μM (Downing and Lee, 1980). Fluorescent histochemistry in other mammals has shown that adrenergic nerves also penetrate medial smooth muscle in cats, rabbits, guinea pigs and hares although the levels of innervation vary between species and some species such as the hedgehog, rat and mouse display no sympathetic fluorescent staining at all in pulmonary arteries (Cech, 1969; Knight et al., 1981) .

Pulmonary arterial smooth muscle cells (PASMCs) express α_1 and α_2-ardrenoceptors (with α_1 mediating vasoconstriction) in addition to β2-adrenoceptors that mediate vasorelaxation (Porcelli and Bergofsky, 1973; Hyman et al., 1981; Hyman et al., 1986).

Release of noradrenaline from sympathetic nerve terminals or an increase in circulating catecholamine levels leads to vasoconstriction through activation of post-synaptic α_1-ardrenoceptors. In the healthy lung, a β2-adrenergic effect helps maintain a vasodilator, anti-proliferative balance (Salvi, 1999) but during periods of stress such as alveolar hypoxia and hypercapnic acidosis, a pro-vasoconstrictor effect results, mediated in part, by α_1-adrenoceptors (Porcelli et al., 1977). α_1-Adrenoceptors in pulmonary arteries show an increased affinity to noradrenaline compared with other vessels (Bevan et al., 1986) and this may be important in the regional regulation of vascular tone within the lung.

Morphological analysis of cholinergic innervation of the pulmonary vasculature has largely relied on acetylcholinesterase staining as the most practical and reliable means of identifying cholinergic nerves. As with adrenergic nerves, cholinergic nerves have been found in association with pulmonary arteries in many species (Downing and Lee, 1980), where they are found in the adventitia and medial smooth muscle (Cech, 1973; Al-Wiel-Bermani et al., 1982; Wojtarowitz et al., 2003) with the greatest density of innervation occurring in larger vessels (Downing and Lee, 1980; Haberberger et al., 1997). Inter-species differences are pronounced, with rats and mice showing little intra-pulmonary cholinergic innervation (Cech, 1973), unlike larger mammals such as rabbits and pigs that have a greater density of cholinergic nerves (Al-Wiel-Bermani et al., 1982; Wojtarowitz et al., 2003). The extent of cholinergic innervation in the human lung is not well understood. Amenta et al., 1983 found cholinergic innervation in human pulmonary arteries where it was found to be denser than in veins. Partanen et al., 1982, however, found no cholinergic nerves associated with blood vessels in the human lung. Regardless of cholinergic innervation, pulmonary arteries in the human and other mammals are responsive to

acetylcholine (Ach). Under conditions of low basal tone, Ach exerts a vasoconstrictor effect on pulmonary arteries. However, when vascular tone is increased, Ach exerts a vasodilator effect (Nandiwada et al., 1983; Hyman and Kadowitz, 1988). Ach induced relaxation is endothelium dependent and involves activation of M_1 and M_3 muscarinic receptors whereas Ach induced vasoconstriction involves activation of M_1 receptors on smooth muscle (Norel et al., 1996).

There are other neuronal influences within the pulmonary vasculature that are neither adrenergic nor cholinergic. These are known as nonadrenergic, non cholinergic (NANC) nerves and can have vasoconstrictor and vasodilator effects (Inoue and Kannaan, 1988; Liu et al., 1992). Adenosine triphosphate (ATP) is involved in both the vasoconstrictor and vasodilator effect while nitric oxide (NO) is involved in the vasodilator response (Inoue and Kannan, 1988; Liu et al., 1992; Gumusel et al., 2001). NO dilates both systemic and pulmonary vessels and is synthesised in the vessel wall from L-arginine by NO synthase (NOS) of which there are three isoforms (endothelial (eNOS), inducible (iNOS) and neuronal (nNOS)) and all three are expressed in the lung (Le Cras and McMurtry, 2001). eNOS is the major isoform responsible for NO synthesis in pulmonary arteries with the other two isoforms making minor contributions (Strange et al., 2002). The extent to which NO produced in the airways can influence vascular tone is not clear. The vasodilator effect of NO is mediated by cyclic guanosine monophosphate (cGMP) and protein kinase G (PKG).

1.1.2.1.2.2 Humoral and endothelial derived factors

In addition to adrenergic, cholinergic and NANC factors the pulmonary vasculature is exposed to many other vasoactive compounds and a tight regulation between those that are vasoconstrictors and those that are vasodilators is essential for maintaining low vascular tone. Important vasoconstrictors of the pulmonary circulation include angiotensin II, a key

effector of the renin-angiotensin system and a potent pulmonary vasoconstrictor. Angiotensin-converting enzyme (ACE) generates angiotensin II from angiotensin I and the capillaries in the lung have a high expression of ACE (Studdy et al., 1983).

Thromboxane A_2 (TXA_2) is derived from arachidonic acid metabolism in platelets and is a constrictor of pulmonary vessels (Buzzard et al., 1993).

In contrast to its role as a vasodilator in the systemic circulation, histamine acts as a vasoconstrictor in pulmonary vessels, acting via H_1 receptors (Tucker et al., 1975).

Endothelin – 1 (ET-1) is a member of a family of vasoconstrictor peptides termed the endothelins that are synthesised from large precursor molecules: the big endothelins, by endothelin-converting enzymes (ECEs) present in endothelial cells (Yanagisawa et al., 1988). Vascular smooth muscle cells can also synthesise ET-1 under inflammatory conditions (Woods et al., 1999). ET-1 acts via ET_A and ET_B receptors to induce vasoconstriction. Which of these receptors mediates the response is dependent on the species being assessed, the size of the blood vessel and the local concentration of ET-1 (Fukuroda et al., 1994; Maclean et al., 1994; McCulloch et al., 1996).

Serotonin (5-HT) which is stored primarily in platelets is a pulmonary vasoconstrictor (Gruetter et al., 1981; McGoon and Vanhoutte, 1984). The major site of serotonin synthesis is in the enterochromaffin cells of the intestine, however serotonin is also released locally in the lung from neuroendocrine and neuroepithelial cells in the airways (MacLean et al., 2000) and from pulmonary arterial endothelial cells (PAECs) (Eddahibi et al., 2006). 5-HT induced vasoconstriction occurs through the $5\text{-}HT_{1B}$ receptor in human pulmonary resistance vessels (Morecroft et al., 1999) with both $5\text{-}HT_{1B}$ and $5\text{-}HT_{2A}$ receptors contributing to vasoconstriction in rats (MacLean et al., 1996).

There are other circulating factors including prostaglandins of the F series and leukotrienes that also act as pulmonary vasoconstrictors (Back et al., 2002; Snetkov et al., 2006).

In addition to NO, whose vasodilator action was discussed in relation to NANC agents, important vasodilators include prostacyclin (PGI_2) which is a vasodilator in all vascular tissues including the lung (Vane and Botting, 1995). Like TXA_2, PGI_2 is derived from arachadonic acid and is, in fact, the major product of this metabolic pathway in all vascular tissues tested (Vane and Botting, 1995). PGI_2 is a short acting but potent vasodilator synthesised in endothelial cells that induces vasodilation through increasing cyclic adenosine monophosphate (cAMP) and protein kinase A (PKA).

Vasoactive intestinal peptide (VIP) belongs to the glucagon-growth hormone-releasing factor secretion superfamily and acts as a pulmonary vasodilator by activating VIP receptors (VPAC-1 and VPAC-2) that are present on the surface of smooth muscle cells in pulmonary blood vessels (Busto et al., 2000). Stimulation of these receptors leads to activation of both cAMP and cGMP.

A related compound with similar sequence homology to VIP is pituitary adenylate cyclase-activating peptide (PACAP) and it possesses similar activity to VIP (Bruch et al., 1998; Cardell et al., 1997). PACAP also binds to VPAC-1 and VPAC-2 in addition to the PAC1 receptor which is widely expressed in the lung (Busto et al., 2000). PAPAC unsurprisingly signals mainly via cAMP but some of its actions appear mediated by other mechanisms (Said, 2006).

Natriuretic peptides (NPs) are a family of peptides comprised of atrial or A-type NP (ANP), brain or B- type NP (BNP) and C-type NP (CNP) that interact with three receptor subtypes, NPR-A, NPR-B and NPR-C and mRNA for all three receptors has been found in the lung (Li et al., 1995). ANP and BNP are responsible for the vasodilation seen in the lung (Lindberg and Andersson, 1988; Zhao et al., 1999), an effect occurring through an increase in cGMP following ligand activation of the guanylate cyclase-linked NPR-A and NPR-B receptors. Knockout mice studies have shown that NPR-A is solely responsible for the vasorelaxant effect of ANP and BNP in this species (Zhao et al., 1999).

Adrenomedullin is a 52-amino acid peptide originally isolated from human pheochromcytoma. It is produced by vascular smooth muscle and endothelial cells (Sugo et al., 1994) in addition to many other cell types (Marutsuka et al., 2003). It is expressed at high levels in the lung (Kitamura et al., 1993) and is a vasodilator in both the pulmonary and systemic circulation (Marutsuka et al., 2003), an action mediated by cAMP.

1.1.2.1.2.3 Oxygen tension

Changes in the partial pressure of O_2 (PO_2) has the opposite effect on the pulmonary circulation to that on the systemic circulation. In the systemic circulation, a decrease in PO_2 (hypoxia) induces vasodilation which increases blood flow to hypoxic regions. In contrast, when alveoli are poorly ventilated resulting in a low PO_2, pulmonary arteries constrict, preventing blood flow to hypoxic regions and diverting blood to better ventilated regions (George et al., 2005). O_2 diffuses through the alveoli wall and into surrounding smooth muscle cells and a reduction in PO_2 in the air spaces of the alveoli results in vasoconstriction of arteries in the hypoxic region (hypoxic pulmonary vasoconstriction; HPV). HPV occurs primarily in small pre-capillary arterioles in the hypoxic region when PO_2 falls below 60 mmHG (Yuan, 2004). The size of the hypoxic region can vary enormously from a lobule to the entire lung. If only a small region is hypoxic, then blood flow can be diverted to more compliant, better perfused areas with only small rises in PAP. If, however, the vast majority of the lung is hypoxic, the result is widespread HPV and a rise in PAP, causing the heart to pump faster to overcome the associated rise in PVR (Yuan, 2004).

In the clinical setting, widespread alveolar hypoxia can occur as acute HPV (AHPV) in rapidly progressing acute lung conditions including asthma, pneumonia and acute respiratory distress syndrome (Dumas et al., 1999). The resulting AHPV can have detrimental effects including reduced CO, increased vascular permeability and right

ventricular failure (Leach and Treacher, 1995). AHPV is rapidly reversible if alveolar oxygenation is restored to normal physiological values.

Widespread, sustained chronic HPV (CHPV) occurs in people living at high altitudes, as a result of diseases associated with decreased ventilation such as chronic obstructive pulmonary disease (COPD) and sleep apnea or in association with diseases that cause progressive lung destruction such as emphysema and fibrosing alveolitis (Dumas et al., 1999). CHPV does not just cause an increase in vascular tone but also vascular remodelling (see section 1.2.3.2.1) and together, they lead to a sustained increase in PAP (pulmonary hypertension) that is not reversible on return to normal alveolar PO_2.

The processes underlying HPV are not well understood and in many experiments HPV presents a biphasic response (depending on experimental preparation and species tested). There is an initial transient vasoconstriction (phase 1) followed by vasorelaxation before a sustained period of vasoconstriction (phase 2). If this sustained vasoconstriction exists for a prolonged period of time then the symptoms associated with CHPV manifest. HPV must involve an O_2 sensor in addition to signal transduction to the contractile machinery of the smooth muscle. Evidence suggests that the O_2 sensor mediating phase 1 HPV is present on the smooth muscle cells themselves as both isolated pulmonary arteries (Madden et al., 1985) and subsequently isolated PASMCs (Madden et al., 1992) were found to contract when exposed to hypoxic conditions. This would rule out neuronal or circulating vasoactive mediators as the O_2 sensor and narrow the search to factors intrinsic to PASMCs. Many factors have been put forward as candidates for the O_2 sensor including O_2-sensitive calcium (Ca^{2+}) channels, O_2-sensitive potassium (K^+) channels and the mitochondria, through changes in mitochondrial reactive oxygen species (ROS) production (Dumas et al., 1999). Whichever mechanism is involved in detecting changes in PO_2, it ultimately leads to an increase in intracellular calcium concentration $[Ca^{2+}]_i$, a critical component of HPV and PASMC contraction in general. There is extensive evidence

supporting the theory that hypoxia inhibits voltage-gated K^+ (Kv) channels causing membrane depolarisation and opening of L-type Ca^{2+} channels causing Ca^{2+} influx across the membrane and into the PASMC. The method by which hypoxia inhibits Kv channels is controversial. Some researchers believe Kv channel inhibition to be a consequence of reduced hydrogen peroxide (H_2O_2). Low H_2O_2 levels result from a reduction in mitochondrial electron transport and superoxide ion production, a direct consequence of decreased O_2 (Aaronson et al., 2006). Evidence exists, however, to support an alternative theory also involving a change in redox state, this time involving an increase in mitochondrial ROS production and release of Ca^{2+} from intracellular stores and store operated Ca^{2+} entry (SOCE) (Aaronson et al., 2006; Waypa and Schumacker, 2005). Despite the differences in these two theories, phase 1 vasoconstriction may be dependent on both voltage-gated Ca^{2+} channels and SOCE (Weigand et al., 2005).

Unlike the transient phase 1, the sustained muscle contraction of phase 2 requires more than a high $[Ca^{2+}]_i$, which stays constant in phase 2 as tension rises (Robertson et al., 2003). Phase 2 is dependent on the presence of endothelium and removal of the endothelium abolishes the sustained phase but not the initial transient phase of HPV. $[Ca^{2+}]_i$ is unaffected by endothelial removal (Robertson et al., 2003). Phase 2 may then involve two processes: one that maintains $[Ca^{2+}]_i$, at the increased level induced during phase 1 and a second, endothelium dependent process, that increases Ca^{2+} sensitisation in PASMCs. An increase in Ca^{2+} sensitisation would mean that agonists binding to G-protein coupled receptors would induce greater smooth muscle contraction at a constant $[Ca^{2+}]_i$. This process is thought to involve Rho-A mediated activation of Rho kinase (ROCK) (Loirand et al., 2006) and inhibition of ROCK with the ROCK antagonist Y-27632 prevents the sustained HPV in CHPV (Robertson et al., 2000). The question then becomes: what is the endothelial derived vasoconstrictor that causes ROCK dependent Ca^{2+} sensitisation and sustained HPV? The primary candidate is ET-1 which can increase

the sensitivity of the contractile apparatus to hypoxic induced increases in $[Ca^{2+}]_i$. (Waypa and Schumacker, 2005). Hypoxia also increases ET-1 expression and secretion in cultured endothelial cells and BQ-123, an ET_A receptor antagonist inhibits CHPV in isolated vessels and whole lung (Waypa and Schumacker, 2005). Additionally, the promoter of the ET-1 gene contains a hypoxia-inducible factor (HIF) response element (HRE; Hu et al., 1998). HIF-1α along with HIF-2α and HIF-3α regulate the expression of most hypoxic-dependent and many hypoxic-independent genes involved in physiological and pathophysiological lung states (Tuder et al., 2007) (See section 1.2.3.2.1). In contrast to a supportive role for ET-1 in CHPV, Roberston et al., 2003 found that CHPV in the rat was unaffected by combined ET_A and ET_B receptor blockade and CHPV in salt-solution perfused lungs is not significantly affected by ET-1 receptor antagonists (Aaronson et al., 2002) so there maybe additional hypoxic induced vasoconstrictor substances released from the endothelium that are yet to be determined and experimental evidence exists to support this idea (Gaine et al., 1998; Robertson et al., 2001).

Research into the underlying mechanisms of HPV over the past 30 years has yielded diverse and often contradictory theories regarding the mediators and modifiers of HPV. Many of these differences are likely due to differences in preparation e.g. location and size of pulmonary artery, species, blood or salt perfused lung, duration of hypoxia and importantly, the level of pre-tone in the vessel being studied. The level of existing tone in a muscle preparation and the stimulus used to induce that tone can have dramatic effects on the subsequent response to hypoxia (Aaronson et al., 2006). Figure 1.3 illustrates the possible mechanisms currently thought to occur in both phase 1 and phase 2 of HPV. In addition to the mediators of HPV outlined in figure 1.3 there are a host of other vasoactive factors already mentioned previously capable of modulating either positively or negatively the degree of HPV. These include NO, prostaglandins, angiotensin II and of particular relevance to this investigation, 5-HT (Dumas et al., 1999; Aaronson et al., 2002). Hypoxia

can increase circulating 5-HT levels (Callebert et al., 2006) and increase expression of tryptophan hydroxylase 1 (TPH1) (Pan et al., 2006), the rate-limiting enzyme in 5-HT synthesis in the periphery. An increase in 5-HT could result in increased activation of the vasoconstrictor receptors, 5-HT_{1B} and 5-HT_{2A}, which can interact with many of the pathways thought to mediate HPV (figure 1.3). (See section 1.2.3.1 for more information on the role of 5-HT in hypoxia). HPV only occurs in the pulmonary circulation and is the most important regulatory mechanism for controlling vascular tone. Sustained HPV over a prolonged period of time (CHPV) is a major contributing factor to pulmonary arterial hypertension (PAH) associated with several hypoxic lung diseases and PAH induced by hypoxia is a model often used to study PAH in rodents and will be used in this investigation (see sections 1.2.3.2.1 and 2.1.5).

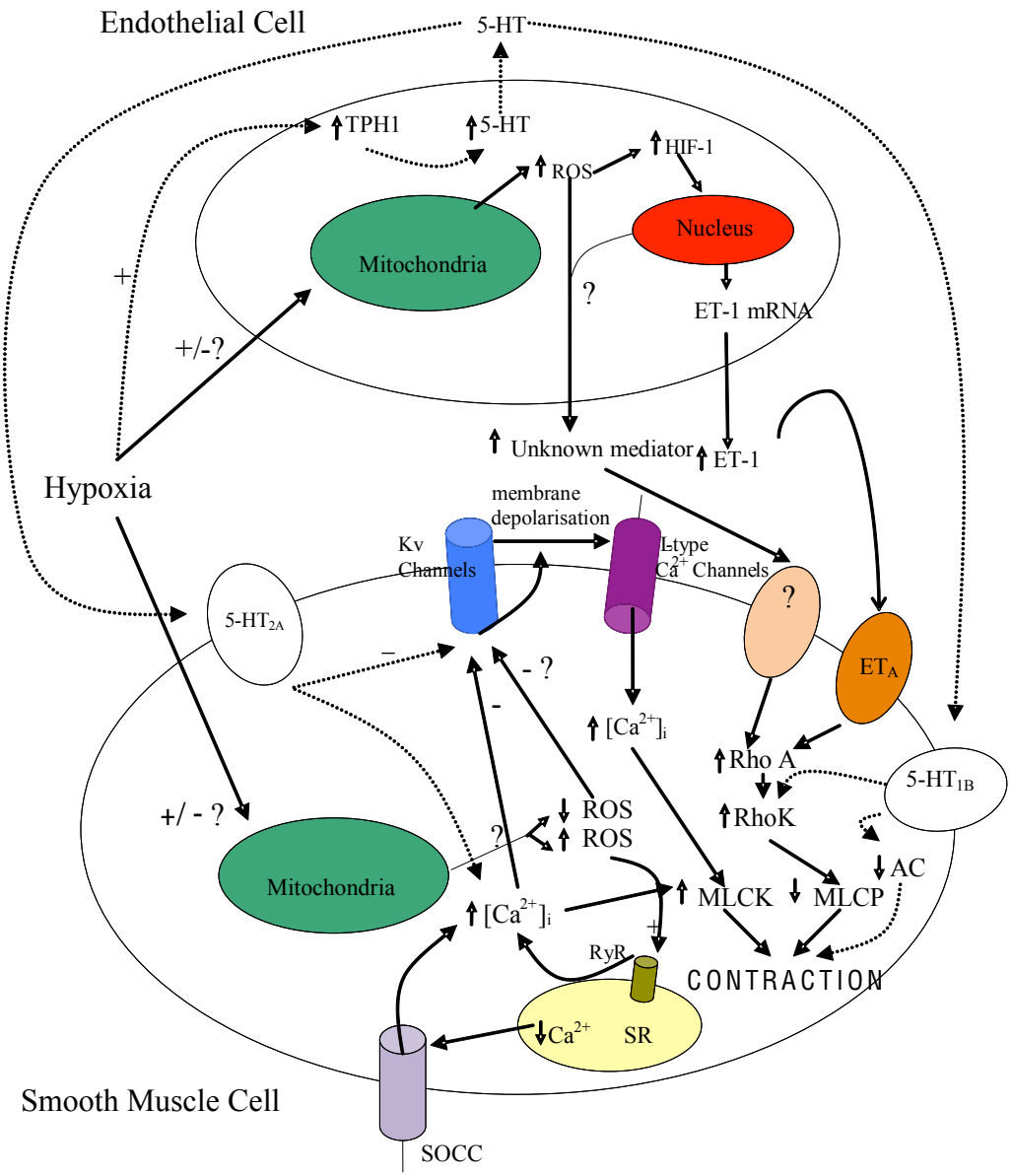


Figure 1.3 Possible signalling mechanisms involved in phase 1 and phase 2 of hypoxic induced vasoconstriction in pulmonary arteries.

Solid arrows represent pathways suggested to contribute to the physiological regulation of pulmonary artery tone during hypoxia. Dashed arrows represent the possible modulating influence of 5-HT signalling on these pathways.

ROS, reactive oxygen species; HIF-1, hypoxia-inducible factor-1; ET-1, endothelin-1; ET_A, endothelin receptor A; RhoK, Rho kinase; MLCK, myosin light chain kinase; MLCP, myosin light chain phosphatase; SR, sarcoplasmic reticulum; SOCC, store-operated calcium channel; RyR, ryanodine receptor; AC, adenylate cyclase; TPH1, tryptophan hydroxylase 1

1.2 Pulmonary arterial hypertension

PAH is characterised by an elevation in PAP and PVR. The raised PVR increases the work load of the right ventricle which is required to overcome the pressure increases. Over time this causes right ventricular failure. Clinically, PAH is defined by a mean PAP > 25 mmHg at rest or > 30 mmHg during exercise (Rich et al., 1987). There are often few symptoms in the early stages but as the disease progresses, patients present with symptoms including dyspnea, fatigue, edema and syncope (Rich et al., 1987). The non specific nature of these symptoms often means diagnosis is delayed and the progressive nature of the disease means, that if left untreated, death usually occurs within 3 years of diagnosis (D'Alonzo et al., 1991). Sporadic PAH is rare with an incidence of 1-2 cases per million per year (Abenhaim et al., 1996; Gaine and Rubin 1998). The mean age at diagnosis is 36 and the disease occurs more frequently (~2:1) in women than in men (Loyd et al., 1984; Rich et al., 1987).

Over five decades ago, the term primary pulmonary hypertension (PPH) was introduced to describe the condition of increased PAP and associated vascular changes occurring exclusively in the pulmonary circulation without an identifiable cause. Additionally, secondary pulmonary hypertension (SPH) was the term used to describe pulmonary hypertension associated with an underlying disease condition. Over the past few decades, advancements in our understanding of both the clinical and pathological aspects of pulmonary hypertension has led to the need for a more useful and accurate classification system. A revised classification for pulmonary hypertension was introduced following a World Health Organisation symposium on the condition in Evian, France in 1998. The term SPH was abandoned and the new classification comprised five groups to reflect similarities in clinical and etiological features. The first group was PAH which included PPH. The other four groups included conditions that predominately affected the venous circulation or conditions that affected the pulmonary circulation through changes in

respiratory structure or function (Mandegar et al., 2004). These groups were pulmonary venous hypertension, pulmonary hypertension associated with disorders of the respiratory system and/or hypoxaemia, pulmonary hypertension due to chronic thrombotic and/or embolic disease and pulmonary hypertension due to disorders affecting the pulmonary vasculature directly. Further advancements in understanding the pathophysiology of pulmonary hypertension including the uncovering of a genetic basis for PAH meant that a second revision to the classification system was necessary and this took place at the third World Conference on Pulmonary Hypertension in Venice, Italy in 2003. The most important revision was the replacement of the term PPH with idiopathic PAH (IPAH) or familial PAH (FPAH) when an identifiable genetic cause is found. All other forms of PAH that are related to risk factors and disease conditions were termed associated PAH (APAH). The revised Venice classification is presented in table 2.1. Despite the different clinical classifications of PAH, it is often difficult, if not impossible, to distinguish PAH of a particular etiology with that from another based on histopathological evidence alone.

1.2.1 Pathophysiology of PAH

Pulmonary arterial vasoconstriction, remodelling of the pulmonary vasculature and in situ thrombosis all contribute to an increase in PVR. As discussed previously, this increase in PVR increases the work load on the right ventricle and progressively leads to right ventricular hypertrophy (RVH) and heart failure. Of these three contributing factors, vascular remodelling is the most important pathological change in PAH and the extent of remodelling at diagnosis will determine the prognosis for the patient.

Revised Clinical Classification of Pulmonary Hypertension (Venice 2003)

1. Pulmonary arterial hypertension (PAH)
1.1. Idiopathic (IPAH)
1.2. Familial (FPAH)
1.3. Associated with (APAH):
    1.3.1. Collagen vascular disease
    1.3.2. Congenital systemic-to-pulmonary shunts
    1.3.3. Portal hypertension
    1.3.4. HIV infection
    1.3.5. Drugs and toxins
    1.3.6. Other (thyroid disorders, glycogen storage disease, Gaucher disease, hereditary hemorrhagic telangiectasia, hemoglobinopathies, myeloproliferative disorders, splenectomy)
1.4. Associated with significant venous or capillary involvement
    1.4.1. Pulmonary veno-occlusive disease
    1.4.2. Pulmonary capillary hemangiomatosis
1.5. Persistent pulmonary hypertension of the newborn

2. Pulmonary hypertension with left heart disease
2.1. Left-sided atrial or ventricular heart disease
2.2. Left-sided valvular heart disease

3. Pulmonary hypertension associated with lung diseases and/or hypoxemia
3.1. Chronic obstructive pulmonary disease
3.2. Interstitial lung disease
3.3. Sleep-disordered breathing
3.4. Alveolar hypoventilation disorders
3.5. Chronic exposure to high altitude
3.6. Developmental abnormalities

4. Pulmonary hypertension due to chronic thrombotic and/or embolic disease
4.1. Thromboembolic obstruction of proximal pulmonary arteries
4.2. Thromboembolic obstruction of distal pulmonary arteries
4.3. Non-thrombotic pulmonary embolism (tumor, parasites, foreign material)

5. Miscellaneous
Sarcoidosis, histiocytosis X, lymphangiomatosis, compression of pulmonary vessels (adenopathy, tumor, fibrosing mediastinitis)

Table 1.1 Revised Clinical Classification of Pulmonary Hypertension (Venice, 2003). The classification system contains groups classified according to clinical and etiological parameters.

1.2.1.1 Pulmonary vascular remodelling

In the non-hypertensive lung, the thickness of the pulmonary arterial wall is maintained relatively constant by the balance between proliferation and apoptosis of the cells present in the vessel wall i.e. PASMCs, PAECs and pulmonary arterial fibroblasts (PAFs). Any disruption in this balance towards a pro-proliferative phenotype will lead to vascular wall thickening, lumen occlusion and loss of vascular compliance (Mandegar et al., 2004). Together, these cellular changes create the vascular remodelling that occurs in PAH and has the ultimate effect of progressively increasing PVR (Mandegar et al., 2004). Morphological analysis of pulmonary hypertensive lungs reveals distal extension of smooth muscle into previously non muscularised vessels, obliteration of small pulmonary arteries associated with adventitial and medial hypertrophy, concentric laminar intimal lesions and fibrosis. Also present may be complex plexiform lesions which occur more frequently in severe PAH and are associated with a poor prognosis for the patient (Strange et al., 2002). The cellular processes governing the vascular remodelling in PAH are still to be fully elucidated with complex vascular changes occurring. These changes include vascularisation of the adventitia and outer media, cell proliferation and differentiation within all levels of the vessel wall, altered apoptosis and recruitment of circulating inflammatory cells. The cellular changes associated with vascular remodelling are now summarised.

1.2.1.1.1 Intimal thickening and plexiform lesions

Intimal thickening may be eccentric or concentric, resulting in narrowing and obliteration of the lumen and occurs most frequently in arteries <200 μm, with no intimal thickening seen in arteries >400 μM (Yi et al., 2000). Intimal thickening is the greatest contributor to the reduction of luminal area seen in PAH with the thickness of the intima increasing, on average, 30 fold (Chazova et al., 1995). Intimal lesions display varying levels of

complexity with localised thrombi thought to contribute to the formation of some, while more advanced lesions can become fibrotic in nature (Tuder et al., 2007). In general, concentric lesions are more pathological than eccentric lesions and characterise severe PAH. Concentric lesions can completely obliterate the vessel lumen whereas eccentric lesions cause only partial luminal obstruction (Yi et al., 2000) (figure 1.4 A-C).

Plexiform lesions are complex intimal lesions that occur at arterial branching points in arteries >200 μM (Chazova et al., 1995) (figure 1.4 D) and are glomeruloid structures containing a network of small vessels lined with PAECs (Tuder et al., 1994). Additionally, myofibroblasts are present in the core of plexiform lesions and there is some debate regarding the relevant importance of both cell types in the origin of these lesions. Smith et al., 1990 and Yi et al., 2000 suggest that myofibroblast proliferation is the key initiator in the formation of the plexiform lesions while others believe dysfunctional PAEC growth following vascular injury or stimulation by growth factors is the essential mediator (Tuder et al., 1994; Cool et al., 1999). There is growing evidence to support the latter viewpoint and that plexiform lesions may be a form of angiogenesis related to phenotypic changes in PAECs (Cool et al., 1999). PAECs within plexiform lesions of IPAH patients display monoclonal expansion whereas expansion is polyclonal in patients with PAH secondary to other diseases (Lee et al., 1998). The polyclonal PAEC expansion in APAH is likely due to PAEC growth induced by high shear stress or an inflammatory response (Lee et al., 1998). The growth factors, platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF) can be increased in vascular endothelium following high shear stress (Malek et al., 1993) and these growth factors may contribute to PAEC growth. FGF and PDGF gene expression increases with increasing shear stress and can be as much as 4.8 fold higher during periods of high shear stress (Malek et al., 1993). The monoclonal PAEC expansion seen in plexiform lesions in IPAH cases results from a somatic mutation in one PAEC that confers a growth advantage. The process therefore resembles the neoplastic growth

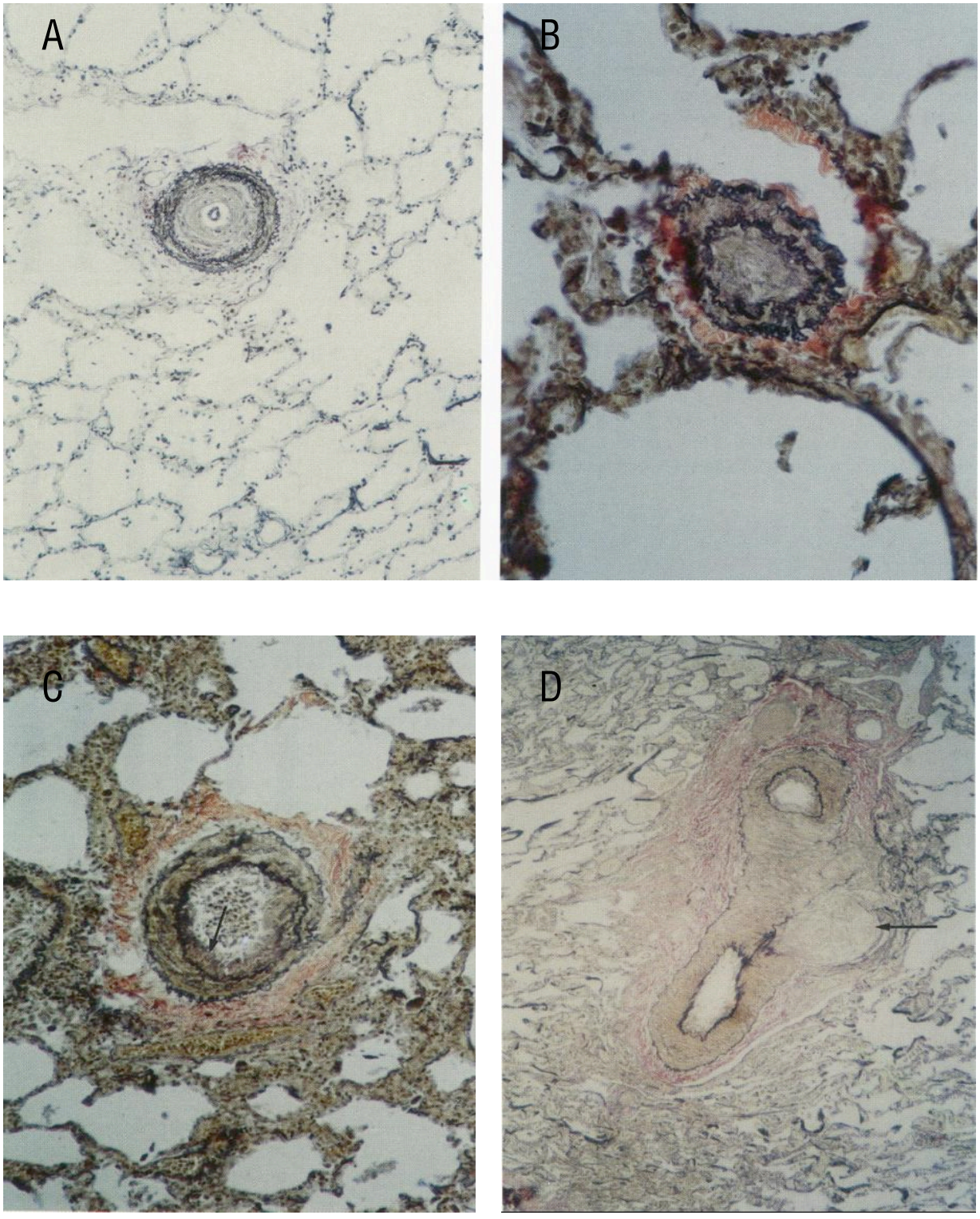


Figure 1.4 Micrographs of pulmonary arteries from pulmonary hypertensive patients. A: Concentric intimal thickening in a pulmonary artery (~180 μM diameter; magnification x 100). B: Obliteration of lumen in a pulmonary artery (~ 80 μM diameter; magnification x 260). C: Eccentric intimal thickening (at arrow) in a pulmonary artery (~220 μM diameter; magnification x 130). D: Plexiform lesion (at arrow) associated with a muscular pulmonary artery (magnification x 110). Reproduced from Chazova et al., 1995.

processes common in cancer and somatic mutations in genes associated with cellular proliferation and apoptosis have indeed been found in PAECs within plexiform lesions from PAH patients (Yeager et al., 2001). Plexiform lesions are extremely detrimental to normal blood flow with a single lesion blocking blood flow for the length of the vessel (Cool et al., 1999). PAECs are also present in the concentric intimal lesions and these occur in association with plexiform lesions (Cool et al., 1997; Cool et al., 1999) often forming hybrid lesions in the pulmonary hypertensive lung that share both concentric and plexiform lesion features (Cool et al., 1997). It may be that rather than existing as distinct lesions, a number of the intimal lesions seen in PAH are lesions at different stages of evolution and that distinct endothelial cell phenotypes determine the development of the lesion (Cool et al., 1999). Contrary to a role for PAECs in concentric lesions, other studies have suggested concentric intimal lesions are composed of myofibroblasts (Atkinson et al., 2002); however, the origin of these myofibroblasts is unknown. Endothelial cells can transdifferentiate into smooth muscle cells when stimulated with growth factors such as transforming growth factor- β (TGF-β) (Frid et al., 2002); therefore, it is possible, although speculative, that the PASMC composed and PAEC composed lesions derive from common progenitor cells. Although the cellular and molecular determinants of the intimal lesions in PAH remains largely unknown they are likely to involve some or all of the following depending on lesion type and form of PAH;

- Injury to vascular endothelium
- Increase proliferation and reduced apoptosis of PAECs
- Smooth muscle cell growth, migration and reduced apoptosis
- Extracellular matrix (ECM) deposition
- Reorganised thrombi

1.2.1.1.2 Medial thickening

The smooth muscle layer that comprises the medial layer of pulmonary arteries provides support and strength to the vessel wall in addition to being the contractile machinery of the arteries. The media is composed of distinct subpopulations of PASMCs that exist in different layers within the media (Frid et al., 1997). The subpopulations are functionally and phenotypically unique, each with different expression levels of contractile and cytoskeletal proteins in addition to inter-population variations in response to hypoxia and growth factors (Frid et al., 1994; Dempsey et al., 1997; Frid et al., 1997). Heterogeneous subpopulations of non-muscle like cells have also been identified in the media that have unique characteristics including a proliferative response to hypoxia, mimicking the *in vivo* effect of hypoxia on vascular remodelling. Certain populations of these cells are also able to secrete mitogens that may directly affect proliferation of 'classical' PASMCs (Frid et al., 1994; Frid et al., 1997).

In PAH, thickening of the media occurs in pulmonary arteries >50 μM with the increase in the medial layer reaching 5 fold that of control values (Chazova et al., 1995). Both hypertrophy and hyperplasia of PASMCs contribute to medial thickening. PASMC hypertrophy is the dominant factor with hyperplasia playing a lesser role (Tuder et al., 2007). In addition to an increase in muscularisation of small pulmonary arteries a layer of smooth muscle also appears in the small, previously non-muscularised pre-capillary vessels <50 μM (Chazova et al., 1995). As there are no existing PASMCs in these arteries, hypertrophy and hyperplasia are unlikely to be essential factors in the distal extension of smooth muscle into previously non-muscularised vessels. One possibility which has already been mentioned in relation to plexiform lesion formation is the transdifferentiation of PAECs into PASMCs. Frid et al., 2002 found that cultured bovine PAECs could transdifferentiate into PASMCs when cell-cell contact was disrupted or when cells were exposed to TGF-β. The process was not dependent on PAEC proliferation. Moreover,

exposure of porcine PAECs to hypoxia for 7 days resulted in the emergence of smooth muscle like cells (Zhu et al., 2006). Only a small number (0.01-0.03%) of PAECs undergo transdifferentiation into PASMCs and it has been postulated that cells undergoing transdifferentiation are bone-marrow derived progenitor cells that have been incorporated into the endothelial monolayer (Frid et al., 2002; Zhu et al., 2006). Other candidates for emergence of smooth muscle in the precapillaries include hypertrophy of pericytes and migration/recruitment of interstitial fibroblasts (Zhu et al., 2006; Tuder et al., 2007) and there is extensive evidence that fibroblasts have a unique and critical role in vascular remodelling (See next section).

Apoptosis is a highly regulated process for maintaining normal vascular structure by removing unwanted cells such as those that have migrated into the vascular lumen or cells that have accumulated within the vessel wall. Cell survival depends on a delicate balance between expression of gene families that are pro-apoptotic or anti-apoptotic. The most important anti-apoptotic family is the Bcl-2 family with important pro-apoptotic genes including Bax and Bad (Gurbanov and Shiliang, 2006). Apoptosis is impaired in PASMCs from PAH patients (Zhang et al., 2003) and Bcl-2 is overexpressed in the lungs of both FPAH and IPAH patients (Geraci et al., 2001). Survivin is a potent inhibitor of apoptosis and is found in pulmonary arteries from pulmonary hypertensive patients but not from control subjects (McMurty et al., 2005). Adenovirus mediated overexpression of survivin in rats results in mild PAH and inhibition of endogenous survivin reduces PAH induced by monocrotaline (MCT) in rats. Tenascin-C, a component of the ECM is an important survival factor for PASMCs and is induced by matrix metalloproteinases (MMPs) and elastase. Tenascin-C is increased in vessels undergoing remodelling in pulmonary hypertensive patients (Jones et al., 1997). Blockade of tenascin-C or its production by the use of elastase inhibitors results in PASMC apoptosis and regression of medial thickening in cultured rat pulmonary arteries (Cowan et al., 1999; Cowan et al., 2000a). Moreover,

elastase inhibitors can reverse remodelling and improve survival in rats with MCT induced PAH (Cowan et al., 2000b). The importance of impaired apoptosis in pulmonary vascular remodelling has further become evident since the discovery that FPAH was related to mutations in the gene for the apoptotic mediator: bone morphogenetic protein receptor 2 (BMPR2) (See section 1.2.2).

1.2.1.1.3 Adventitial thickening

The adventitia is a connective tissue layer containing elastic and collagen fibres and is populated by PAFs. The adventitial thickening associated with vascular remodelling is associated with PAF hyperplasia and collagen deposition (Chazova et al., 1995; Sartore et al., 2001). The adventitia can reach 6 times the thickness of normal in PAH patients (Chazova et al., 1995). PAFs have important roles in response to injury (Sartore et al., 2001) and following such an injury, they undergo significant changes. Such changes are possible due to PAFs being less differentiated than PASMCs or PAECs and they can undergo proliferation, migration and transdifferentiation into myofibroblasts in addition to synthesising cytokines and ECM proteins (Mandegar et al., 2004). Myofibroblasts which express PASMC contractile proteins are able to migrate into the media where they may contribute to remodelling.

Hypoxia is a potent stimulus for PAFs and can modulate most of the PAF functions outlined above. PAF proliferation is the most pronounced feature of early hypoxic induced PAH in animal models (Belknap et al., 1997). The number of PAFs in the vessel wall of the hypoxic rat has shown to be increased 3 fold within 24 hours of hypoxic treatment and this was followed by transdifferentiation into 'transitional cells' displaying both smooth muscle and fibroblast characteristics before complete transdifferentiation into mature PASMCs by the 7^{th} day of hypoxia (Sobin et al., 1983). Further work on hypoxic rats has shown that PAFs from these animals show an enhanced proliferative phenotype that

continues even if the cells are returned to normoxic conditions (Welsh et al., 2001). Hypoxia also triggers the release of paracrine mitogenic agents from PAFs that induce proliferation of PASMCs (Rose et al., 2002). The specific mitogenic compounds released by the PAFs are not known but may include ROS, PDGF and FGF (Rose et al., 2002; Stenmark et al., 2006). Like the PASMCs in the media, PAFs appear to exist as heterogeneous subpopulations (at least in the neonatal calf) with varying sensitivities to hypoxia (Das et al., 2002).

The vasa vasorum micro-circulation borders the adventitia of pulmonary arteries and brings oxygenated blood and nutrients to the outside of the vessel wall. Hypoxia increases the size of the vasa vasorum with the greatest increase occurring in more distal regions of the arterial tree. This vascularisation results in incomplete and fragile vessels which may allow plasma proteins, blood cells and circulating progenitor cells into the adventitia (Davie et al., 2004). Progenitor cells derived from the bone marrow could infiltrate the vascular wall and contribute to the adventitial thickening observed in hypoxic PAH. Indeed bone marrow derived cells expressing myofibroblast or smooth muscle like phenotype have been found in the adventitia of hypertensive pulmonary arteries where they contribute to vascular remodelling (Hayashida et al., 2005).This process is thus similar to the theory discussed in the previous section proposing a role for bone marrow derived progenitor cells in smooth muscle formation.

1.2.1.1.4 Contribution of inflammatory cells to vascular remodelling

T- and B- lymphocytes, in addition to macrophages have been found in plexiform lesions, supporting a role for these inflammatory cells in PAH (Tuder et al., 1994; Cool et al., 1999). Pro-inflammatory cytokines including interleukin-1 (IL-1) and interleukin-6 (IL-6) are increased in the serum of pulmonary hypertensive patients (Humbert et al., 1995) and IL-6 is increased in both the hypoxic and MCT animal models of PAH (Bhargava et al.,

1999; Wang et al., 2003). Additional evidence for an inflammatory component to pulmonary vascular remodelling comes from the increased expression of the chemoattractant RANTES that is predominately expressed in vascular lesions composed of proliferating PAECs (Dorfmuller et al., 2002).

The recently discovered chemokine: fractalkine (FKN) has the unique properties of existing either as a soluble form where it acts as a chemokine or as a membrane bound form on PAECs (that have been activated by inflammatory cytokines) where it acts as an adhesion molecule (Bazan et al., 1997). The membrane bound form mediates the recruitment and capture of leukocytes expressing the FKN receptor CX3CR1 (Foussat et al., 2000). The FKN-CX3CR1 interaction between endothelium and leukocytes is sufficient to cause cellular adhesion with no apparent involvement of integrins or other adhesion molecules even under conditions of high blood flow (Fong et al., 1998). FKN appears to be unique amongst chemokines in being able to mediate firm leukocyte adhesion and has, therefore, come under attention in the investigation of several vascular disorders. Two polymorphisms (V249I and T280M) have been found in CX3CR1 resulting in reduced FKN binding to CX3CR1 and decreased risk for atherosclerosis (McDermott et al., 2001) and coronary artery disease (Moatti et al., 2001) indicating a possible pathological role for CX3CR1 in these diseases. More recently, it was discovered that circulating T-lymphocytes from PAH patients show elevated expression of CX3CR1 (Balabanian et al., 2002) and contrary to the protective role of the V249I and T280M polymorphisms in the diseases just mentioned, they are associated with an increased risk of systemic sclerosis associated PAH (Marasini et al., 2005). The reasons why the two polymorphisms are protective in atherosclerosis and coronary artery disease but are associated with an increased risk of systemic sclerosis PAH is not known but the situation is more complex than first thought. The presence of V249I and T280M does not protect against ischaemic stoke (Hattori et al., 2005) or peripheral artery disease (Gugl et al., 2003)

and the V249I variant has been shown to display enhanced adhesive properties (Daoudi et al., 2004). Tissue specific differences in the role/response to CX3CR1 in addition to the large individual differences noted in the expression of CX3CR1 within groups with the same genotype may account for some of the differences observed (Marasini et al., 2005).

The excess infiltration of leukocytes and the release of inflammatory mediators may contribute to vascular remodelling. Inhibition of IL-6 attenuates MCT induced PAH in rats (Bhargava et al., 1999) and mice deficient in the macrophage derived leukotriene: 5-lipooxygenase (5-LO), develop less severe hypoxic PAH than control mice (Voelkel et al., 1996). RANTES may indirectly modulate vascular tone/remodelling through release of ET-1 (Molet et al., 2000) and the CX3CR1 receptor has been found on PASMCs in MCT induced PAH where its activation induced PASMC proliferation (Perros et al., 2007). The enhanced immune response, both at the cellular and molecular level coupled with the close relationship often found between PAH and inflammatory/auto-immune conditions provides compelling evidence for at least a modulating role for inflammation in PAH.

1.2.1.2 Pulmonary vasoconstriction

Vasoconstriction of pulmonary arteries can contribute significantly to an increase in PAP and PVR. The importance of vasoconstriction in the pathology of PAH is evident by the response of some patients to vasodilators such as prostacyclin, NO and endothelin receptor antagonists (See section 1.2.3.2.5). The majority of patients, however, do not respond to current vasodilator treatment. This may be a consequence of vascular remodelling that is so advanced by diagnosis and the commencement of treatment that vascular reactivity is impaired or it may be that in these patients, vasoconstriction does not contribute significantly to PVR. At this stage in our understanding of the disease, it is unknown if vasoconstriction is involved in the initiation of PAH or if it merely acts as a contributing factor. As mentioned previously, sustained vasoconstriction in HPV results in vascular

remodelling but even in the absence of hypoxia, increased PAP such as that induced by vasoconstriction can induce vascular smooth muscle cell proliferation (Hishikawa et al., 1994).

1.2.1.3 In situ thrombosis

In situ thrombosis in PAH is thought to occur following abnormal changes to the vascular endothelium and/or changes in platelets and clotting factors (Humbert et al., 2004). Thrombotic lesions are found in lung samples from up to 50% of PAH patients and are often associated with plexiform lesions (Welsh et al., 1996). There are noticeable differences in procoagulant and fibrinolytic agents in PAH patients with a decrease in natural anti-coagulation factors in association with a decrease in fibrinolysis (Welsh et al., 1996). The coagulation promoting agent von Willebrand factor is upregulated in PAH and high circulating plasma levels are correlated with lower life expectancy (Lopes et al., 1998). Von Willebrand factor promotes platelet aggregation and the increased levels of thromboxane metabolites seen in PAH patients are evidence of platelet aggregation (Christman et al., 1992). Thrombotic lesions are not thought to be an initiating factor in PAH but to occur secondary to endothelial injury and to gain in severity with disease duration (Wagenvoort et al., 1993). The accumulation of platelets at a vascular lesion can enhance vascular remodelling by the release of vasoconstrictor and mitogenic mediators including TXA_2, 5-HT, vascular endothelial growth factor (VEGF), PDGF and TGF-β (Humbert et al., 2004).

1.2.2 The genetic basis of PAH

A familial basis for PAH was first proposed in 1954 but it was not until 1984 that a detailed account was produced that indicated many cases previously thought to be idiopathic did in fact have a familial basis (Loyd et al., 1984). Observations emerged that

illustrated why the familial basis of PAH had not been well characterised previously. The disease showed an autosomal dominant pattern of inheritance with incomplete penetrance which varied significantly between families. An interesting observation in this study was that the disease showed a female to male predominance of 2:1 (Loyd et al., 1984). The inheritance patterns just outlined in addition to incomplete family records makes a precise calculation of the number of familial cases difficult; however, a national prospective study in 1984 involving analysis of 187 families with PAH identified ~6% of cases as familial i.e. having two or more family members with PAH (Rich et al., 1984). The accumulation of family registries allowed linkage analysis studies that localised a marker for the disease to chromosome 2q31-32 (Nichols et al., 1997; Morse et al., 1997) and candidate genes were tested and identified by DNA sequencing. This resulted in the discovery of mutations in the BMPR2 gene. To date, over 140 mutations have been discovered in up to 70% of familial cases (Machado et al., 2001; Morisaki et al., 2004; Aldred et al., 2006) and 11 - 40% of apparently idiopathic cases (Thomson et al., 2001; Morisaki et al., 2004; Koehler et al., 2004). The relatively large number of IPAH patients with BMPR2 mutations is partly related to the incorrect classification of patients as having IPAH when in fact a genetic basis does exist (Newman et al., 2001). Other mutations are likely to be *de novo* mutations (Aldred et al., 2006; Morrell et al., 2006). The large number of BMPR2 mutations in both FPAH and other non familial forms coupled with the complete absence of these mutations in the control population (Deng et al., 2000; Lane et al., 2000; Machado et al., 2001) makes the presence of a BMPR2 mutation the largest known risk factor for the development of PAH. The mutations uncovered occur along the entire length of the gene, affecting the extracellular domain, transmembrane domain, kinase domain and the long cytoplasmic domain (Machado et al., 2006). About 30% of coding region mutations are missense mutations (Machado et al., 2006) occurring in highly conserved amino acid sequences, resulting in a failure of protein trafficking to the cell membrane or impaired signalling in

those proteins that do integrate into the cell membrane (Machado et al., 2001; Nishihara et al., 2002; Rudarakanchana et al., 2002; Machado et al., 2006). 70% of coding region mutations are frame shift or nonsense mutations that would result in premature truncation of the protein and loss through nonsense-mediated mRNA decay (Machado et al., 2006; Morrell, 2006). No gain of function mutation in BMPR2 has been discovered (Newman et al., 2008). The heterozygous loss of function mutations in carriers with a BMPR2 mutation supports haploinsufficiency as the predominant genetic process in inherited PAH. Not every case of FPAH has an identifiable BMPR2 mutation. This may be due to mutations in exonic coding regions yet to be studied or in intronic or regulatory regions. Recently, an analysis of familial and idiopathic patients who were negative for coding region mutations identified gene rearrangements in 28% of FPAH and in 5.7% of IPAH patients (Aldred et al., 2006). These were predicted to have deleterious effects on protein structure and function and it is likely that with further investigation, the number of familial PAH cases linked to BMPR2 mutations will rise still further.

1.2.2.1 BMPR2

Bone morphogenetic proteins (BMPs) are the largest group of growth factors belonging to the TGF-β superfamily. In addition to BMPs, this family also includes the TGF-β isoforms (TGF-β 1-3), activins, Mullerian inhibiting substance (MIS) and other growth and differentiation factors (Kingsley, 1994). BMPs were first discovered as molecules that induce bone and cartilage formation but are also now known to be involved in proliferation, differentiation and apoptosis of many cell types and have a vital role in embryogenesis (Morrell et al., 2006). This latter role explains why BMPR2 mutations are only found in the heterozygous state, as BMPR2 has a critical role in early vascular development. This is confirmed by the death of $BMPR2^{-/-}$ mice before gastrulation (Beppu et al., 2000).

Members of the TGF-β superfamily bind to two forms of cell surface receptors, termed type I and type II receptors, that both contain serine/threonine kinase domains. Both type I and type II receptors are required for signal transduction and they exist as either oligomeric complexes or in monomeric form on the cell surface. As oligomers, they may be type I homomers, type II homomers or type I-type II heteromers (Miyazono et al., 2001). BMPs bind to three different type I receptors (BMPR1A/ALK-3, BMPR1B/ALK-6 and ActR-IA/ALK-2) and three different type II receptors (BMPR2, ActR-IIa and ActR-IIb) and different tissues display different expression levels of the receptor types. Like other type II receptors, BMPR2 is a constitutively active serine/threonine kinase. It exists as two isoforms: a long form and a short form. The long form is unique within the TGF-β family in having a long cytoplasmic tail region rich in serine and threonine residues (Kawabata et al., 1998).

There are over 30 members of the BMP family with binding affinities to type I and type II receptors varying significantly amongst member proteins. The most well known BMPs; BMP-2, BMP-4, BMP-7, growth differentiation factor-5 (GDF-5) and growth differentiation factor-6 (GDF-6) bind with high affinity to BMPR1A and BMPR1B but with low affinity to BMPR2 (Morrell et al., 2006). Their respective affinities for type I receptors also differ, with BMP-7 and GDF-5 having higher affinities for BMPR1B than BMPR1A (Nishitoh et al., 1996). Other BMPs including BMP-5 and BMP-6 show higher affinity for BMPR2 over type I receptors (Yu et al., 2005; Morrell et al., 2006) and BMP-2 and BMP-7 can also bind the activin receptors ActRIIa and ActRIIb albeit with lower affinity than activins (Kawabata et al., 1998). Although ActRIIa and ActRIIb can bind BMPs and transduce signals in co-operation with type I receptors, it is BMPR2 that is the predominant type II receptor mediating BMP signalling under normal physiological conditions (Yu et al., 2005). Over recent years it is emerging that such receptor selectivity and differences in spatial expression patterns of each receptor allows a high degree of

plasticity in BMP signalling which likely underlies the tissue specific functions of BMPs in addition to their role in pathological conditions.

The TGF-β superfamily signal through a remarkably simple phosphorylation cascade that is highly conserved across mammals and involves the major TGF-β superfamily specific signalling molecules: the Smads. BMPs acting via type I and type II receptors are able to signal via this well documented Smad dependent pathway but also via Smad independent pathways that have been linked to mitogen-activated protein kinase (MAPK) activation (Nohe et al., 2002). Which of these pathways is activated is related to the nature of ligand binding to receptor complex. As mentioned, type I and type II receptors can exist as oligomers on the cell surface in addition to existing in free form. If a ligand binds to a preformed heterodimeric complex of one type I receptor and one type II receptor (predominately BMPR2) then signalling occurs via the 'traditional' Smad pathway. If, however, a ligand binds to a monomeric type I receptor, then this is thought to cause homodimerisation with another type I receptor and subsequent recruitment of a type II receptor. Therefore the receptor signalling complex is distinct from the preformed heterodimeric complex of type I and type II receptors and this ligand-heteromeric complex results in Smad independent signalling via MAP kinases (figure 1.5) (Nohe et al., 2002).

1.2.2.1.1 Smad dependent signalling

Following ligand binding to receptor complex, BMPR2 phosphorylates a glycine-serine rich domain on the proximal intracellular region of the type I receptor causing activation of the type I receptor kinase domain. The type I receptor phosphorylates and activates a group of Smad proteins termed 'receptor activated' or R-Smads. This group is composed of Smad1, Smad5 and Smad8 that are often grouped together as Smad1/5/8 as the functional differences between them are not well characterised. Smad1/5/8 must form a complex with the common partner Smad (co-Smad), Smad4 to allow translocation to the nucleus

where they regulate gene transcription in association with transcriptional activators and co-repressors (Humbert et al., 2004).

1.2.2.1.2 Smad independent signalling

Signalling via phosphorylated Smad1/5/8 (p-Smad1/5/8) cannot explain all the cellular effects of BMPs and it is now recognised that other Smad independent pathways exist. Following ligand binding to a type I receptor followed by homodimerisation and recruitment of a type II receptor, a p38 MAPK dependent pathway is activated (Nohe et al., 2002). Activation of p38 is thought to be dependent on the prior activation of the MAPK, TAK1, whose activity is enhanced by interaction with the TAK binding protein, TAB1. TAB1 may provide the link to the BMPR1A/1B receptors through the adaptor protein XIAP (Nohe et al., 2004). In addition to p38, another major MAPK, extracellular regulated kinase (ERK) can be activated by BMPs (Lai and Cheng, 2002; Yang et al., 2005) although the molecular mechanism responsible for p-ERK1/2 activation is currently unknown. The long cytoplasmic tail region of BMPR2 is capable of binding a multitude of proteins (Hassel et al., 2004) of which several have been investigated in relation to BMPR2 signalling (Foletta et al., 2003; Machado et al., 2003; Wong et al., 2005; Chan et al., 2007) and these provide another level of complexity to BMPR2 mediated intracellular signalling (figure 1.5).

1.2.2.1.3 Regulation of BMP signalling

There are several levels of regulation to BMP signalling including the BMP antagonists noggin, chordin and members of the DAN family, the presence of accessory receptors such as the endoglin receptor or inhibitory proteins such as BAMBI (BMP and activin membrane bound inhibitor) and Smurf-1 (Smad ubiquitination regulatory factor-1) (Massague and Chen, 2000). Moreover, phosphatases have recently been discovered

capable of dephosphorylating and inactivating Smad-1 (Chen et al., 2006). In addition to Rmads and co-smads that are a third group termed inhibitory Smads comprised of Smad6 and Smad7. Smad6 inhibits BMP signalling through competition with co-Smad4 for Smad1 binding yielding inactive Smad1-Smad6 complexes. Smad6 and Smad7 are thought to function as a negative feedback system to control signal strength (Massague and Chen, 2000).

1.2.2.2 Role of BMPR2 in the pathogenesis of PAH

Mutations and dysfunctional TGF-β receptors have been associated with many diseases involving deregulated cellular proliferation including over a dozen cancers, pulmonary fibrosis and atherosclerosis (Miyazono et al., 2001), so it was perhaps not surprising that BMPR2 as a member of the TGF-β superfamily should be implicated in PAH. Unlike mutations in TGF-β receptors that are implicated in many disease conditions, BMPR2 mutations have only been associated with PAH. As already stated, $BMPR2^{-/-}$ mice die during early embryogenesis and Smad5 mutant embryos have malformed vasculature associated with defective angiogenesis (Yang et al., 1999) illustrating the importance of the BMPR2/Smad pathway in vascular development. BMPR2 is expressed on PAEC with lower levels of expression in PASMCs and macrophages (Atkinson et al., 2002). BMPR2 expression is decreased in FPAH/IPAH with the greatest reductions found in patients harbouring BMPR2 mutations or in those patients that display evidence of linkage to 2q33. Patients with PAH secondary to other illnesses also express BMPR2 at lower levels than control patients (Atkinson et al., 2002) indicating a pathological role for dysfunctional BMPR2 in pulmonary hypertensive patients not carrying a BMPR2 mutation. The low level expression of BMPR2 in PAH patients who are wild-type (WT) BMPR2 carriers

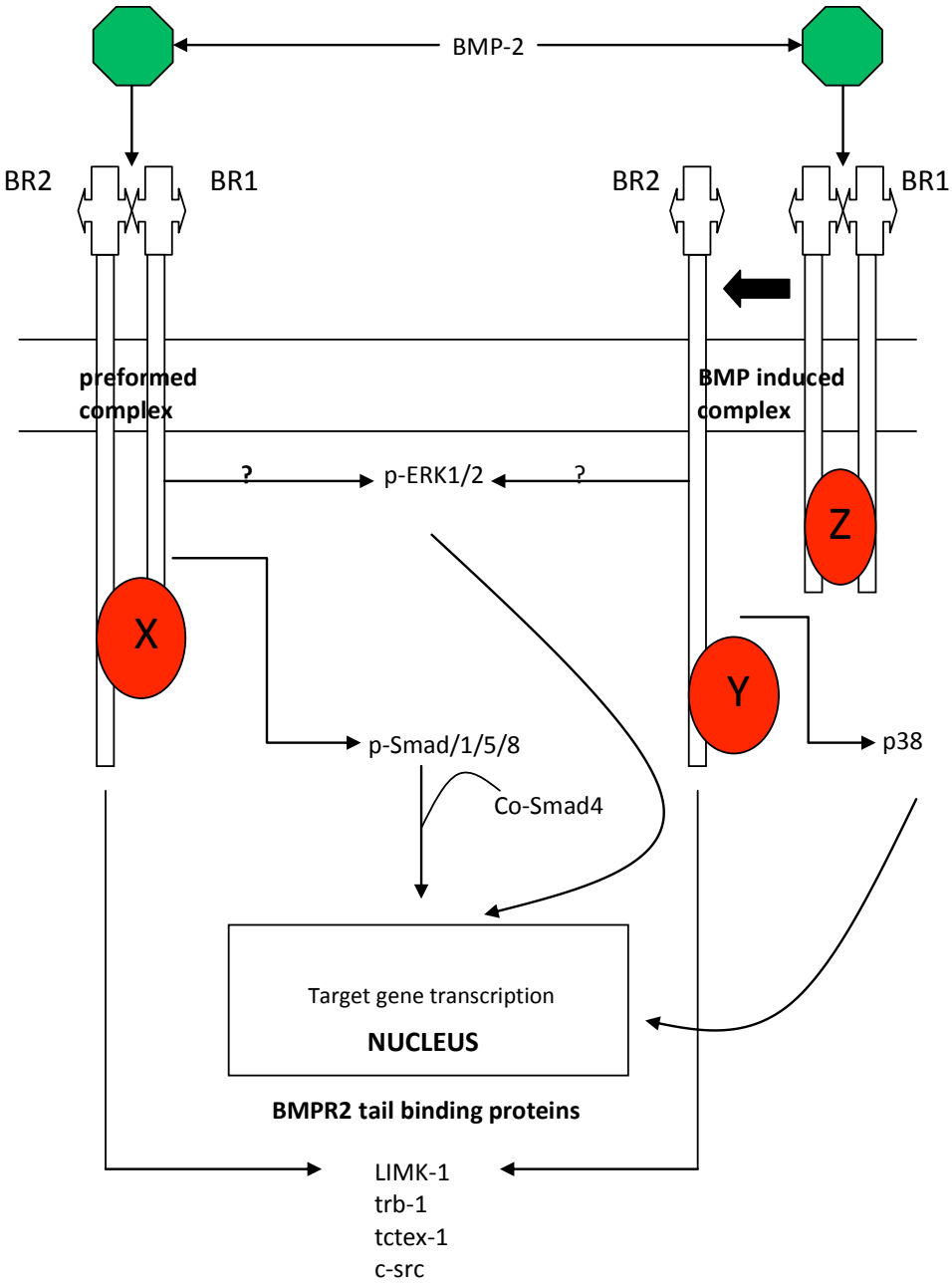


Figure 1.5 Possible signalling mechanisms of BMPR2.
A BMP (in this case BMP-2) may bind to a preformed receptor complex of type I (BR1) and type II (BR2) receptors inducing a Smad dependent pathway. Alternatively, a ligand may bind to a type I receptor causing homodimerisation and subsequent recruitment of a type II receptor. This activates the p38 dependent pathway. Both pathways may utilise additional adaptor proteins (X, Y and Z). p-ERK1/2 can also be activated by BMP ligands, however, it is not known which receptor complex mediates this activation. The cytoplasmic tail of BMPR2 interacts with cytoplasmic signalling molecules offering the potential for additional signalling pathways.

coupled with levels of BMPR2 lower than that predicted by haploinsufficiency in mutant carriers indicate there are other, as yet undetermined factors, that suppress BMPR2 expression in PAH. BMPR1A receptors are also downregulated in patients with PAH of various etiologies (Du et al., 2003). It is not just receptor expression that is reduced but also the activated (phosphorylated) forms of downstream Smads. In FPAH and IPAH patients (with and without BMPR2 mutations) p-Smad1 was found to be reduced in the media and intima of small pulmonary arteries (Yang et al., 2005). In contrast to these results, Richter et al., 2004 found no difference in p-Smad1/5/8 between PAH and control patients but did note a lack of expression in PAECs at the core of plexiform lesions which supports the concept that PAECs involved in vascular lesions have phenotypic heterogeneity.

Investigations of both the hypoxic and MCT animal models of PAH have yielded diverse results with regard to BMP signalling molecules. Hypoxia has been shown to increase p-smad1/5/8 and inhibitor of differentiation (Id1), a downstream target of BMP signalling in mice (Frank et al., 2005; Long et al., 2006) but to decrease these parameters in hypoxic rats (Takahashi, 2007). Moreover, BMPR2 is reduced in the lungs of rats following 1-3 weeks of hypoxia (Takahashi et al., 2005). Hypoxia induced PAH can also be attenuated through gene delivery of BMPR2 into the vascular endothelium resulting in an overexpression of BMPR2 (Reynolds et al., 2007). Such an approach however was unable to attenuate MCT induced PAH (McMurty et al., 2007). BMPR1B, BMPR2, p-Smad1 and Smads 4,5,6 and 8 have all been found to be decreased in MCT PAH however ERK1/2 and p38 levels were found to be unchanged (McMurty et al., 2007; Morty et al., 2007).

Work on cells isolated from PAH and control patients has helped understand the role that BMPs, BMP receptors and downstream effectors may play in PAH. One important conclusion that can be drawn from the work to date is that the effect of BMP signalling is highly context specific. The nature of ligand, cell type, cellular location within vessel and

species all determine the response recorded. BMP-2, BMP-4 and BMP-7 induce apoptosis of PASMCs from non hypertensive patients (Zhang et al., 2003), a process involving phosphorylation of Smad1, activation of caspases and downregulation of Bcl-2 (Zhang et al., 2003; Lagna et al., 2006). BMPs also inhibit proliferation of PASMCs in healthy patients (Morrell et al., 2001; Takeda et al., 2004). The effects of BMPs are, however, very different in PASMCs from pulmonary hypertensive patients. Zhang et al., 2003 found that PASMCs from FPAH/IPAH but not APAH patients were more resistant to BMP induced apoptosis and PASMCs expressing a mutant form of BMPR2 are resistant to BMP induced apoptosis (Lagna et al., 2006). The anti-proliferative effect of BMPs are also lost in PASMCs from patients with PAH (Morrell et al., 2001) with BMP-2 and BMP-7 actually inducing proliferation of pulmonary hypertensive PASMCs, a process dependent on p38 and ERK1/2 (Takeda et al., 2004). It has been proposed that p38 and p-ERK1/2 signalling opposes p-Smad1/5/8 signalling and that an imbalance between proliferative p38/p-ERK1/2 pathways and the apoptotic p-Smad1/5/8 pathway contributes to proliferation in BMPR2 mutant PASMCs (Yang et al., 2005).

Generally speaking, BMPs have the opposite effect on PAECs than they do on PASMCs, namely a decrease in apoptosis and stimulation of proliferation (Valdimarsdottir et al., 2002; Teichert-Kuliszewska et al., 2006). One speculative theory regarding the initiation of PAH in the presence of dysfunctional BMPR2 is that apoptosis of PAECs leads to endothelial damage and exposure of underlying smooth muscle to mitogenic agents such as TGF-β (Morrell et al., 2006). The smooth muscle cells would undergo proliferation and perhaps migration, unchecked by the pro-apoptotic effect of BMPR2 mediating signalling. In addition, rapid apoptosis of PAECs could lead to the emergence of apoptosis resistant cell populations which would contribute to intimal lesions.

1.2.3 Modifying factors in PAH

The discovery of BMPR2 mutations in patients with PAH was a significant breakthrough in the understanding of the disease. The low penetrance of the disease in families harbouring BMPR2 mutations coupled with the fact that the majority of IPAH patients do not have BMPR2 mutations suggest that dysfunctional BMPR2 alone is insufficient to induce PAH. Current thinking points towards a disease created by 'multiple hits'; that is, an underlying genetic pre-disposition coupled with other endogenous or environmental factors that act in concert to create the disease phenotype. The search for an additional modifying gene for PAH has focused intensively on the 5-HT transporter (5-HTT).

1.2.3.1 5-HT and PAH

As mentioned previously, 5-HT is synthesised primarily in the enterochromaffin cells of the intestine. However, the lung also contains several sites for local 5-HT synthesis including the neuroendocrine and neuroepithelial cells in the airways (MacLean et al., 2000) and PAECs (Eddahibi et al., 2006). 5-HT is a product of the essential amino acid L-tryptophan. In 5-HT producing cells, L-tryptophan undergoes hydroxylation of the benzene ring by TPH to yield 5-hydroxytryptophan (5-HTP). 5-HTP then undergoes decarboxylation via L-amino acid decarboxylase to form 5-HT. 5-HT is a pulmonary vasoconstrictor (MacLean et al., 1996; Morecroft et al., 1999) and PASMC mitogen (Fanburg et al., 1997); however, normal physiological plasma concentrations are kept low (nanomolar concentrations) due to uptake and storage in platelets. The first indicator that elevated plasma levels of 5-HT may be a contributing factor in the pathology of PAH came following the association of PAH with ingestion of the anorexigen, aminorex fumurate (aminorex). Aminorex is a substituted amphetamine that was used in several European countries during the 1960s. It soon became clear that people ingesting aminorex were 20 times more likely to develop PAH than the general population (Kay et al., 1971) and 5

years after its discontinuation, levels of PAH had returned to the levels seen prior to the introduction of aminorex (Kramer and Lane, 1998). While the exact mechanism of action of aminorex was not known, it was discovered that aminorex released 5-HT from platelets to a greater degree than other sympathomimetics and phenethylamines. It was also found to be a monoamine oxidase (MAO) inhibitor (Fristrom et al., 1977). Further support for the role of 5-HT in PAH came from the fawn-hooded rat, a strain with a genetic deficiency in platelet storage and that has high circulating plasma levels of 5-HT. The fawn-hooded rat develops spontaneous PAH (Kentera et al., 1988; Ashmore et al., 1991) and an exaggerated pulmonary hypertensive response to hypoxia compared with other rat strains (Nagaoko et al., 2001). A causative role for 5-HT in the fawn hooded rats' heightened response to hypoxia was supported when it was shown that infusion of 5-HT for 2 weeks aggravates hypoxic PAH in rats (Eddahibi et al., 1997). In 1990 PAH was discovered in a patient with a rare familial platelet storage disease that results in elevated plasma 5-HT levels (Herve et al., 1990). Elevated plasma 5-HT levels were subsequently found in a group of FPAH/IPAH patients that persisted even after heart-lung transplant indicating the increased plasma [5-HT] was not a secondary phenomenon (Herve et al., 1995). This data coupled with another outbreak of PAH related to an anorexigen with serotonergic effects, this time dexfenfluramine (Dfen) (see section 1.2.3.2.3), formed the basis of the 'serotonin hypothesis' of PAH in which 5-HT plays a causative or modulating role in the disease pathology. The mechanisms by which 5-HT contributes to the vascular changes in PAH are still being elucidated but both 5-HT receptors and 5-HTT are thought to contribute.

1.2.3.1.1 5-HT receptors in PAH

5-HT induced vasoconstriction occurs through the 5-HT_{1B} receptor in human pulmonary resistance vessels (Morecroft et al., 1999) with both 5-HT_{1B} and 5-HT_{2A} receptors contributing to vasoconstriction in rats (MacLean et al., 1996). The 5-HT_{1B} mediated

contraction is greater in the pulmonary vessels of chronic hypoxic rats, an effect attenuated by the 5-$HT_{1B/1D}$ antagonist GR127935 (Keegan et al., 2001). This same antagonist also reduced the pulmonary vascular remodelling that occurs in hypoxic rats. The $5HT_{1B/1D}$ knockout mouse paralleled the antagonist studies with reduced vascular remodelling and attenuated vasoconstriction compared to WT hypoxic mice (Keegan et al., 2001). In addition to its role as a mediator of vasoconstriction in rats, the 5-HT_{2A} receptor also contributes to hypoxic induced fibroblast proliferation (Welsh et al., 2004). The 5-HT_{2B} receptor has also been implicated in the pathogenesis of PAH with 5-HT_{2B} knockout mice showing reduced parameters of vascular remodelling following hypoxia compared with WT hypoxic animals and the major metabolite of Dfen, nordexfenfluramine (NDfen) is a high affinity 5-HT_{2B} agonist (Launay et al., 2002). The effect of the 5-HT_{2B} receptor on the pulmonary circulation appears to be species dependent as the 5-HT_{2B} receptor is associated with a vasodilator effect in pigs (Glusa and Pertz, 2000). Support for a role for this receptor in PAH comes from the finding that a loss of function 5-HT_{2B} receptor mutation was found in a patient with Dfen associated PAH. The mutation was not found in 80 control patients (Blanpain et al., 2003). The contribution of 5-HT receptors to the pathogenesis of PAH is likely to involve the vasoconstriction element of the disease more than vascular remodelling; however, recent evidence indicates that stimulation of 5-HT receptors may be necessary for vascular remodelling when coupled with 5-HTT activation (see section 1.2.3.1.3) and there is convincing evidence that the 5-HTT is critical to 5-HT induced vascular remodelling.

1.2.3.1.2 The 5-HTT and PAH

The ‘serotonin hypothesis’ initially came into being through studies implicating a rise in circulating 5-HT as a factor in PAH; however, the data regarding plasma 5-HT levels in PAH is not clear. In contrast to earlier studies, Lederer et al., 2007 found that plasma 5-HT

levels were normal in PAH patients. People with carcinoid tumours of the 5-HT producing enterochromaffin cells have high plasma [5-HT] but are not at an increased risk of PAH and neither are users of selective serotonin reuptake inhibitors (SSRIs) which increase plasma 5-HT levels. If, however, 5-HT induced pulmonary hypertension is dependent on cellular internalisation of 5-HT via the 5-HTT then the expression and activity of the 5-HTT may be a better indicator of the involvement of the serotonergic system in PAH than plasma 5-HT levels and may also explain why SSRIs are not associated with an elevated risk of the disease.

The 5-HTT belongs to a large family of membrane proteins including dopaminergic, α-aminobutyric acid, noradrenaline and other transporters. The principal action of these transporters is to mediate cellular uptake of neurotransmitters. The 5-HTT is expressed on several cell types including neurons, platelets, PAECs, PASMC and PAFs. Experiments in bovine and rat PASMCs have demonstrated that the mitogenic action of 5-HT on these cells is dependent on uptake via the 5-HTT (Lee et al., 1991; Eddahibi et al., 1999) and the proliferative effect of 5-HT + serum on PAFs is blocked by the 5-HTT inhibitor, fluoxetine (Welsh et al., 2004). Levels of the 5-HTT are increased in the pulmonary arteries of hypoxic rats and hypoxic PASMCs have increased expression of 5-HTT coupled with an increased proliferative effect to 5-HT (Eddahibi et al., 1999). 5-HTT inhibitors attenuate vascular remodelling in hypoxic induced pulmonary hypertensive mice (Marcos et al., 2003) and mice lacking the 5-HTT ($5\text{-HTT}^{-/-}$ mice) also develop less severe hypoxic PAH than WT mice (Eddahibi et al., 2000). Conversely, mice overexpressing the 5-HTT (5-HTT+ mice) have higher pulmonary pressures and a greater degree of remodelling and right ventricular hypertrophy following hypoxia than WT mice. Interestingly, normoxic 5-HTT+ mice have a higher (up to 3 fold) right ventricular pressure (RVP) than WT mice (MacLean et al., 2004).

Human data has helped support the animal studies, with 5-HTT mRNA levels two-fold

greater in FPAH/IPAH patients than in control subjects (Eddahibi et al., 2001). 5-HTT expression was shown to be greater in vessels undergoing remodelling, particularly in the medial smooth muscle and in regions of concentric intimal lesions associated with proliferating PASMCs. PASMCs isolated from the pulmonary hypertensive patients showed a doubling of proliferation to 5-HT + serum, an effect that was abolished by fluoxetine and another 5-HTT inhibitor, citalopram. The proliferative response was unique to 5-HT with several other growth factors including PDGF and TGF-β having similar effects on PASMCs from PAH patients and control subjects (Eddahibi et al., 2001).

The reasons for the increased levels of the 5-HTT in pulmonary hypertensive patients are unclear. A polymorphism exists in the promoter region of the human 5-HTT gene that alters its transcriptional activity. The polymorphism involves two alleles, a 44 base pair insertion and deletion, termed the L and S allele respectively. The L allele is associated with a 2 to 3 fold higher level of 5-HTT transcription than the S allele. Eddahibi et al., 2001 found that the L/L allele was present in 27% of the control population but was present in 69% of patients with either FPAH, IPAH or PAH associated with Dfen. The L/L genotype is also present in the majority of lung transplant patients with PAH secondary to other diseases (Marcos et al., 2004). This data suggests that the L/L genotype confers a genetic susceptibility to PAH. Two recent studies, however, found no difference in allele distribution between FPAH/IPAH patients and control subjects (Willers et al., 2006; Machado et al., 2006) but Willers et al., 2006 did find that the L/L genotype correlated with an earlier age of diagnosis in FPAH patients, possibly indicating an interaction between overexpression of 5-HTT with a BMPR2 mutation. The L/L genotype is correlated with higher PAP in patients suffering from PAH associated with COPD (Eddahibi et al., 2003) and PAH associated with heart failure (Olson et al., 2007) supporting a role for the 5-HTT in modulating disease severity.

1.2.3.1.3 5-HT signalling in the pulmonary vasculature

5-HT can activate several signalling systems depending on whether 5-HT acts via one of the many 5-HT receptors or through the 5-HTT. The relative contribution of 5-HT receptors and 5-HTT to pulmonary vasoconstriction and remodelling is uncertain and likely differs between species.

The 5-HT receptors mentioned above in relation to PAH are all G-protein coupled receptors. The 5-HT_{1B} and 5-HT_{2A} receptors are thought to be the most important 5-HT receptors with regards to PAH and these receptors are expressed on pulmonary smooth muscle with lower expression on endothelium (Marcos et al., 2004). 5-HT_{1B} and 5-HT_{2A} receptors signal via distinct pathways. 5-HT_{1B} receptors are coupled to Gi and inhibit adenylate cyclase and cAMP production resulting in vasoconstriction (Weir et al., 2004). 5-HT_{2A} receptors couple to Gq and activate phospholipase C (PLC) leading to an increase in inositol triphosphate (IP_3) levels which induces release of Ca^{2+} from intracellular stores causing vasoconstriction (Weir et al., 2004). Alternatively, 5-HT can act intracellularly following uptake via the 5-HTT. The 5-HTT parallels 5-HT_{1B} and 5-HT_{2A} expression in pulmonary arteries with the 5-HTT being expressed predominately in smooth muscle with weaker expression in the endothelium (Eddahibi et al., 2001; Marcos et al., 2004). 5-HT, once taken up into cells via the 5-HTT can be degraded via MAO, packaged into vesicles or it can initiate signalling pathways leading to proliferation and possibly vasoconstriction. Experiments in bovine PASMCs have shown that once inside a PASMC, 5-HT activates ROS which phosphorylates and activates ERK1/2 which enters the nucleus and induces proliferation (Lee et al., 1999) via the transcription factor, GATA-4 (Suzuki et al., 2003). For ERK1/2 to translocate to the nucleus it requires the presence of ROCK and ROCK is increased by 5-HT stimulation of $5\text{-HT}_{1B/1D}$ receptors (Liu et al., 2004). Not only does this provide an additional mechanism of 5-HT induced vasoconstriction through ROCK mediated Ca^{2+} sensitisation, it also allows cross-talk between the $5\text{-HT}_{1B/1D}$ receptor and 5-

HTT in relation to PASMC proliferation (figure 1.6). In fact, blockade of either 5-$HT_{1B/1D}$ receptors or 5-HTT prevents 5-HT mediated proliferation of bovine PASMCs (Liu et al., 2004). The situation appears different, however, in human PASMCs. In these cells it is the 5-HTT that is required for nuclear translocation of ERK1/2 and 5-HT_{1B} receptors that activate ERK1/2 (Lawrie et al., 2005). It may be that cellular and species differences are important in the relative contributions made by receptors and 5-HTT to PASMC proliferation. In human lung samples and isolated PASMCs from PAH patients, the 5-HTT was overexpressed, whereas the 5-HT_{1B}, 5-HT_{2A} and 5-HT_{2B} receptors were expressed at similar levels to samples from control patients. Inhibition of the 5-HTT, but not the receptors prevented the augmented proliferative response to 5-HT in PASMCs from PAH patients (Marcos et al., 2004). Like the BMPR2 receptor, the expression level of the 5-HTT in PAH patients is different to that predicted by genetic factors alone. In the case of the 5-HTT, PASMCs from PAH patients have higher levels of 5-HTT than control patients with the same genotype indicating that other factors must act to increase 5-HTT expression in PAH (Marcos et al., 2004).

1.2.3.1.4 Tryptophan hydroxylase 1

There are two isoforms of TPH. The first, TPH1, is present in the periphery and is the rate limiting enzyme for 5-HT synthesis outside the central nervous system (CNS). The second, TPH2, is present only in the CNS and is the rate-limiting enzyme for central 5-HT synthesis. There exists, therefore, two independent 5-HT systems allowing the generation of viable $Tph1^{-/-}$ mice that lack peripheral 5-HT but have only minor reductions in brain 5-HT (Walther and Bader, 2003). PAECs express TPH1 and levels of TPH1 are increased in PAECs from IPAH patients compared to control subjects and this is associated with increased 5-HT release (Eddahibi et al., 2006). Hypoxia increases TPH1 expression in rabbit lung 3 fold (Pan et al., 2006) and hypoxic PAH is attenuated in $Tph1^{-/-}$ mice

(Morecroft et al., 2007; Izikki et al., 2007) indicating the importance of TPH1 and 5-HT in the development of hypoxic PAH.

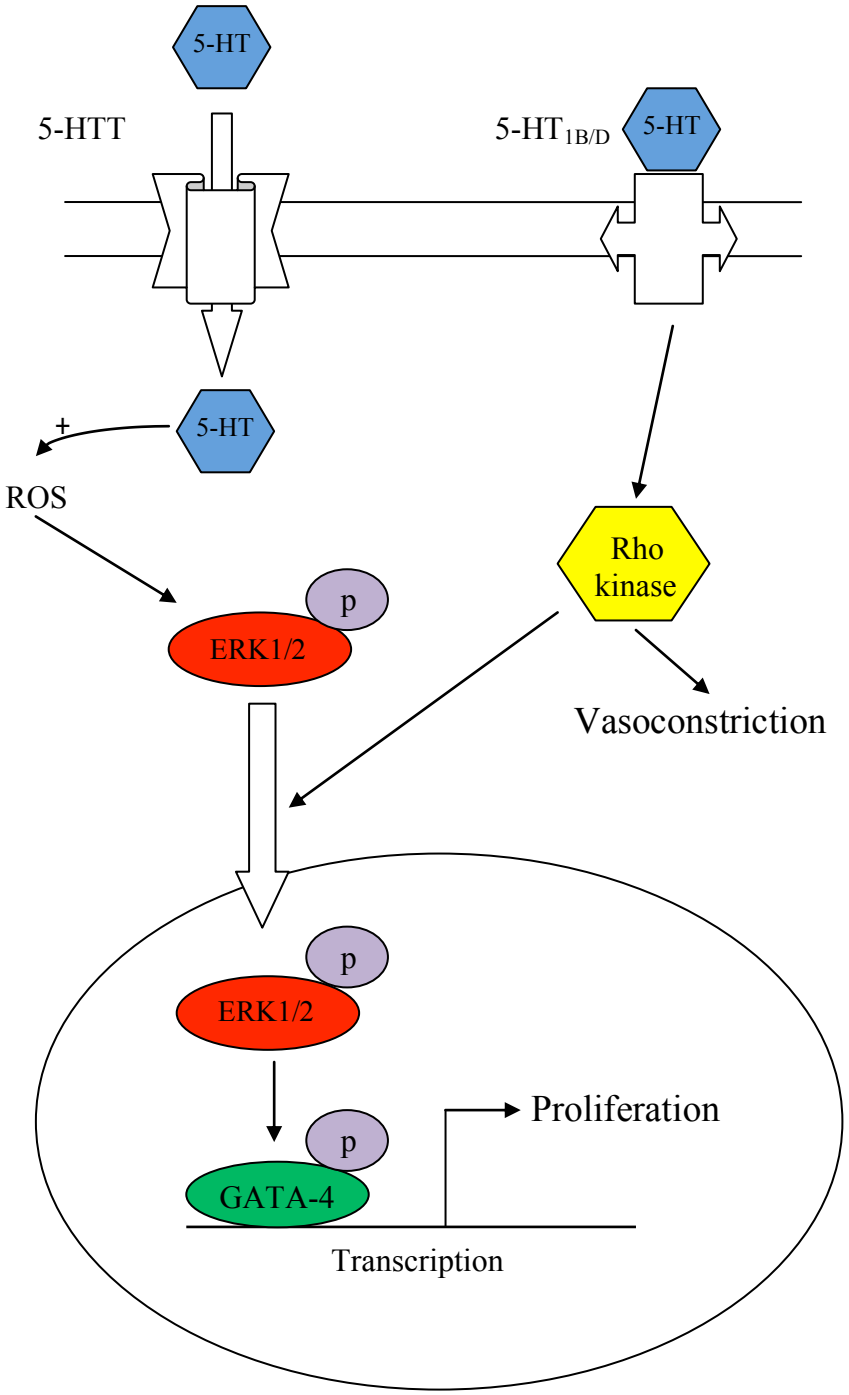


Figure 1.6 Cross-talk between the 5-HTT and 5-HT$_{1B/1D}$ receptor in 5-HT induced proliferation in bovine PASMCs. Following cellular uptake by the 5-HTT, 5-HT activates reactive oxygen species (ROS) which phosphorylates and activates the MAPK, ERK1/2. Rho Kinase (ROCK) which is increased by 5-HT$_{1B/1D}$ receptor activation enables p-ERK1/2 translocation to the nucleus and subsequent induction of proliferation via GATA-4.

1.2.3.2 Endogenous and Exogenous Modifying Factors in PAH

1.2.3.2.1 Hypoxia

As mentioned previously, hypoxic induced pulmonary vasoconstriction is a physiological regulatory mechanism for maintaining adequate oxygenation of blood. Sustained HPV and the development of chronic HPV are associated with several lung diseases including COPD and sleep apnea. Hypoxic induced PAH is also an important model for studying PAH. This process involves exposing rodents to 50% atmospheric oxygen levels for a period of at least 2 weeks. The result of the hypoxic exposure is an increase in RVP, RVH and vascular remodelling (MacLean et al., 2004). The extent of the vascular changes observed is dependent on sex, species and developmental stage of the animal (Stenmark et al., 2006). Hypoxia has profound effects on cells within pulmonary arteries. PAECs undergo phenotypic changes involving the upregulation of inflammatory markers, increased release of growth factors and ECM proteins. Cell surface receptor expression and plasma membrane structure also undergo pronounced alterations. The net result of the PAEC changes are an increase in production of growth factors and vasoconstrictors such as 5-HT and ET-1 and decreased production of vasodilators including prostacyclin and NO. The release of interleukins and leukocyte adhesion molecules facilitates attraction and capture of leukocytes to the vessel wall and recruitment of circulating progenitor cells (Stenmark et al., 2006; Nicolls and Voelkel, 2007). PASMCs also change towards a proliferative phenotype with many of the changes observed being a direct consequence of the action of the endothelial derived mitogenic factors. Two important effects of hypoxia on PASMCs have already been mentioned, namely the effect on cellular ROS production and subsequent K^+ channel inhibition (See next section) and the effect on the 5-HTT. The role of PAFs in hypoxic PAH was briefly discussed earlier and as an early mediator of the hypoxic response they are involved in promoting PASMC growth and contraction through the release of the paracrine growth factors outlined in section 1.2.1.1.3. The mitogenic

effect that PAFs exert on PASMCs is dependent on HIF supporting a role for PAFs as an oxygen sensor in hypoxic PAH (Rose et al., 2002). There are three HIF subtypes known (HIF-1α, HIF-2α and HIF-3α) all acting via the HRE to induce gene transcription. HIF-1α is expressed in the lung under normoxic conditions and is upregulated during hypoxia. HIF-2α is only expressed under hypoxic conditions and HIF-3α is expressed at lower levels than HIF-1α under normoxia but increases under hypoxia (Rose et al., 2002). The importance of HIF-1α in the role of PAH was evaluated in studies using heterozygous mice (HIF-1$\alpha^{+/-}$). These mice were found to develop less severe hypoxic PAH than WT mice (Yu et al., 1999). Subsequently, it was also shown that HIF-2$\alpha^{+/-}$ mice were protected against hypoxic PAH (Brusselmans et al., 2003). The proliferative effect that hypoxia has on PAFs seems to be dependent exclusively on HIF-2α with both HIF-1α and HIF-2α contributing to PAF migration (Eul et al., 2005).

The hypoxic model of PAH mimics many of the vascular changes of severe PAH in human patients but often lacks the complex intimal lesions seen in these patients. Plexiform lesions, for example, are not a feature of hypoxic PAH in animal models but HIF-1α is overexpressed in PAECs from plexiform lesions in PAH patients. HIF-1α is necessary for the production of VEGF, which is also upregulated in plexiform lesions indicating an angiogenic like process within the lesion. It is not clear, however, whether HIF-1α expression in plexiform lesions is increased by hypoxia or by other factors such as growth factors or mutations (Tuder et al., 2001).

1.2.3.2.2 K^+ channels

Cell membrane potential (V_m) is regulated by the permeability of Kv channels. Inhibition of Kv channel activity reduces K^+ efflux causing depolarisation of the cell membrane resulting in increased cytosolic [Ca^{2+}] caused by Ca^{2+} entry through voltage-dependent calcium channels (VDCC) and Ca^{2+} release from the sarcoplasmic reticulum (SR). As

discussed previously, K^+ channel inhibition by hypoxia is involved in HPV and HPV is reduced in mice deficient in Kv1.5 channels (Archer et al., 2001). Both Kv1.5 and Kv2.1 channels are present on PASMCs and both are downregulated in hypoxia causing reduced Kv current (Archer et al., 1998a). The importance of Kv1.5 channels in hypoxic PAH was revealed when gene transfer of Kv1.5 into hypoxic rats reduced pulmonary hypertensive parameters including RVH and arterial remodelling, offering a new potential avenue for therapeutic investigation (Pozeg et al., 2003). It is not clear why some Kv channels are altered by hypoxia and not others but the mechanism appears to involve HIF-1α. HIF-1$\alpha^{+/-}$ mice do not have dysfunctional Kv currents following hypoxia and inhibition of HIF-1α restores Kv1.5 channel current in PASMCs from pulmonary hypertensive fawn-hooded rats (Bonnet et al., 2006). It maybe the case that certain Kv channels have HREs in their promoter that, when activated by HIF-1α, cause a decrease in gene transcription.

PASMCs from FPAH/IPAH patients have a higher cytosolic [Ca^{2+}] than cells from APAH patients or control subjects. This is associated with decreased Kv current and cell membrane depolarisation (Yuan et al., 1998). The elevated [$Ca2^+$] concentration in the PASMCs would facilitate both agonist induced vasoconstriction and cellular proliferation.

In addition to their role in regulating intracellular [Ca^{2+}] and hence vasoconstriction and proliferation, K^+ channels also regulate apoptosis. K^+ channels control the rate of apoptotic volume decrease (AVD), an early feature of apoptosis. Cellular volume is controlled by ion concentration gradients and K^+, being the major cation in the cytosol, has a critical role in determining cell volume. When K^+ channels are activated, K^+ ions pass out of the cell into the intercellular space. This results in a corresponding efflux of chloride ions (Cl^-) out of the cell along its electrochemical gradient, a process that is further enhanced by the membrane hyperpolarisation caused by the K^+ efflux. The accumulation of KCl outside the cell shifts the osmotic balance so that H_20 is drawn out of the cell causing AVD, a process which results in activation of cytochrome-c, caspases and apoptosis (Platoshyn et al., 2002;

Remillard and Yuan, 2004). Staurosporine (ST), a known inducer of apoptosis activates Kv currents in PASMCs and Bcl-2, an anti-apoptotic protein prevents apoptosis by reducing Kv current (Ekhterae et al., 2001). A decrease in K^+ channel function would, therefore, prevent the reduction in cell volume thus preventing apoptosis. In PAH this is likely to alter the balance between proliferation/apoptosis in PASMCs towards proliferation and subsequent vascular remodelling. Conversely, restoration of Kv current by Kv1.5 gene transfer reduces PVR and remodelling after 14 days in rats despite continuing hypoxia (Pozeg et al., 2003). Any deviation in Kv channel function and current can influence three major contributors to PAH, namely vasoconstriction, cellular proliferation and apoptosis so Kv channels are likely to be important mediators in the disease.

There are three groupings of Kv channels. The first group, containing Kv1-6 and Kv8-9 is the group that has been most studied in relation to PAH. Another group is comprised of Kv10-12 and the final group is composed of Kv7.1-7.5, also called KCNQ channels. In PASMCs the K^+ current is composed of many Kv channels with similar attributes and the relative contribution of the various Kv channel subunits to K^+ current differs between cell types (Bonnet and Archer, 2007). The development of more specific Kv channel antagonists and inactivating antibodies is helping to unravel the composition of the whole cell K^+ current which will enable better targeting of therapeutics. Although Kv1.5 and Kv2.1 are well established as important contributors to PASMC K^+ current, other Kv channels also contribute and recently, KCNQ channels were found to contribute to the resting V_m of PASMCs. This information was derived from the use of two selective KCNQ channel inhibitors (linopirdine and XE991) that caused powerful vasoconstriction of isolated pulmonary arteries, an effect caused by a direct action on the PASMCs (Joshi et al., 2006). KCNQ channels have recently been found to also contribute to the resting V_m of rat mesenteric arteries (Mackie et al., 2008). It is only within the last five years that KCNQ channels have been thought of as among the group of functional K^+ channels in vascular

smooth muscle cells and hence their functional status or role in PAH is unknown.

1.2.3.2.3 Appetite suppressants

Appetite suppressants, specifically substituted amphetamine appetite suppressants are important risk factors for the development of PAH. Aminorex was the first such drug to be associated with the disease and gave the first indication that 5-HT may be involved in the pathophysiology of PAH. Subsequently, it was found that fenfluramine and its more active dextro-isomer, Dfen were associated with an increased risk of PAH. In people who used fenfluramines for more than three months, the risk of PAH increased 23 fold (Abenhaim et al., 1996). While the risk of PAH increases with increasing exposure, even short term use (<1 month) can induce fatal PAH (Mark et al., 1997). Dfen is now known to have a complicated mechanism of action that is not fully understood. Along with other common appetite suppressants, Dfen is a 5-HTT substrate causing 5-HT cellular efflux while being taken up by the 5-HTT into the cell. Similar compounds to Dfen that are not 5-HTT substrates are not associated with PAH and so it seems likely that Dfens mechanism of action is, at least in part, due to its role as a 5-HTT substrate (Rothman et al., 1999). 5-HTT substrates also cause far greater release of 5-HT than non substrate 5-HTT inhibitors such as fluoxetine (Zolkowska et al., 2006) adding further support to the serotonin hypothesis of PAH. In addition to its interaction with the 5-HTT, Dfen also inhibits K^+ channels in PASMCs (Weir et al., 1996; Perchenet et al., 2001) and the Dfen metabolite NDfen is an arterial vasoconstrictor acting via $5\text{-}HT_{2A}$ receptors (Ni et al., 2005). Only a minority of Dfen users develop PAH and of those that do, some develop the disease after a relatively short exposure time while others may only develop the disease after years of use. This suggests that other underlying susceptibilities exist that predispose certain individuals to Dfen associated PAH. BMPR2 mutations have been found in 9% of fenfluramine users with PAH and these patients develop PAH after a relatively short exposure time (Humbert

et al., 2002). This suggests an interaction between genetic pre-disposition and Dfen to initiate the disease in these patients.

1.2.3.2.4 Female gender as a risk factor for PAH

PAH occurs more commonly in women than men (Loyd et al., 1984: Rich et al., 1987; Loyd et al., 1995), a situation paralleled in other lung conditions including idiopathic pulmonary fibrosis, COPD in non smokers and asthma (Carey et al., 2007). Interestingly, the epidemiological data for asthma show that there is a higher prevalence in males during childhood but this reverses to a female predominance at the time of puberty and continues till the fifth or sex decade after which the female predominance disappears (Carey et al., 2007). This strongly suggests an influence of female sex hormones on the progression of asthma. The fact that the majority of PAH cases occur in women of child-bearing age has also caused speculation that female sex hormones may be responsible for the increased risk of PAH seen in this population. Experiments that have assessed the role of female sex hormones in animal models of PAH have, more often than not, shown that female hormones actually reduce the symptoms of PAH. For example, 17β-estradiol (estradiol) protects rats against MCT induced PAH (Farhat et al., 1993) and estradiol attenuates persistent PAH in fetal lambs (Parker et al., 2000). 2-methoxyestradiol (2-ME), a major metabolite of estradiol is involved in the protective effect that female hormones have in protecting animals against MCT induced PAH (Tofovic et al., 2005; Tofovic et al., 2006). Gender differences have also been noted in the response to hypoxia. During one study, female mice were found to be more resistant to hypoxia than males and administration of estradiol prolonged survival times under hypoxia (Stupfel et al., 1984). In relation to PAH, female rats were shown to develop less severe PAH following hypoxia than males (Rabinovitch et al., 1981) and ovariectomized rats develop more severe hypoxic PAH than rats with intact ovaries (Resta et al., 2001).

It is not known how female sex hormones may influence the progression of PAH in animal models of PAH or in human PAH patients. Estradiol and its metabolites can influence vascular tone, cellular proliferation and apoptosis through both genomic and non genomic mechanisms.

1.2.3.2.5 Endothelial derived factors

PAECs secrete a wide variety of vasoactive mediators that directly influence vasoconstriction and cellular growth in pulmonary arteries. Some of these have been mentioned previously and many are altered in PAH. A tight balance between vasoconstrictors and mitogenic agents against vasodilators and pro-apoptotic factors controls vascular homeostasis, low vascular tone, inflammation and tissue repair. In PAH, the balance is thought to favour proliferation and vasoconstriction and there have been many experimental observations that support this view. This is a brief summary of some of those observations:

1.2.3.2.5.1 Nitric oxide

As an important regulator of vascular tone (Cooper et al., 1996), NO has come under attention from PAH researchers but the expression and role of NOS in the pulmonary hypertensive lung is not clear. One study found NOS to be dramatically downregulated in the lungs of PAH patients compared with control subjects (Giaid and Saleh, 1995). Other human studies have found increased expression of eNOS in small resistance arteries in pulmonary hypertensive patients although with large individual variability (Mason et al., 1998). In this study, plexiform lesions were found to have high levels of eNOS, perhaps indicating proliferating PAECs. It is uncertain what the role of NO might be in the endothelium of plexiform lesions and whether its effects are beneficial or detrimental (Mason et al., 1998). eNOS deficient mice have increased PAP and PVR compared with

WT mice (Steudel et al., 1997). Mice lacking eNOS develop severe PAH following mild hypoxia (Fagan et al., 1999) and conversely, increased levels of eNOS attenuate hypoxic PAH in mice (Ozaki et al., 2001). Administration of the NO precursor, L-arginine reduces PAH following hypoxia and MCT in rats (Mitani et al., 1997) and reduces PAP and PVR in human patients with PAH (Mehta et al., 1995; Nagaya et al., 2001).

While there is some uncertainty as to any pathological role NO dysfunction may play in the development of PAH, its potent effect as a pulmonary vasodilator has led to NO inhalation being tested as a therapy for PAH (Pepke-Zaba et al., 1991; Goldman et al., 1995; Hoeper et al., 2000; Krasuski et al., 2000) with results often comparable to other treatments currently used as inhalable vasodilators such as certain prostacyclin analogues. cGMP is the main signalling molecule mediating the vasodilator effect of NO and is degraded in the lung by phosphodiesterase type 5, (PDE5). Sildenafil, a PDE5 inhibitor is currently a therapeutic candidate for the treatment of PAH. Sildenafil inhibits hypoxic PAH in humans and mice (Zhao et al., 2001) and can reduce PVR in humans with chronic PAH (Ghofrani et al., 2004).

1.2.3.2.5.2 Prostacyclin

PGI_2 is a potent vasodilator that acts to counteract vasoconstriction and proliferation (Budhiraja et al., 2004). It is produced by prostacyclin synthase (PGI_2-S) and this enzyme is decreased in pulmonary arteries from PAH patients, with the most pronounced reduction occurring in vessels with concentric intimal lesions (Tuder et al., 1999). Overexpression of PGI_2-S protects mice against hypoxic PAH (Geraci et al., 1999) and administration of PGI_2-S via gene transfer attenuates MCT induced PAH in rats (Nagaya et al., 2000). Prostacyclin and its analogues have been the most successful treatments for attenuating the symptoms of PAH in humans to date. Epoprostenol (intravenous PGI_2 analogue), beraprost (orally active PGI_2 analogue), iloprost (inhalation carbacyclin PGI_2 analogue) and

treprostenil (PGI_2 analogue that is room temperature stable allowing subcutaneous infusion) have all been used successfully on PAH patients in all clinical categories (Saji et al., 1996; McLaughlin et al., 1998; Nagaya et al., 1999; Rosenzweig et al., 1999; Robbins et al., 2000; Olschewski et al., 2000; Hoeper et al., 2000; Simonneau et al., 2002).

1.2.3.2.5.3 Endothelin-1

ET-1, a pulmonary vasoconstrictor (Deuchar et al., 2002) and PASMC mitogen (Davie et al., 2002) is upregulated in both hypoxic (Li et al., 1994) and MCT (Frasch et al., 1999) animal models of PAH and also in the endothelium of remodelled vessels in PAH patients (Giaid et al., 1993). ET-1 receptor antagonists such as bosentan and sitaxsentan have shown promise in improving symptoms and exercise capacity in PAH patients (Channick et al., 2001; Rubin et al., 2002; Barst et al., 2002).

1.2.3.2.5.4 Thromboxane

Produced by PAECs and platelets, TXA_2 is a pulmonary vasoconstrictor, PASMC mitogen and activator of platelet aggregation (Tuder and Hassoun, 2004). Patients with PAH show elevated excretion rates of 11-dehydro-thromboxane B_2, a metabolite of TXA_2, in addition to decreased excretion of the PGI_2 metabolite, 2,3-dinor-6-keto-prostaglandin F1α. (Christman et al., 1992). This is evidence for an imbalance between vasoconstrictors and vasodilators in PAH. Terbogrel, a combined TXA_2 synthetase inhibitor and TXA_2 receptor antagonist was tested unsuccessfully in one clinical trial as a treatment for PAH and caused significant side effects leading to premature termination of the study (Langleben et al., 2002).

1.2.3.2.6 Non-endothelial derived factors

1.2.3.2.6.1 Vascular endothelial growth factor

VEGF is highly expressed in the lungs and is the most important growth factor for PAECs, promoting PAEC survival and tissue homeostasis (Said, 2006). VEGF is upregulated by hypoxia in human hepatoma cells via HIF-1α activation of a HRE (Forsythe et al., 1996). The role of VEGF in hypoxic PAH, however, is uncertain. Two groups found that circulating levels of VEGF and lung VEGF mRNA levels are increased in hypoxic rats (Christou et al., 1998) and that overexpression of VEGF protects rats against hypoxic PAH (Partovian et al., 2000). Contradictory findings include unchanged VEGF expression in rat lung following hypoxia and a 90% decrease in rat lungs following MCT (Partovian et al., 1998). Pfeifer and colleagues could also find no change in VEGF gene expression in hypoxic rat lungs (Pfeifer et al., 1998). Due to the critical role VEGF has in preventing PAEC apoptosis, it is reasonable to assume that loss of VEGF would promote PAEC apoptosis, thus having a detrimental effect on vascular integrity. Indeed, it has been demonstrated that blockade of the VEGF receptor induces mild PAH in rats and when combined with hypoxia induces severe irreversible PAH associated with initial PAEC apoptosis, followed by proliferation of apoptosis resistant cell populations and formation of obliterative intimal lesions (Taraseviciene-Stewart et al., 2001). There is also evidence that VEGF receptor blockade can induce transdifferentiation of endothelial derived precursor cells into PASMC like cells (Sakao et al., 2007) which is also thought to occur in the vascular remodelling associated with PAH.

1.2.3.2.6.2 Platelet-derived growth factor

PDGF is a PASMC mitogen (Yu et al., 2003) and its expression is increased in the lungs of PAH patients compared with control subjects (Perros et al., 2008). The PDGF receptor antagonist, imatinib reverses both hypoxic and MCT induced PAH in rats (Schermuly et

al., 2005) and imatinib has been used successfully in the treatment of one isolated case of severe PAH (Ghofrani et al., 2005).

1.2.3.2.6.3 Adrenomedullin

Adrenomedullin was first isolated in 1993 from human pheochromocytoma cells (Kitamura et al., 1993). It has now been found in a multitude of tissues in both animals and humans, including the lung, which also contains a dense number of adrenomedullin receptors, a number that is unusually high for peptide receptors (Ichiki et al., 1994; Sakata et al., 1994; Owji et al., 1995). Adrenomedullin is a systemic and pulmonary vasodilator, acting via cAMP and is also an inhibitor of vascular smooth muscle cell proliferation (Kano et al., 1996). Plasma levels of adrenomedullin are increased in patients with PAH (Kakishita et al., 1999), the reasons for which are unclear. It perhaps acts as a compensatory mechanism against the increased PVR. The high potency of adrenomedullin coupled with its high expression in the lung led to its testing in animal models of PAH. Both subcutaneous and inhaled adrenomedullin attenuates MCT induced PAH in rats (Yoshihara et al., 1998; Nagaya et al., 2003). Adrenomedullin is also effective in reducing the parameters of PAH in hypoxic rats (Qi et al., 2006) and mice deficient in adrenomedullin develop more severe PAH following hypoxia than WT mice (Matsui et al., 2004). In human IPAH patients, adrenomedullin improves pulmonary haemodynamics and exercise capacity (Nagaya et al., 2004) and is a more effective treatment used in combination with NO than NO alone (Dani et al., 2007).

1.2.3.2.6.4 Vasoactive intestinal peptide

VIP is a pulmonary artery vasodilator (Sago and Said, 1984; Shahbazian et al., 2007) and PASMC growth inhibitor (Petkov et al., 2003). Pre-treatment with VIP attenuates MCT induced PAH in rabbits (Gunaydin et al., 2002) and also reduces PVR in pulmonary

hypertensive piglets (Haydar et al., 2007). There is growing evidence that VIP has a role in controlling vascular stasis in the lung. VIP knockout mice ($VIP^{-/-}$) develop pulmonary arterial remodelling and RVH in normal air and the symptoms are reduced in mice receiving VIP (Said et al., 2007). VIP serum levels are lower in PAH patients than control subjects (Haberl et al., 2007) and VIP, which is moderately expressed in pulmonary arteries from control subjects, is absent in pulmonary arteries from PAH patients. This was associated with an upregulation of VPAC-1 and VPAC-2 (Petkov et al., 2003). Petkov and colleagues also administered inhalation VIP to PAH patients daily for 3 months. This resulted in significant improvements in PAP, CO and a 50% reduction in PVR. A corresponding increase in exercise tolerance was also measured. The success of this preliminary study coupled with the lack of side effects has led to the undertaking of further clinical trials studying the therapeutic potential for VIP in PAH (Said, 2006).

1.2.3.2.6.5 Angiopoietin-1

Angiopoitein-1 is secreted by smooth muscle cells during development and is involved in angiogenesis. It binds to the endothelial-specific tyrosine kinase (TIE2) receptor which is present only on vascular endothelial cells and helps co-ordinate cells during vascular development. In 2003, Du and colleagues (Du et al., 2003) found that angiopoietin-1 protein and mRNA expression were increased in the lungs of patients with PAH and that angiopoietin-1 levels correlated with disease severity. Angiopoietin-1 also decreased BMPR1A expression when incubated in cultured PAECs. Further work from this group showed that overexpression of angiopoietin-1 in rat lungs induced muscularisation of pulmonary arteries and PAH (Sullivan et al., 2003). Cell culture experiments using human PASMCs and PAECs showed that angiopoietin-1 induced PAECs to release 5-HT that induced proliferation of PASMCs. The growth of the PASMCs was inhibited by fluoxetine (Sullivan et al., 2003). In contrast to these results, Zhao et al., 2003 put forward evidence

for a protective role for angiopoietin-1 in PAH. They found that angiopoietin-1 gene transfer into rat PASMCs increased eNOS expression while reducing RVP, EC apoptosis and mortality in rats with MCT induced PAH. More recent studies support the former hypothesis for a deleterious action of angiopoietin-1 in PAH. Dewachter et al., 2006 also obtained the result that angiopoietin-1 induces PAECs from both IPAH patients and control subjects to release 5-HT with more 5-HT being released from PAECs from IPAH patients. PAECs from IPAH patients also released ET-1 following angiopoietin-1. The greater release of growth factors from PAECs was attributable to heightened expression of the TIE2 receptor (Dewachter et al., 2006).

1.3 Aim of thesis

It is generally accepted that PAH is a multi-factorial disease with known genetic risk factors such as mutations in BMPR2 coupled with possible genetic risk factors (5-HTT+ polymorphisms) interacting with one or several of the physiological and exogenous modifying factors outlined in the previous sections. The aim of this thesis is to investigate modifying factors that may interact with genetic predisposition to trigger the phenotypic changes associated with PAH. This will involve a combination of *in vivo*, *in vitro* pharmacological and biochemical/histological techniques. Transgenic (TG) mice overexpressing the 5-HTT (5-HTT+), deficient in BMPR2 ($BMPR2^{+/-}$) or a double (cross) transgenic 5-HTT+/$BMPR2^{+/-}$ (Cross TG) will be employed in addition to mice lacking peripheral 5-HT ($Tph1^{-/-}$). Additional modifying factors assessed in these transgenic models were:

- Hypoxia
- Dfen and NDfen
- 5-HT
- BMP-2

- KCNQ channels
- Gender

Chapter 2

Materials and methods

2.1 Animals

All animals were bred in the Central Research Facility, University of Glasgow and when required for experimental use, were housed in a holding room with a controlled environment: temperature (21°C), humidity (60%) and regular cycling of filtered air. A 12 hour light/dark cycle was maintained. Animals were kept in cages with a suitable supply of water and diet and checked daily. All experiments were conducted under a HO Project Licence number 60/3773 granted to Prof M MacLean.

2.1.1 WT mice

WT mice were C57BL/6 strain.

2.1.2 The 5-HTT+ mouse

5-HTT+ mice were donated to Prof M MacLean by Prof A Harmar, Edinburgh University. The C57BL/6xCBA WT strain was employed to generate the 5-HTT+ mouse. The transgene was a 500-kb yeast artificial chromosome (YAC35D8) containing the h5-HTT gene flanked by 150 kb of 5' and 300 kb of 3' sequence with the short allele of the 5-HTTLPR in the promoter region and the 10-repeat allele of the variable-number tandem repeat (VNTR) in intron 2 (Shen et al., 2000a). YAC35D8 was modified to include a hemagglutinin epitope tag at the C-terminus of the 5-HTT protein and a *lacZ* promoter gene downstream of an internal ribosomal entry site as described previously (Shen et al., 2000b). The phenotype was verified by RT-PCR by Mrs Lynn Loughlin in Prof MacLean's laboratory.

2.1.3 The BMPR2$^{+/-}$ mouse

The BMPR2$^{+/-}$ mice were donated to Prof M MacLean by Prof N Morrell, University of Cambridge.

The C57BL/6 WT strain was employed to generate the BMPR2$^{+/-}$ mouse. A replacement gene targeting vector was constructed with exons 4 and 5 (which encode the entire transmembrane domain and the amino-terminal region of the kinase domain respectively) deleted and replaced by a cassette of SE IRED/LacZ/loxP/pgk neo/loxP. The targeting vector was transfected into embryonic stem cells by electroporation. Positive recombinant clones were injected into blastocysts from C57BL/6 mice. These chimeric mice were then bred with C57BL/6 females and offspring analysed for presence of the BMPR2 mutation by southern blotting. Mice positive for the BMPR2 mutation were BMPR2$^{+/-}$ (Beppu et al., 2000). The phenotype was verified by RT-PCR by Mrs Lynn Loughlin in Prof MacLean's laboratory.

2.1.4 The 5-HTT+ x BMPR2$^{+/-}$ mouse

5-HTT+ and BMPR2$^{+/-}$ mice were bred together (usually 1 male to 2 females) to produce a double (cross) TG strain that are 5-HTT+/BMPR2$^{+/-}$ and will be referred to in this thesis as Cross TG mice. This cross breeding was carried out under the supervison of Dr Yvonne Dempsie and Dr Ian Morecroft and the phenotype verified by Mrs Lynn Loughlin in Prof MacLean's laboratory.

2.1.5 The Tph1$^{-/-}$ mouse

Tph1$^{-/-}$ mice were donated by Prof M Bader, Max-Delbrück-Center for Molecular Medicine Berlin-Buch, Germany.

The C57BL/6 WT strain was employed to generate the Tph1$^{-/-}$ mouse. A 1 kb (short arm) and 5.2 kb (long arm) fragment were used to construct a targeting vector containing a neomycin-resistance cassette and a thymidine kinase cassette. The construct was transfected in embryonic stem cells by electroporation. Positive recombinant clones were injected into blastocysts from C57BL/6 mice. These chimeric mice were then bred with

C57BL/6 females and offspring analysed for presence of the targeted Tph1 mutation by southern blotting. Mating of the resulting $Tph1^{+/-}$ mice generated $Tph1^{-/-}$ mice (Walther et al., 2003).

2.1.6 Animal weights

2-3 months mice: 15-40 grams

5-6 months mice: 25- 50 grams

2.2 Hypoxic model of PAH

As covered extensively during the preceding chapter, hypoxia is one of two principal methods commonly used to induce PAH in animals. For the purposes of this investigation hypoxia involved housing mice in an atmosphere of approximately 10% O_2 for a period of 14 days. To achieve hypoxia, mice were kept in a hypobaric chamber. The chamber is built from perspex with a removable door. Caged mice were placed into the hypobaric chamber and a pump activated that continuously removes air at a constant rate. The chamber door is sealed by the pressure gradient between the chamber and outside environment which is created by a small controllable inlet valve on the door that controls air flow into the chamber. By opening and closing the valve, chamber pressure can be raised and lowered respectively. As the atmospheric pressure of the chamber decreases, the partial pressure of the chamber gases decreases and so controlling chamber pressure allows the control of O_2 concentration. The chamber pressure was lowered by 50 mbar per hour over two days to 550 mbar at which point, the O_2 concentration is ~ 10%. The chamber was in an animal holding room which was continuously ventilated with room air at 45 L per minute to prevent accumulation of CO_2 and to maintain low humidity. Chamber temperature was monitored by two thermometers, one inside the chamber and one resting on the outside of the chamber. Internal chamber temperature was within 2°C of the outside temperature

which was maintained at approximately 21°C. Chamber conditions and status of mice were checked daily and information logged. The chamber was briefly recompressed every 3 days to clean cages and replenish food and water.

2.3 *In vivo* haemodynamic measurements

Anaesthesia of mice was induced by 3% isoflurane in O_2 and body weight measured. Mice were then placed in a supine position with limbs secured by tape. Anaesthesia was maintained by 1 – 1.5% isoflurane in O_2 supplied via a face mask. Sufficient level of anaesthesia was determined by absence of hind limb and tail reflex.

A small area of fur and skin was removed over the sternum in preparation for transdiaphragmatic cardiac puncture that would allow recording of RVP. Right ventricular haemodynamic function is the most critical factor in determining survival in patients with PAH (D'alonzo et al., 1991) and elevated RVP has been used extensively as an index of PAH (Eddahibi et al., 2000; Launay et al., 2002; Maclean et al., 2004). These reasons coupled with the relative simplicity of measuring RVP through cardiac puncture compared with measuring PAP led to RVP being the haemodynamic measurement of choice in this thesis. The procedure employs a polypropylene hypodermic needle (25 gauge) attached to a an E751A pressure transducer (Elcomatic, Glasgow, UK) which is mounted onto a micromanipulator (Stoelting Co., Wood Dale, IL). The needle and transducer are filled with 0.9% saline solution containing heparin (25 units/ml) (LEO Pharma Inc, Princes Risborough, UK). The tip of the needle was aligned with the mid point of the sternum and then adjusted approximately 2 mm to the right. The needle was advanced under the rib cage, through the diaphragm and into the right ventricle (RV). Entry of the needle into the RV was confirmed by the waveform trace on the computer screen.

Systemic arterial pressure (SAP) was measured by carotid artery cannulation. A small area of fur was removed at the top of the thorax and blunt dissection was used to expose and

isolate the right carotid artery. Surgical thread was used to tie off the distal end of the artery. A small clip was placed over the proximal end of the artery to prevent blood loss following arterial incision. The incision was made towards the distal end of the artery and a cannula (0.75 mm OD, Portex) was advanced in the proximal direction. A second piece of thread was used to tie around the cannula and artery and the arterial clip removed to allow blood flow into the vessel. Both SAP and RVP were recorded by a MP100 data acquisition system (BIOPAC Systems Inc, Santa Barbra, CA) and relayed to a computer. Following completion of the haemodynamic measurements, the animal was euthanised by 5% isoflurane in O_2 followed by cervical dislocation. The thorax was opened and hearts and lungs from each animal were removed and placed in ice cold Krebs solution (Composition (mM): NaCL 118, $NaHCO_3$ 25, D-glucose 11, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2 (pH7.4)) for further analysis.

2.3.1 Data analysis of *in vivo* data

Results were analysed using the Acquiknowledge 3.7 software package (BIOPAC Systems Inc, Santa Barbra, CA). A minimum of 5 minutes telemetry data was acquired per animal for both RVP and SAP. Figure 2.1 displays a sample screenshot of approximately 15 seconds of telemetry data. Data acquisition involved the analysis of data in three separate 10 second segments spread over the 5 minutes of telemetry. The highlighted area in figure 2.1 represents one of these 10 second segments. The top half of the screen displays RVP and the bottom SAP. Systolic RVP (sRVP) was calculated by recording the value of 4 peaks during systole in each of the three 10 second segments giving 12 values in total which were averaged to give the final sRVP value. Mean RVP (mRVP) was taken from the automatic calculation, provided by the software, for each three segments and then averaged to give the final mRVP. Heart rate (HR) was calculated by highlighting 10 systolic peaks within the 10 second segment and recording the time in seconds for 10 systolic peaks

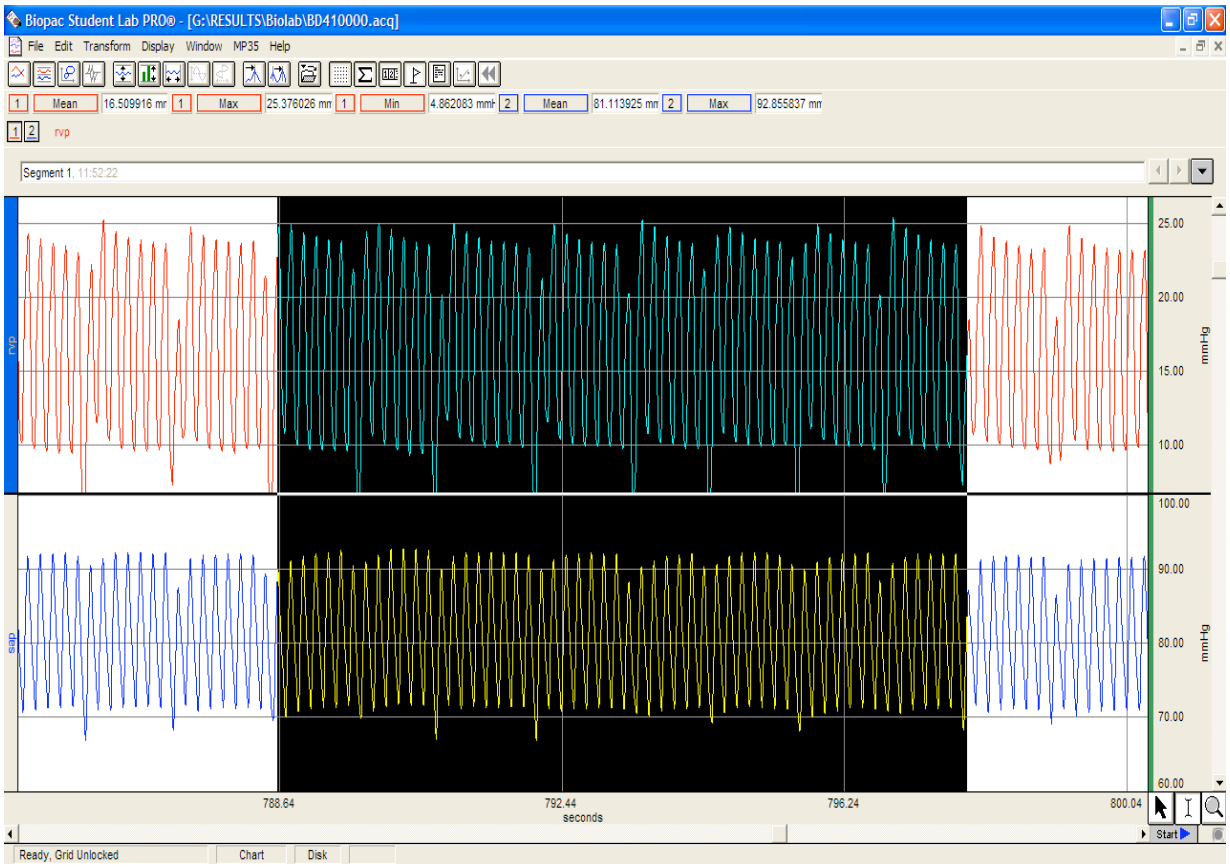


Figure 2.1 Sample of RVP and SAP telemetry trace.
RVP is displayed in red and SAP in blue. Highlighted segment is approximately 10 seconds of data. Three of these segments taken during 5 minutes of data recording were used to derive sRVP, mRVP, mSAP and HR.

(beats) to occur. By dividing 60 (seconds) by this value and multiplying by 10 gives the HR in beats per minute. This was repeated for the two additional segments and averaged to give the final HR. mSAP was recorded in the same manner as mRVP.

2.4 Measurement of right ventricular hypertrophy

Adipose and connective tissue were carefully removed from around the heart and lungs and hearts separated from the lungs. Atria and large calibre blood vessels were dissected from the rest of the heart. The RV free wall was then dissected away from the left ventricle (LV) and septum. The LV + septum along with the RV were blotted dry and weighed separately. The ratio of RV/(LV+S) was used as an index of RVH.

2.5 Lung Histology

One sagittal section was obtained from either the left or right lungs. Sections were paraffin wax fixed and stained with Elastica-Van Gieson stain (by Mrs M Nilsen in Prof MacLean's laboratory) and microscopically assessed for evidence of muscularisation of small pulmonary arteries (<80 μm diameter). Arteries were considered muscularised if they possessed a distinct double-elastic lamina visible around at least 70% of the vessel circumference. At least 150 vessels per section were counted. The percentage of muscularised (remodelled) vessels was calculated as the number of remodelled vessels/total number of vessels counted x 100.

2.6 In vitro pharmacological studies

2.6.1 Wire myography

Myography is a technique for pharmacological investigation of small vessels and was first described by Bevan and Osher (1972) and developed in 1976 by Mulvany and Halpern, allowing analysis of contractile response in vessels with 100-400 μM internal diameter.

Vessels of this size are studied by wire myography, a relatively atraumatic technique providing accurate measurements of muscle contraction.

2.6.1.1 Wire myography equipment

For this study, the Mulvany/Harpern small vessel wire myograph (model 610M) was used; purchased from Danish Myo-technology Ltd, Aarhus, Denmark. The unit has four individual stainless steel organ chamber baths, each capable of holding one vessel. Each organ bath chamber contains two vessel support heads, one is attached to a highly sensitive isometric force transducer and the other to a metal support arm. The metal support arm is connected to a micropositioner which allows adjustment of the support arm and hence the distance between the two support heads. When a vessel is mounted in the chamber, the alteration in distance between support heads alters the tension applied to the vessel. Each chamber has a moveable metal swing arm that enters the top of the chamber and has a dual function. The first is a pipe to control gas flow into the organ bath and the second is a suction pipe for removing fluid from the chamber. Myograph chamber temperature control (± 0.1°C) is maintained by electronic internal heating units. Temperature and force from each chamber are digitally displayed on the base unit. The myograph is connected to a computer and all information is recorded using Myodaq20 software (Danish Myo-technology Ltd, Aarhus, Denmark) and analysed using Myodata (Danish Myo-technology Ltd, Aarhus, Denmark).

2.6.1.2 Intralobar pulmonary artery dissection

Lungs were obtained from freshly culled mice (intraperitoneal injection of 200 mg/kg sodium pentobarbitone (Euthatal) (Merial Ltd, Harlow, UK) or from mice following *in vivo* procedure. The larger left lung was separated from the rest of the lungs and fixed to a dissecting dish using pins. The lung was orientated with the parietal surface lying in an

inferior position. The tissue was regularly washed with ice cold Krebs during dissection to keep tissue hydrated. Using a dissecting microscope (Zeiss), an incision was made at the opening of the airway, cutting down the airway length from the large proximal airway, along the bronchial tree to the distal bronchiole. Remaining bronchiole tissue lying directly above the intralobar pulmonary artery (IPA) was carefully removed exposing the underlying IPA. Surrounding connective tissue was dissected free and up to two 2 mm strips of IPA were cut from the lung attached IPA and placed into ice cold Krebs solution.

2.6.1.3 Vessel mounting procedure

Each myograph chamber was filled with 5mls of Krebs solution and bubbled with a gas mixture containing 16% O_2/ 5% CO_2 (balance N_2). This mixture is set to mimic the arterial partial pressure of each gas *in vivo*. Gas was fed to each myograph chamber from a pre-filled Douglas bag via an electronic Dymax 30 pump (Charles Austen Pumps Ltd, Byfleet, UK). 2 mm sections of IPA dissected previously were placed into a petri dish containing cold Krebs solution. A 40 μm steel wire was slowly advanced through the vessel lumen using two pairs of fine forceps, one pair holding the steel wire and the other gently holding the other end of the vessel. The wire and vessel were then placed into the myograph organ chamber and positioned between the two mounting head jaws. The wire was secured in place by advancing the adjustable mounting head towards the other till there was sufficient pressure between the two sets of jaws to hold the wire in place. The vessel is situated in a space between the mounting head jaws. The steel wire was then secured at each end of the left mounting head (the head attached to the micropositioner) by wrapping each end of the wire around top and bottom screws which were tightened to create tension (figure 2.2A). The two mounting heads were then separated and another section of 40 μm steel wire inserted through the vessel lumen (figure 2.2B). The two heads were once again brought together until the second wire was securely fixed (figure 2.2C) and the wire secured

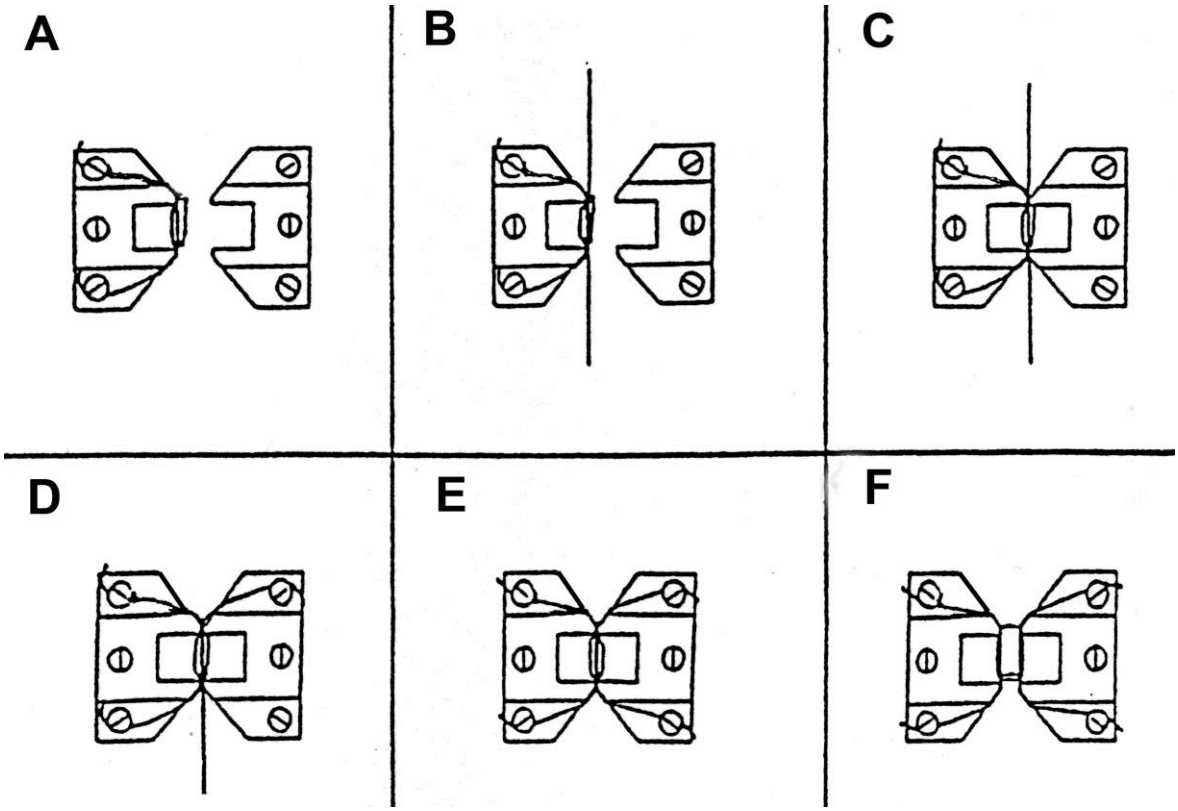


Figure 2.2 Mounting procedure for a vessel on a wire myograph. A 2mm section of IPA containing a steel wire through vessel lumen is secured between mounting jaws to mounting head (A). A second wire is threaded through vessel lumen (B) and attached to other mounting head (C-E). Mounting jaws are then separated slightly in preparation for normalisation.

in place around the two screws on the right mounting head (the head attached to the force transducer) (figure 2.2D and E). The heads were then separated slightly (figure 2.2F) before the chamber was replaced back onto the myograph base unit and the heating unit initialised to maintain temperature at 37°C.

2.6.1.4 Vessel normalisation

Normalisation is a procedure for setting vessels to standard initial conditions to mimic, as closely as possible, the *in vivo* environment. This is achieved by setting an optimal transmural pressure that matches the *in vivo* transmural pressure of the vessel studied. The Laplace equation, which links the wall tension in a cylinder with its internal radius and transmural pressure, can be modified to convey the relationship between variables for a blood vessel on a myograph (equation 2.1).

Equation 2.1) $$Pi = \frac{T}{IC/2\pi}$$

Equation 2.1 Laplace equation for calculation of effective pressure in a cylinder
Pi = effective pressure (the pressure required to extend vessel to the measured internal diameter,) T = wall tension, IC = internal circumference

Wall tension is calculated from the following equation:

Equation 2.2) $$T = \frac{F}{2L}$$

Equation 2.2 Calculation of vessel wall tension
T = wall tension, F = force, L = vessel wall length
Each vessel has an upper and lower wall thus the equation requires vessel length to be multiplied by 2.

The internal circumference is calculated from the micropositioner readings and by incorporating the size of diameter of each steel wire within the vessel lumen (40 μm):

Equation **2.3)** $$IC = (\pi + 2)d + 2(Xi - Xo)$$

Equation 2.3 Calculation of vessel internal circumference
IC = internal circumference, d = diameter of wire, (Xi - Xo) = distance between the 40 μm steel wires

To calculate Xo, the two mounting head jaws are brought together until they gently touch causing a negative pressure trace on the computer screen. The reading is taken at this point from the reading on the micropositioner. Xi is the value recorded from the micropositioner at any given amount of stretch applied to the vessel by adjusting the distance between mounting jaws.

Equation 2.1 can be rearranged to give:

Equation 2.4) $$Pi = \frac{2\pi T}{IC}$$

Equation 2.4 Rearranged Laplace equation for calculation of effective pressure

Using the value of IC calculated above, this can be used, together with the substituted values for wall tension to give the following equation:

Equation 2.5) $$Pi = \frac{2\pi F}{2L \times IC}$$

Equation 2.5 Calculation of transmural pressure of vessel by its relationship to wall tension and internal circumference

This equation allows the calculation of transmural pressure for a vessel. By adjusting the level of stretch on a vessel by small increments, pressure can be increased or decreased until the optimum physiological pressure is achieved which in mouse pulmonary vessels is ~15 mmHg. A range of 12-16 mmHg was considered acceptable.

2.6.1.5 General experimental procedure

Following mounting and normalisation, vessels were left to equilibrate for approximately 30 minutes in Krebs solution (37ºC; 16% O_2/ 5% CO_2 (balance N_2) gas mixture infused). A final bath concentration (FBC) of 50 mM potassium chloride (KCl) was added to each chamber to stimulate vessels and to assess their viability. Once contractile response was maximal and stable, the vessels were washed 6-8 times with fresh Krebs solution and left to return to baseline tension. The KCl procedure was then repeated and this contractile response was used as a reference response. Further experiment specific details can be found in the methods section of individual results chapters.

2.6.1.6 Data analysis of pharmacological studies

The output from the myograph was recorded on a computer using Myodaq20 software (Danish Myo-technology Ltd, Aarhus, Denmark) and analysis of the data performed using Myodata software (Danish Myo-technology Ltd, Aarhus, Denmark). When data produced sigmoidal concentration-response curves, analysis of the data was performed by fitting the data with a Hill equation model using Graphpad Prism 4 (GraphPad Software Inc, CA, USA) This provided values for pEC50 (a measure of drug potency) and Emax (the maximum response induced by a drug). The Hill equation also provides a value derived from the steepness of the slope of the curve. This is called the slope function or *Hill coefficient*. The Hill coefficient was found to be generally uniform across experiments. In those instances where significant differences were observed, these are stated in the results section. Where concentration-response curves clearly did not follow the classical sigmoidal shape or where maximal contractile response was not achieved at concentrations tested, the contractile response at each concentration tested was measured and analysed for intergroup differences.

For all the above measurements, individual concentration-response curves were

constructed for each experimental group and mean ± SEM calculated for each group of vessels.

2.7 Biochemical analysis

2.7.1 Preparation of tissue

2.7.1.1 WT and Tph1$^{-/-}$ male mice (for p-ERK1/2 and p-Smad1/5/8 analysis)

Each mouse was killed by intraperitoneal injection of 200 mg/kg Euthatal and heart and lungs immediately dissected out and placed in ice cold Krebs solution. Heart and lungs were fixed with pins to a dissecting dish and washed regularly with ice cold Krebs. Adipose and connective tissue were dissected from around the heart and lungs to expose the main pulmonary artery. The main pulmonary artery was cut at the entry point to the heart and at the entry point of both the left and right lung. The artery was then transferred into several drops of ice cold Krebs on a petri dish. The lungs were placed into a beaker of ice cold Krebs and the heart and remaining tissue discarded. Once in the petri dish, the main artery was cut into small pieces using dissection scissors before being transferred into a small plastic dish containing lysis buffer (20 mM Tris (Fisher Scientific, Loughborough, UK), 1 mM dithiothreitol (Melford Laboratories Ltd, Ipswich, UK) and a Complete, Mini, EDTA-free serine/cysteine protease inhibitor (Roche Applied Science, Burgess Hill, UK)). The IPA from the left lung was then dissected out as described previously, placed into a few drops of Krebs on a petri dish, cut into small segments and placed into the ice cold lysis buffer. The IPA from the right lung was processed in a similar manner. This process was completed for 24 WT animals and 24 Tph1$^{-/-}$ animals so that there were 3 groups of 8 animals for each genotype. Each group contained IPAs + main pulmonary arteries in 200 µl of lysis buffer. The lysis buffer + tissue samples were transferred into 12 x 75 mm culture tubes (WWR International, Lutterworth, UK) and homogenised using a Miccra-D1 homogeniser (ART Moderne Labortechnik, Mulheim, Germany) for 30 seconds. Samples

were then placed in ice cold sealed eppendorf tubes and stored at - 80°C.

2.7.1.2 WT and Tph1$^{-/-}$ male mice (for BMPR2 analysis)

Procedure as above except lysis buffer was composed of 125 mM Tris, 10% glycerol (Riedel-de Haen, Seelze, Germany), 2% Sodium dodecyl sulphate (SDS) (Fisher Scientific, Loughborough, UK) and Complete, Mini, EDTA-free serine/cysteine protease inhibitor. Experiment utilised 3 groups of 4 animals for each genotype. Quantity of lysis buffer used was reduced proportionally.

2.7.1.3 Male and female WT mice (for p-ERK1/2, p-p38 and p-Smad1/5/8 analysis)

Each mouse was killed by intraperitoneal injection of 200 mg/kg of Euthatal and heart and lungs immediately dissected out and placed in ice cold Krebs solution. Heart and lungs were placed into a petri dish containing a small quantity of cold Krebs solution. The left lung alone would yield sufficient tissue for analysis (personal communication, Lynn Loughlin). The left lung was dissected free of the remaining heart and lung tissue and placed onto a separate petri dish containing a small quantity of cold Krebs. The left lung was cut into small pieces using scissors and transferred into 250 μl of lysis buffer (20 mM Tris, 1 mM dithiothreitol and a Complete, Mini, EDTA-free serine/cysteine protease inhibitor). The lysis buffer + left lobe was transferred into a 12 x 75 mm culture tube and homogenised for 30 seconds. Sample was placed into an ice cold sealed eppendorf tube and stored at -80°C. This process was repeated to give a total of 6 male and 6 female tissue samples.

2.7.2 Preparation of samples and running of acrylamide gel

It was not possible to determine protein concentration of tissue samples using the Bradford assay. Protein concentration was found to be below the level of detection. Subsequently the

plate reader was found to be malfunctioning and further attempts to obtain protein concentration were not possible. It was deemed sufficient to use GAPDH and α – tubulin as loading controls to which western results could be normalised to. The volume of tissue sample + lysis buffer and sample buffer used in the experiments outlined below have previously been shown to be appropriate for reliable detection of proteins (personal communication, Lynn Loughlin).

Tissue samples were removed from -80°C freezer and kept on ice (0°C). To pre-cooled, labelled, top-pierced eppendorf tubes 8 μl (for experiments performed in the smaller volume 12 well polyacrylamide gels) or 10 μl (for experiments performed in the larger volume 10 well polyacrylamide gels) of Nupage 4x LDS sample buffer (Invitrogen, Paisley, UK) was added followed by 3 μl of Nupage (10x) sample reducing agent (Invitrogen, Paisley, UK). Finally, protein samples were added to the eppendorf tubes so that protein sample and sample buffer were in a 1:1 ratio. Samples were then placed in a heating block (Grant Instruments, Cambridge, UK) pre-heated to 70°C for 10 minutes before being centrifuged (Biofuge 15 centrifuge, Heraeus Sepatech GmbH, Osterode, Germany) at 10,000 rpm for 60 seconds. Samples were then placed back on ice in preparation for gel loading. Running buffer containing 5% NuPage MOPS SDS running buffer (20x) (Invitrogen, Paisley, UK) and 95% water was prepared. 200 mls of buffer was placed into a separate beaker and 500 μl of antioxidant (Invitrogen, Paisley, UK) added.

The electrophoresis cell (X Cell, Novex, San Diego, CA) was prepared according to the manufacturer's instructions and contained either 1 or 2 10 well or 12 well Nupage 10-12% Bis-Tris polyacrylamide gels (Invitrogen, Paisley, UK) depending on experimental requirements. Gels were removed from their protective packaging and briefly rinsed with cold water. The casting comb was gently removed and wells washed with running buffer + antioxidant before being placed into the cell. The outer chamber of the cell contained the running buffer and the inner chamber containing the gels contained running buffer +

antioxidant. Using long gel loading pipette tips 10 μl of SeeBlue® Plus2 prestained standard (1x) (Invitrogen, Paisley, UK) followed by 19 μl (12 well gels) or 23 μl (10 well gels) of tissue sample/sample buffer/reducing agent was loaded to the gel. The lid was placed over the cell electrodes and attached to an E861 electrophoresis power supply (Consort, Belgium). The gel was run at 150 volts until the dye had reached the bottom of the gel. This process usually required 1 – 1½ hours.

2.7.3 Protein transfer

This protocol is for experiments using 2 gels in one cell.

In preparation for protein transfer from gel to transfer membrane, 4 sheets of 3mm Whatman filter paper (Whatman plc, Maidstone, UK) and 6 foam sponges were pre-soaked in transfer buffer (750 mls distilled water, 50 mls Nupage transfer buffer (20x), 200 mls methanol (Riedel-de Haen, Seelze, Germany) and 1ml Nupage antioxidant). Two 8cm x 8 cm sections of Immobilon-P polyvinylidene difluoride (PVDF) Transfer Membrane (Sigma-Aldrich, Poole, UK) were cut and activated by immersion in methanol for 1 minute before being transferred into the transfer buffer. In an X Cell II transfer module (Novex, San Diego, CA) 2 soaked sponges were placed. Gel 1 was removed from its plastic casing and placed onto a sheet of Whatman paper with the PVDF membrane placed on the other side of the gel and placed face up into the cassette. A second sheet of Whatman paper was placed over the PVDF membrane, followed by 2 more sponges. Gel 2 was then removed from its casing and orientated on top of the sponges in the same manner as Gel 1. To complete the setup two final sponges were placed over the final piece of Whatman paper on top of the second PVDF membrane and the module lid was fitted in place and the entire transfer module placed into the electrophoresis cell. Both the inner and outer chambers of the cell were filled with transfer buffer. The lid was fitted and the gels run at 30 volts for 1 hour.

2.7.4 Blocking of PVDF membrane

Tris buffered saline (TBS) solution was prepared from a TBS stock (0.2 M Tris, 1.37 M sodium chloride (Riedel-de Haen, Seelze, Germany) in 5 litres of distilled water). The TBS stock solution was diluted 1:10 with distilled water and the pH adjusted to 7.6 using concentrated 37% hydrochloride acid (Riedel-de Haen, Seelze, Germany). Tween (Sigma-Aldrich, Poole, UK) was added at 0.1% giving a Tween-TBS solution (TBST) that would be used as a buffer for washing the PVDF membrane.

Following protein transfer, the gels were safely disposed of and the PVDF membranes were washed in TBST for 5 minutes before blocking of unoccupied protein binding sites in 5% skimmed milk powder (Marvel, Premier Brands, Spalding, UK) in TBST for 1 hour at room temperature under gentle rocking. Membranes were then washed (3 x 15 minutes) in TBST.

2.7.5 Detection

After blocking, primary antibody was added at the appropriate dilution and in the appropriate dilutent (table 2.1). The incubation was carried out overnight at 4°C under moderate agitation. Membranes were then washed (3 x 15 minutes) in TBST before being incubated with the appropriate secondary antibody (table 2.1) diluted in 5% Marvel at room temperature for 1 hour under gentle rocking. Membranes then had a final round of TBST washing (3 x 15 minutes) before being immersed and shaken gently for 1 minute in ECL Western Blotting chemoluminescent substrate (Pierce, Rockford, IL). Membranes were then placed in a hypercassette (Amersham International plc, Little Chalfont, UK) and in a dark room were exposed to x-ray film (Kodak, Hemel Hempstead, UK) for ~1-5 s (α-Tubulin), ~5-60 s (GAPDH), ~10-60 s (p-ERK1/2) and ~5-30 mins (p-p38 and BMPR2) before being developed by a Kodak X-omat imaging machine.

2.7.6 Data analysis of western blots

For WT and Tph1$^{-/-}$ comparison experiments (p-ERK1/2 and p-Smad1/58) there were three lots of samples for each genotype, each containing IPAs and main pulmonary arteries from 8 mice. Each of these samples was run 4 times giving a total of 12. For WT and Tph1$^{-/-}$ comparison experiments (BMPR2) there were 3 lots of samples for each genotype, each containing IPAs and main pulmonary arteries from 4 mice. Each of these samples was run 3 times giving a total of 9. Not all results were useable, however, due to poor detection of protein bands or too high a background signal. This is reflected in the n numbers for experiments being lower than 12 and 9 for respective experiments. For male and female comparison experiments whole lung was used for efficiency, yielding a greater volume of tissue for analysis. In these experiments there were 6 males and 6 females, each run on separate lanes in the one experiment yielding an n of 6 of each gender. Densitometric analysis of films following exposure was performed to determine the normalised level of protein expression using TotalLab version 2 (TotalLab Ltd, Newcastle, UK).

2.8 Statistical analysis

When comparing two groups of data, a Student's unpaired two-tailed t-test was used. When comparing three or more groups of data, analysis of variance (ANOVA) was used. ANOVA was either one-way (when comparing one variable) or two-way (when comparing two variables). Newman-Keuls multiple comparison post test was applied to investigate statistical differences following one-way ANOVA and Bonferroni multiple comparison post test was applied to investigate statistical differences following two-way ANOVA. $P<0.05$ was deemed to indicate statistical significance. All statistics were calculated using GraphPad Prism 4.

1° Antibody	Company	Dilution	Dilutent	2° Antibody	Company	Dilution
Phospho-ERK1/2	Cell Signalling Technology Inc, Beverly, MA	1:1000	TBST/BSA*	Anti-rabbit IgG	Sigma-Aldrich Ltd, Poole, UK	1:1500
Phospho-p38	Cell Signalling Technology Inc, Beverly, MA	1:500	TBST/BSA*	Anti-rabbit IgG	Sigma-Aldrich Ltd, Poole, UK	1:1500
Phospho-Smad 1/5/8	Millipore (UK) Ltd, Croxley, UK	1:1000	TBST/BSA*	Anti-rabbit IgG	Sigma-Aldrich Ltd, Poole, UK	1:1500
BMPR2	BD Biosciences, Oxford, UK	1:500	5% marvel	Anti-mouse IgG	Sigma-Aldrich Ltd, Poole, UK	1:3000
α - Tubulin	Abcam Ltd, Cambridge, UK	1:5000	5% marvel	Anti-mouse IgG	Sigma-Aldrich Ltd, Poole, UK	1:5000
GAPDH	Abcam Ltd, Cambridge, UK	1:20000	5% marvel	Anti-mouse IgG	Sigma-Aldrich Ltd, Poole, UK	1:5000

* 1.5 mls TBST + 1.5 mls (10% bovine serum albumin (BSA) made in TBST)

Table 2.1 Primary and secondary antibodies used for Western blotting.
p-Smad1/5/8 and BMPRR2 1º and 2º dilutions were pre-determined by a series of optimisation experiments until distinct protein bands were observed against a clear background. p-ERK1/2, p-p38, α-tubulin and GAPDH optimal dilutions had been experimentally derived previously (personal communication, Yvonne Dempsie).

Chapter 3

Effect of hypoxia on transgenic mice

3.1 Introduction

Genetic manipulation of mice has provided a vast amount of information for the study of human disease and many genes implicated in PAH have been altered in mice to assess what, if any, role they may have in the pathogenesis of PAH. As discussed previously, these include NOS (Fagan et al., 1999; Ozaki et al., 2001), PGI_2-S (Geraci et al., 1999), adrenomedullin (Matsui et al., 2004), VIP (Said et al., 2007) and K^+ channels (Archer et al., 2001; Pozeg et al., 2003). Of importance to this investigation has been the development of $BMPR2^{+/-}$ mice and 5-HTT+ mice. The $BMPR2^{+/-}$ mouse developed by Beppu et al., 2000 that is used in this investigation and another BMPR2 deficient heterozygous mouse line (carrying an in-frame deletion of exon 2 resulting in the loss of the extracellular ligand binding domain) (Delot et al., 2003) both may mimic the heterozygous loss of BMPR2 function associated with PAH and hence provide a suitable genetic model for studying the disease. Results from the $BMPR2^{+/-}$ mouse used in this investigation to date have been inconclusive with an early study indicating a mild pulmonary hypertensive phenotype in normal air (Beppu et al., 2004) but a later study by a different group finding no evidence of PAH under normoxia (Long et al., 2006). There have also been some discrepancies in data between Beppu et al., 2004 and Long et al., 2006 derived from $BMPR2^{+/-}$ mice following exposure to hypoxia. Neither of these studies, however, found a greater susceptibility to hypoxic PAH in $BMPR2^{+/-}$ mice compared with WT.

Given the conflicting results obtained so far with this $BMPR2^{+/-}$ mouse and the importance that both BMPR2 deficiency and hypoxia have in PAH, there is a need for greater characterisation of the $BMPR2^{+/-}$ mouse phenotype under both normoxia and hypoxia and this investigation will continue the analysis of these mice for susceptibility to PAH. The normoxic 5-HTT+ mouse has increased PAP and under hypoxia, has elevated PAP, vascular remodelling and RVH compared with WT mice (MacLean et al., 2004). This investigation will analyse the 5-HTT+ mice again for evidence of PAH.

The limited animal data gathered so far on the BMPR2$^{+/-}$ mouse does not suggest a strong association with a pulmonary hypertensive phenotype in either normoxia or hypoxia. The human and animal data outlined previously supporting a modulating role for the 5-HTT in the development of PAH created the rationale of developing a mouse model that was BMPR2$^{+/-}$ and 5-HTT+, mimicking a human with BMPR2 mutation and who had the L/L genotype for the 5-HTT. This model would allow assessment of whether the two genetic factors act synergistically to increase the risk of PAH under normoxia or under challenge from an environmental stimulus, in this case hypoxia.

One important aspect that has to be taken into account when interpreting the *in vivo* results just outlined in relation to the BMPR2$^{+/-}$ and 5-HTT+ mice is the role that gender plays in determining the phenotypic outcome. Beppu et al., 2004 used both males and females with the majority being females and found no gender differences in results. Long et al., 2006 used exclusively males. The 5-HTT+ mice used by MacLean et al., 2004 were exclusively females. In this study, both male and female WT, 5-HTT+, BMPR2$^{+/-}$ and Cross TG mice will be assessed allowing the interaction of gender with genetic predisposition to be determined.

3.2 RESULTS

All mice were age 5-6 months.

3.2.1 Pulmonary and systemic haemodynamics in male mice

sRVP and mRVP were assessed in male mice under both normoxic and hypoxic conditions. The results are shown in figure 3.1. WT mice had higher sRVP than Cross TG mice under normoxia (WT: 25.6 ± 0.9 mmHg, n=5, Cross TG: 15.8 ± 1.0 mmHg, n=6) and 5-HTT+ mice had significantly higher sRVP (23.5 ± 2.4 mmHg, n=5) than Cross TG normoxic animals (5-HTT+: 23.5 ± 2.4 mmHg, n=5, Cross TG: 15.8 ± 1.0 mmHg, n=6). Inter-group differences were also observed for sRVP in hypoxic mice. WT mice (38.4 ± 2.2 mmHg, n=6) had significantly higher sRVP than both $BMPR2^{+/-}$ (15.1 ± 1.0 mmHg, n=5) and Cross TG (25.3 ± 2.2 mmHg, n=6) mice. 5-HTT+ hypoxic mice had similar sRVP (39.2 ± 1.5 mmHg, n=8) to WT mice which was greater than the sRVP recorded in $BMPR2^{+/-}$ and Cross TG animals. No significant inter-group differences in mRVP were found for either normoxic or hypoxic animals.

Hypoxia caused a significant rise in sRVP in WT, 5-HTT+ and Cross TG mice (WT: $p<0.001$, 5-HTT+: $p<0.001$, Cross TG: $p<0.01$) but not in $BMPR2^{+/-}$ mice (figure 3.1A). Similar results were obtained for mRVP with hypoxia causing a significant increase in mRVP in WT ($p<0.001$), 5-HTT+ ($p<0.001$) and Cross TG ($p<0.05$) mice (figure 3.1B).

No inter-group differences were observed for either mSAP or HR and these parameters were not significantly altered by hypoxia (figure 3.2)

3.2.2 Pulmonary and systemic haemodynamics in female mice

The results are shown in figure 3.3. In normoxic conditions, unlike male mice, 5-HTT+ female mice had higher sRVP than both WT and $BMPR2^{+/-}$ (5-HTT+: 29.0 ± 3.4 mmHg, n=6, WT: 15.9 ± 2.1 mmHg, n=4, $BMPR2^{+/-}$: 16.8 ± 3.2, n=4) mice. No inter-group

differences were observed in hypoxic animals. The only significant difference in mRVP was between normoxic 5-HTT+ and WT with the 5-HTT+ group having the higher pressure (5-HTT+: 19.8 ± 3.1 mmHg, n=6, WT: 8.3 ± 1.9, n=4).

Hypoxia increased sRVP in WT ($p<0.01$), $BMPR2^{+/-}$ ($p<0.05$) and Cross TG ($p<0.05$) mice (figure 3.3A) and mRVP was increased in WT ($p<0.01$), 5-HTT+ ($p<0.05$) and Cross TG ($p<0.05$) mice (figure 3.3B).

No significant differences were observed in mSAP under normoxia. Under hypoxia, 5-HTT+ mice had elevated mSAP compared with the other three groups (5-HTT+: 99.0 ± 3.6 mmHg, n=6, WT: 84.5 ± 2.2 mmHg, n=6, $BMPR2^{+/-}$: 75.1 ± 3.8 mmHg, n=4, Cross TG: 83.1 ± 4.9 mmHg, n=4). Hypoxia only affected mSAP in $BMPR2^{+/-}$ mice where it caused a decrease in pressure ($p<0.05$) (figure 3.4A).

HR was similar amongst groups under normoxia (figure 3.4B). Under hypoxia, however, $BMPR2^{+/-}$ mice had significantly lower HR compared with the other groups ($BMPR2^{+/-}$: 297.8 ± 4.4 bpm, n=9, WT: 438.0 ± 12.6 bpm, n=9, 5-HTT+: 445.2 ± 18.3 bpm, n=9, Cross TG: 409.0 ± 43.7 bpm, n=4). Similar to the mSAP results, hypoxia caused a decrease in HR in $BMPR2^{+/-}$ mice ($p<0.05$) while the other groups were unchanged.

3.2.3 Right ventricular hypertrophy and vascular remodelling in male mice

RVH and vascular remodelling data are presented in figure 3.5. There were no inter-group differences in RVH in either normoxic or hypoxic conditions. Hypoxia significantly increased RVH in $BMPR2^{+/-}$ ($p<0.01$) and Cross TG ($p<0.01$) mice. Remodelling was similar between groups in both normoxia and hypoxia. When comparing normoxic and hypoxic groups, there was a greater than 5 fold difference in each of the 4 genotype ($p<0.001$ for each group except $BMPR2^{+/-}$ where significance was $p<0.01$) groups.

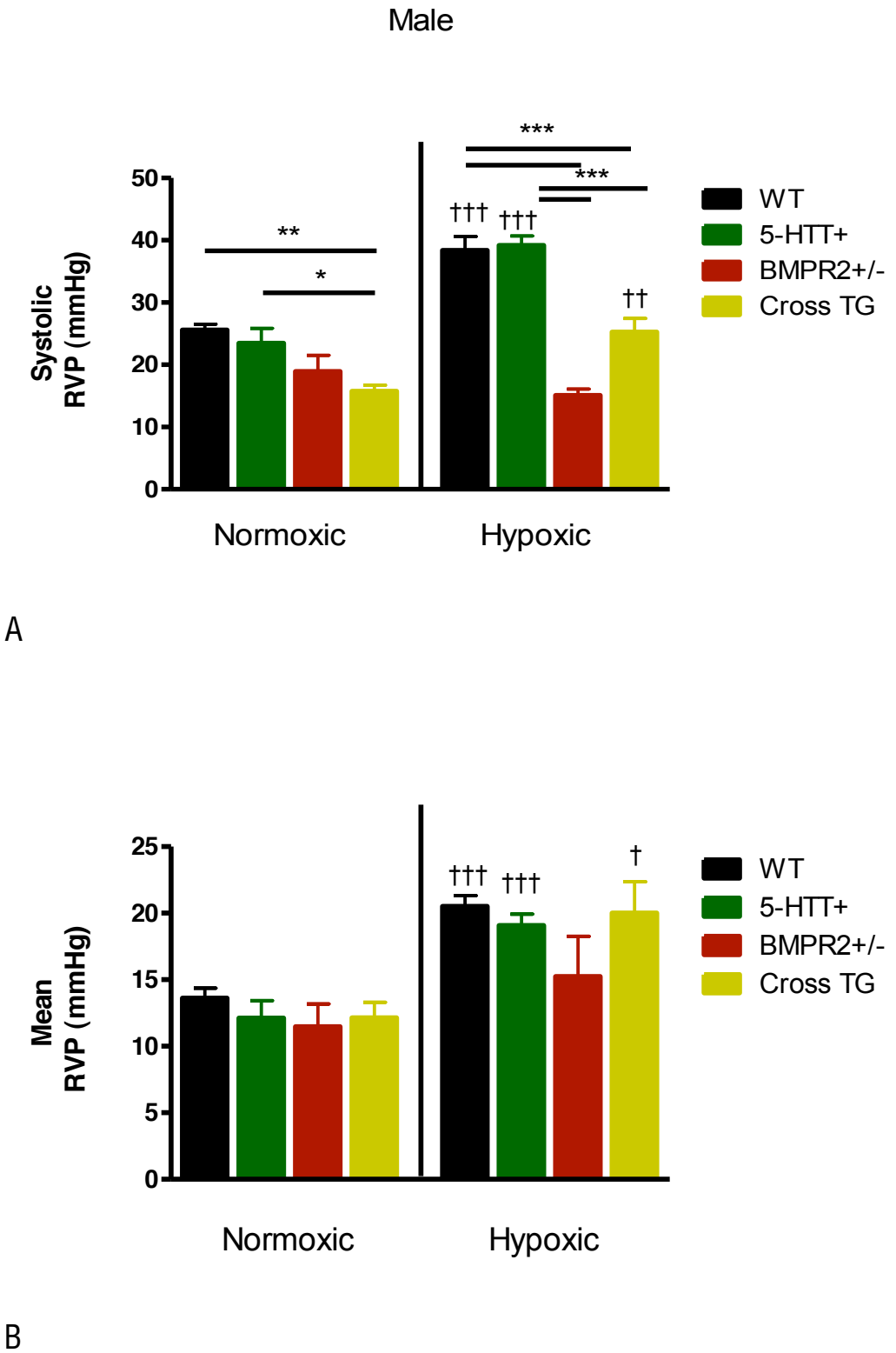
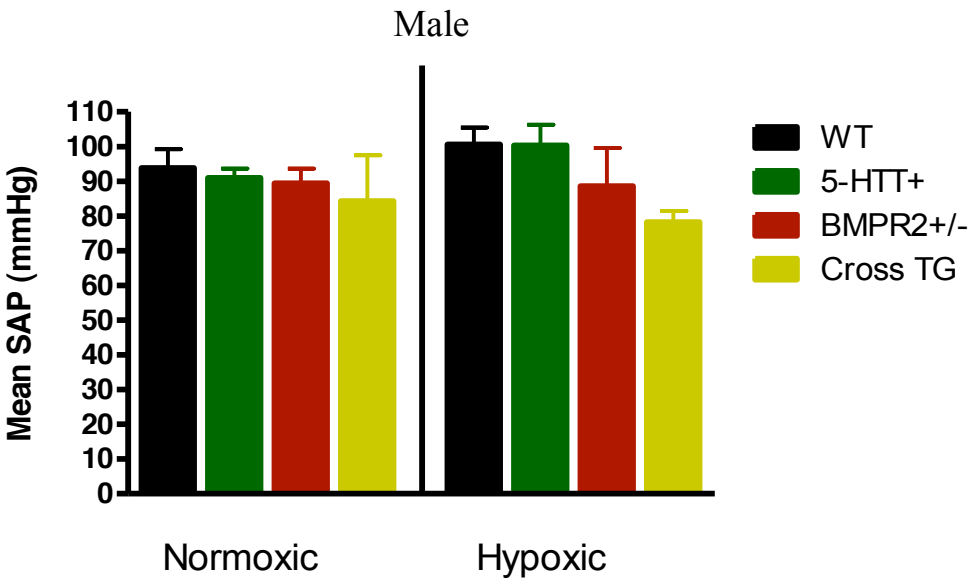
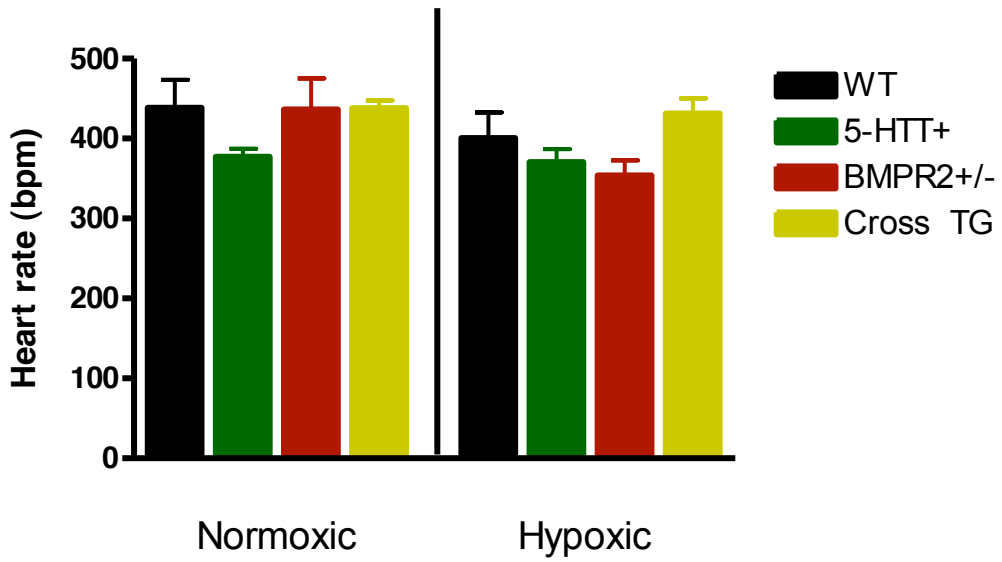


Figure 3.1 Effect of 14 days hypoxia in WT, 5-HTT+, BMPR2$^{+/-}$ and Cross TG male mice (n=4 to 8 mice per group) on sRVP (A) and mRVP (B). Results expressed as mean ± SEM. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ using Newman-Keuls multiple comparison test. † $p<0.05$, †† $p<0.01$, ††† $p<0.001$ versus corresponding normoxic value using an unpaired two-tailed t-test.

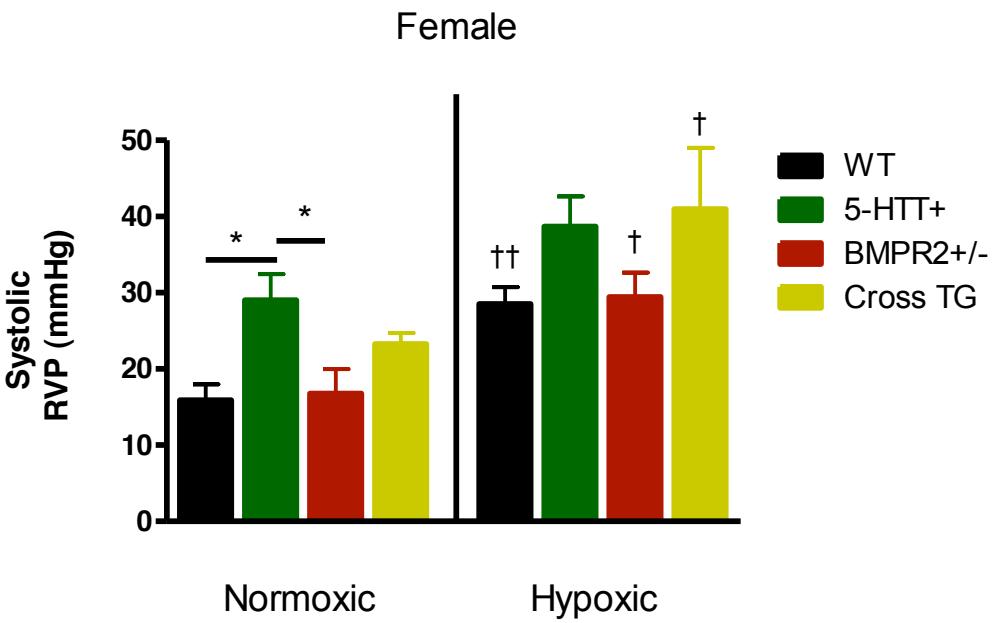


A



B

Figure 3.2 Effect of 14 days hypoxia in WT, 5-HTT+, BMPR2$^{+/-}$ and Cross TG male mice (n=2 to 8 mice per group) on mSAP (A) and HR (B). Results expressed as mean ± SEM. Newman-Keuls multiple comparison test of inter-genotype differences and an unpaired two-tailed t-test comparison of normoxic and hypoxic groups revealed no significant differences.



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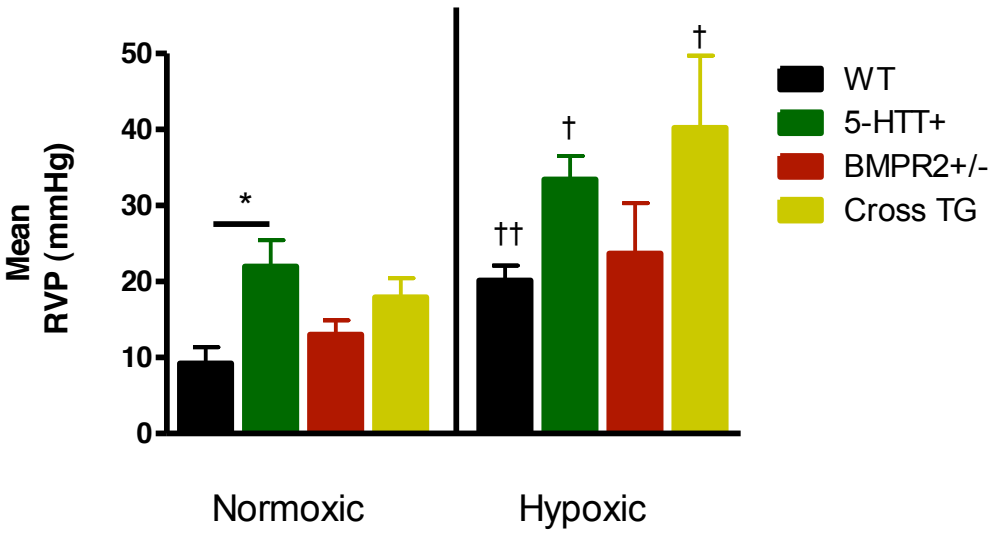
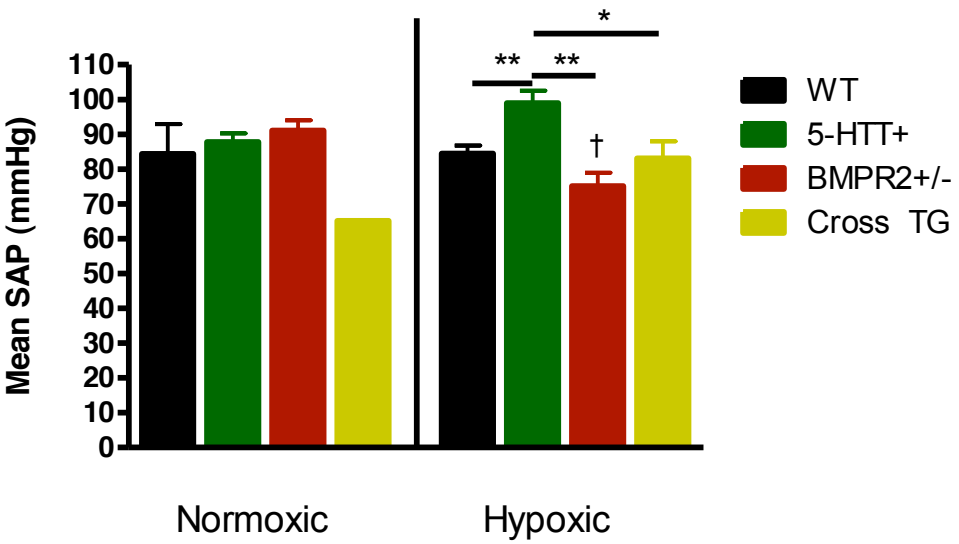


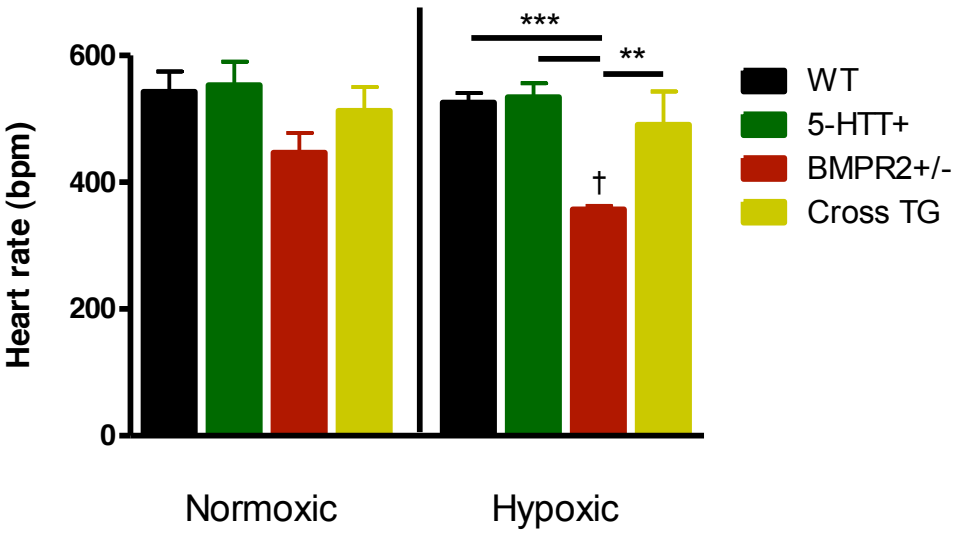
B

Figure 3.3 Effect of 14 days hypoxia in WT, 5-HTT+, BMPR2$^{+/-}$ and Cross TG female mice (n=4 to 6 mice per group) on sRVP (A) and mRVP (B). Results expressed as mean ± SEM. * $p<0.05$ using Newman-Keuls multiple comparison test. † $p<0.05$, †† $p<0.01$ versus corresponding normoxic value using an unpaired two-tailed t-test.

Female



A



B

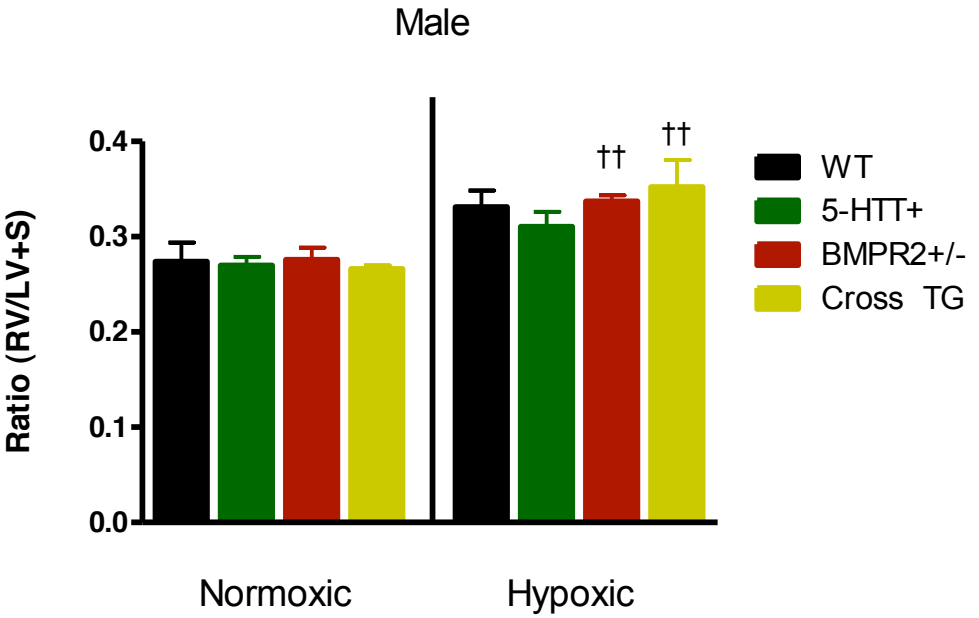
Figure 3.4 Effect of 14 days hypoxia in WT, 5-HTT+, BMPR2$^{+/-}$ and Cross TG female mice (n=2 to 9 mice per group) on mSAP (A) and HR (B). Results expressed as mean ± SEM. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ using Newman-Keuls multiple comparison test. † $p<0.05$ versus corresponding normoxic value using an unpaired two-tailed t-test.

3.2.4 Right ventricular hypertrophy and vascular remodelling in female mice

RVH and remodelling data for female mice are presented in figure 3.6. No significant differences were noted for RVH between groups under normoxia. Under hypoxia, 5-HTT+ mice had a higher degree of RVH compared with WT and Cross TG (WT: 0.35 ± 0.01, n=19, 5-HTT+: 0.39 ± 0.01, n=18, Cross TG: 0.33 ± 0.02, n=7) mice. Hypoxia increased RVH in WT ($p<0.001$), 5-HTT+ ($p<0.001$), $BMPR2^{+/-}$ ($p<0.001$) and Cross TG ($p<0.05$) mice. 5-HTT+ normoxic mice had more remodelled vessels than WT and $BMPR2^{+/-}$ (WT: 3.3 ± 0.6%, n=4, 5-HTT+: 8.2 ± 1.2%, n=4, $BMPR2^{+/-}$: 3.7 ± 0.7%, n=4) mice. There was considerable inter-group variability in remodelling in the hypoxic animals. 5-HTT+ mice had more remodelled vessels than WT and $BMPR2^{+/-}$ mice had more remodelled vessels than WT and 5-HTT+ mice. Cross TG mice had a similar level of remodelling to the $BMPR2^{+/-}$ mice and this value was significantly greater than WT and 5-HTT+ mice (WT: 18.3 ± 1.1%, n=10, 5-HTT+: 24.7 ± 1.5%, n=10, BMPR2+/-: 36.3 ± 5.0%, n=4, Cross TG: 35.4 ± 2.2%, n=4). Hypoxia increased remodelling in all groups ($p<0.001$) compared with respective normoxic animals.

3.2.5 Gender differences

To determine if any gender differences existed, male vs female results were plotted for sRVP, mRVP, mSAP, HR, RVH and vascular remodelling (figures 3.7 to 3.12). Under normoxic conditions, WT female mice had lower sRVP compared with WT males (male: 25.6 ± 0.9 mmHg, n=5, female: 15.9 ± 2.1 mmHg, n=4) (figure 3.7). A similar difference was noted under hypoxia (male: 38.4 ± 2.2 mmHg, n=6, female: 28.5 ± 2.2 mmHg, n=5). Cross TG female mice had higher sRVP under normoxia compared to Cross TG males (male: 15.8 ± 1.0 mmHg, n=6, female: 23.3 ± 1.5 mmHg, n=5) and although female Cross TG mice had higher sSVP compared with males following hypoxia, it did not reach



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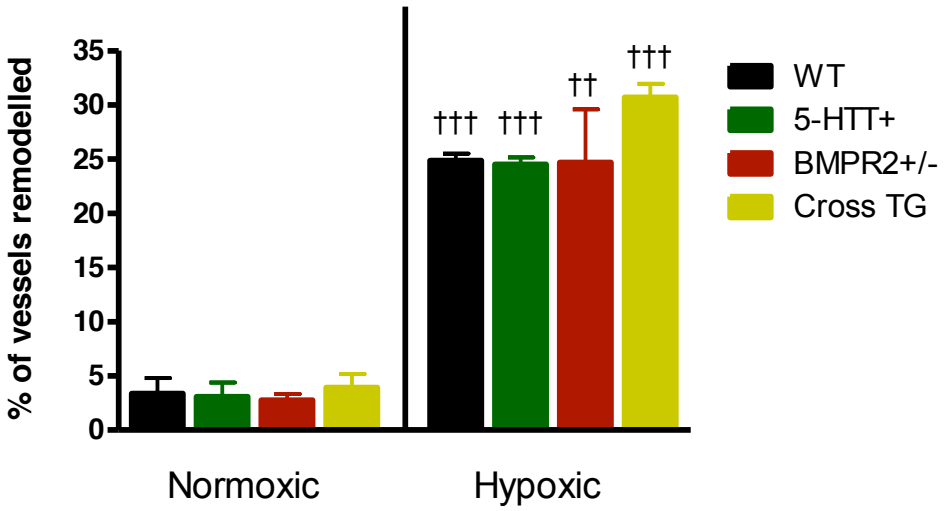
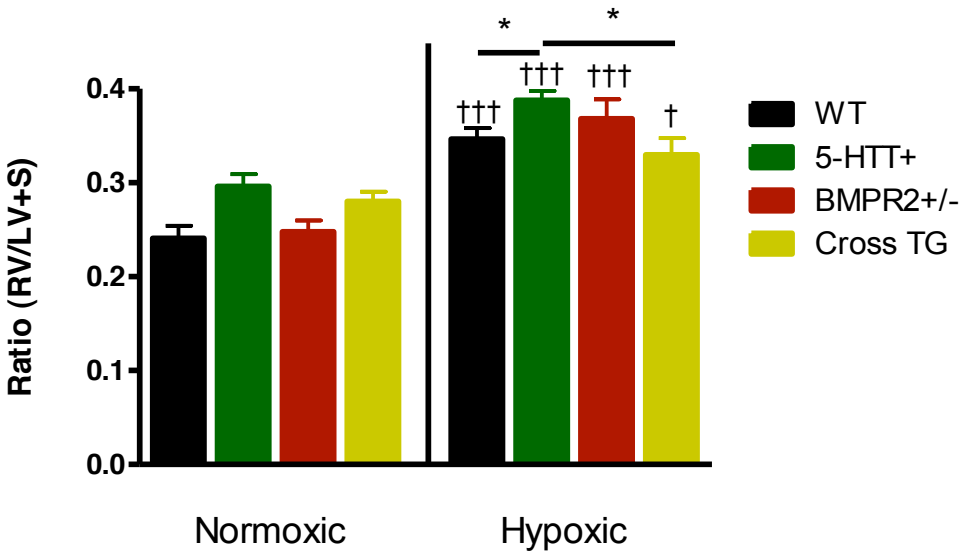


B

Figure 3.5 Effect of 14 days hypoxia in WT, 5-HTT+, BMPR2$^{+/-}$ and Cross TG male mice (n=4 to 8 mice per group) on RVH (A) and remodelling (B). Results expressed as mean ± SEM. Newman-Keuls multiple comparison test of inter-genotype differences revealed no significant differences. †† $p<0.01$, ††† $p<0.001$ versus corresponding normoxic value using an unpaired two-tailed t-test.

Female



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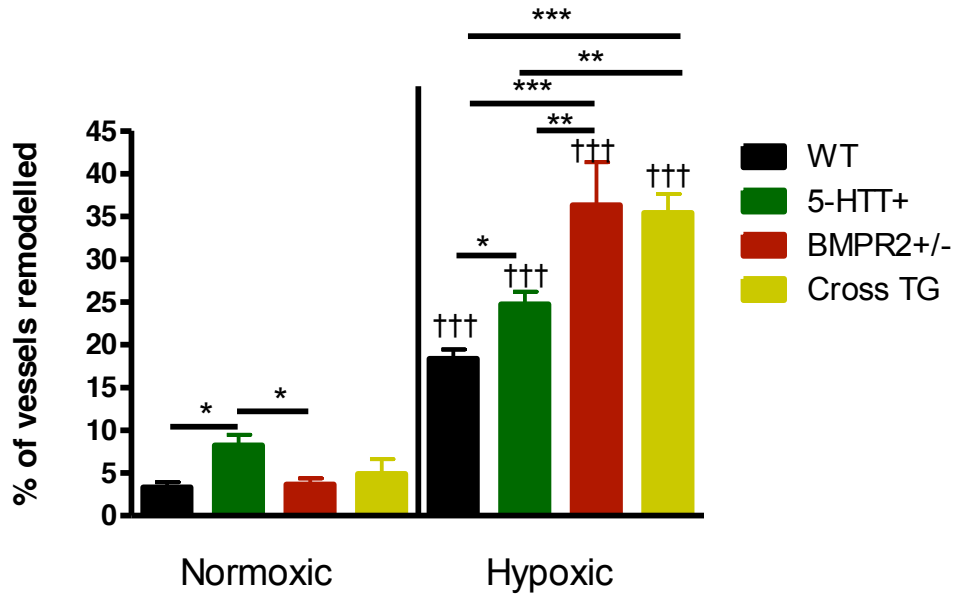


B

Figure 3.6 Effect of 14 days hypoxia in WT, 5-HTT+, BMPR2$^{+/-}$ and Cross TG female mice (n=4 to 19 mice per group) on RVH (A) and remodelling (B). Results expressed as mean ± SEM. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ using Newman-Keuls multiple comparison test. ††† $p<0.001$ versus corresponding normoxic value using an unpaired two-tailed t-test.

significance (figure 3.7). There was a significant difference, however, between $BMPR2^{+/-}$ male and female mice under hypoxia with female mice having higher pressures (male: 15.1 ± 1.0 mmHg, n=5, female: 29.5 ± 3.2 mmHg, n=5). No such difference between male and female $BMPR2^{+/-}$ mice was observed under normoxia. Mean RVP data is presented in figure 3.8. Only for WT mice under normoxia, did the mean RVP closely parallel the sRVP results.

Mean RVP was significantly lower in WT female mice compared with males (male: 13.6 ± 0.7 mmHg, n=5, female: 8.3 ± 1.9 mmHg, n=4; figure 3.8). There was no difference between male and female WT mice under hypoxia but there was a substantial rise in mRVP in 5-HTT+ female mice compared with males under hypoxia (male: 19.1 ± 0.9 mmHg, n=8, female: 30.1 ± 2.8 mmHg, n=5). No significant differences were observed between males and females for either $BMPR2^{+/-}$ or Cross TG mice under normoxia or hypoxia (figure 3.8).

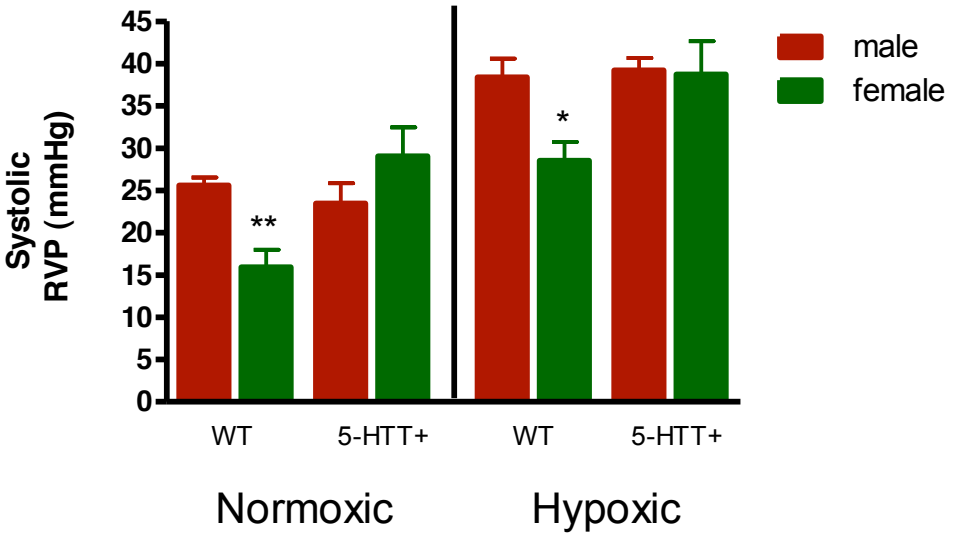
Mean SAP was similar between male and female in all groups studied except WT mice under hypoxia where females had lower mean SAP than males (male: 100.6 ± 4.8 mmHg, n=5, female: 84.5 ± 2.2 mmHg, n=6) (figure 3.9).

Figure 3.10 displays HR by gender. 5-HTT+ female mice had a higher HR than males under normoxia (male: 377.2 ± 10.1 bpm, n=5, female: 461.2 ± 30.9 bpm) and hypoxia (male: 370.8 ± 16.3 bpm, n=8. female: 445.2 ± 18.3 bpm, n=9). There was also a small, but significant difference in HR in $BMPR2^{+/-}$ mice under hypoxia (male: 353.8 ± 18.8 bpm, n=5, female: 297.8 ± 4.4 bpm, n=4).

RVH data is presented in figure 3.11. The only gender difference noted was in 5-HTT+ hypoxic animals with female mice having a greater degree of RVH than males (male: 0.31 ± 0.02, n=8, female: 0.39 ± 0.01, n=18).

The remodelling results are outlined in figure 3.12. 5-HTT+ female mice had more remodelled vessels than males under normoxia (male: 3.1 ± 1.3%, n=4, female: 8.2 ± 1.2,

n=4) but not hypoxia. The only other gender difference observed was for WT mice under hypoxia where female mice had less remodelling than males (male: 24.9 ± 0.7%, n=4, female: 18.3 ± 1.1%, n=10).



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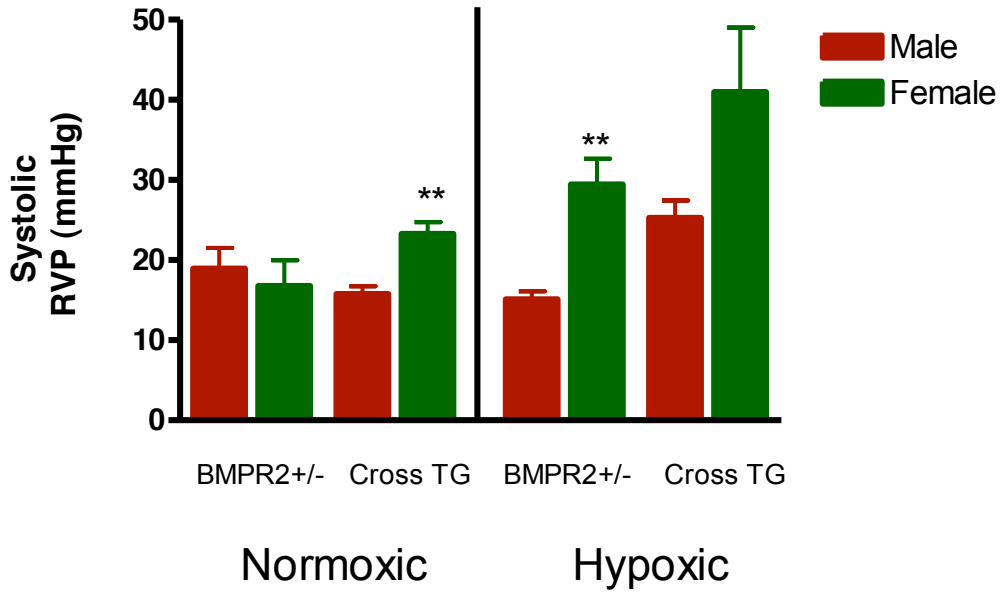
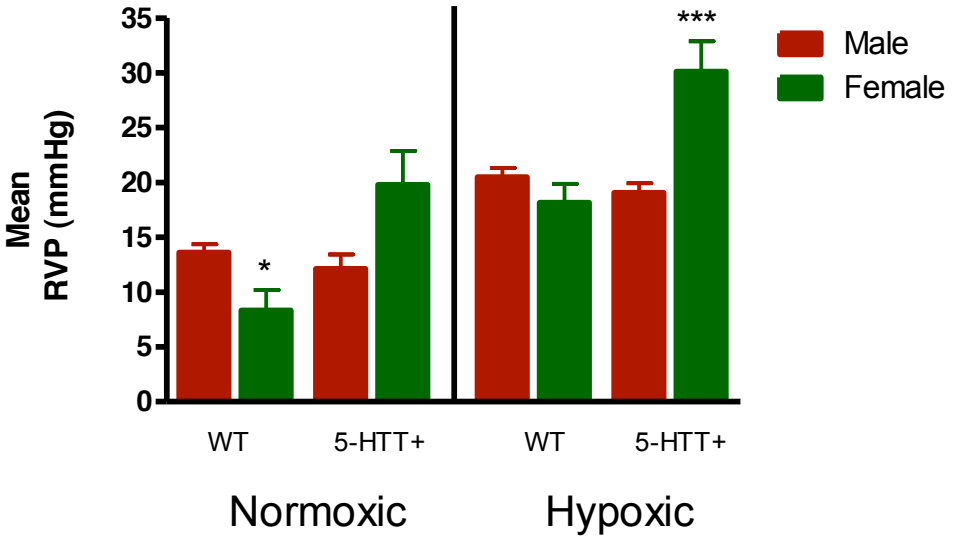


B

Figure 3.7 Comparison of male and female sRVP in WT and 5-HTT+ mice (A) and in BMPR2$^{+/-}$ and Cross TG mice (B) (n= 4 to 8 mice per group) under both normoxic and hypoxic conditions. Results expressed as mean ± SEM. * $p<0.05$, ** $p<0.01$ versus male using an unpaired two-tailed t-test.



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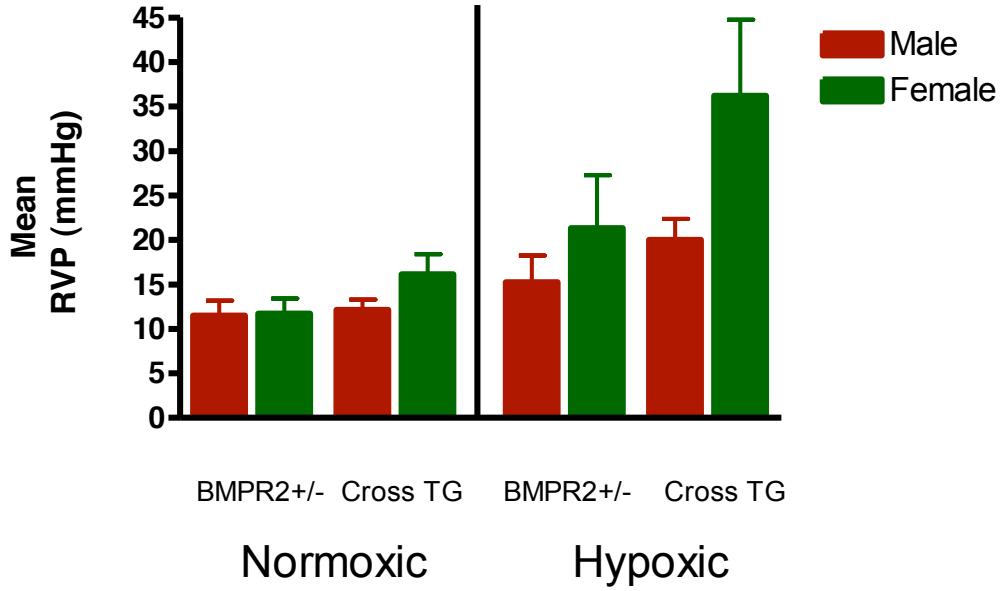
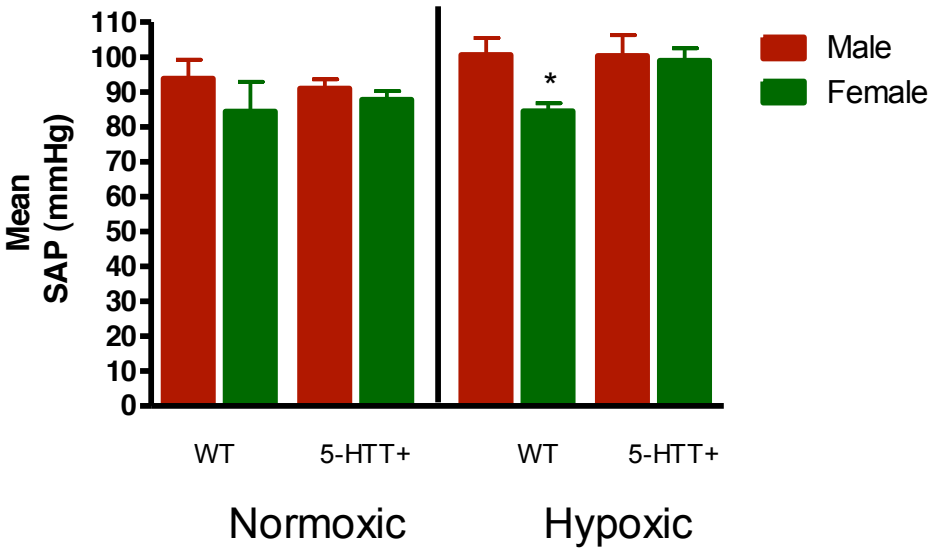


B

Figure 3.8 Comparison of male and female mRVP in WT and 5-HTT+ mice (A) and in BMPR2$^{+/-}$ and Cross TG mice (B) (n= 4 to 8 mice per group) under both normoxic and hypoxic conditions. Results expressed as mean ± SEM. * $p<0.05$, *** $p<0.001$ versus male using an unpaired two-tailed t-test.



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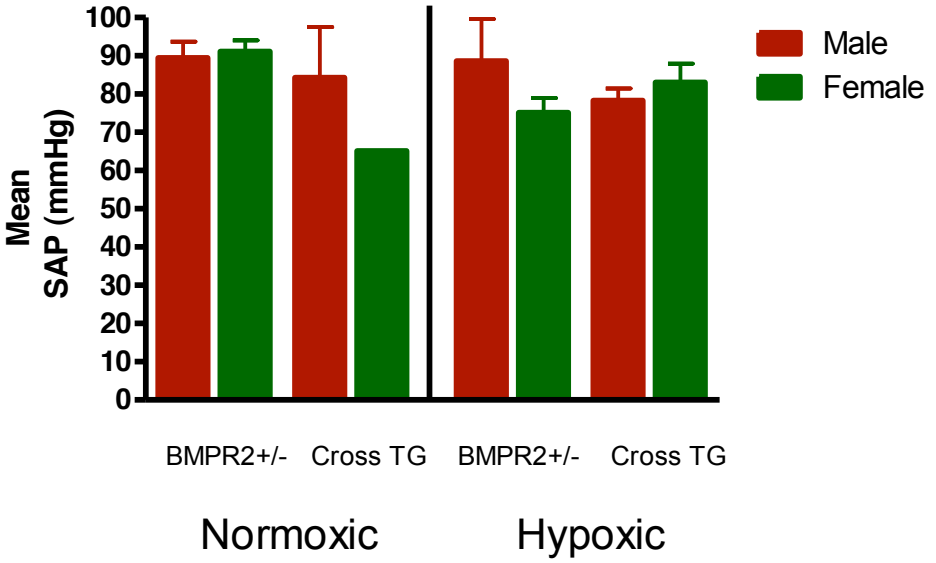
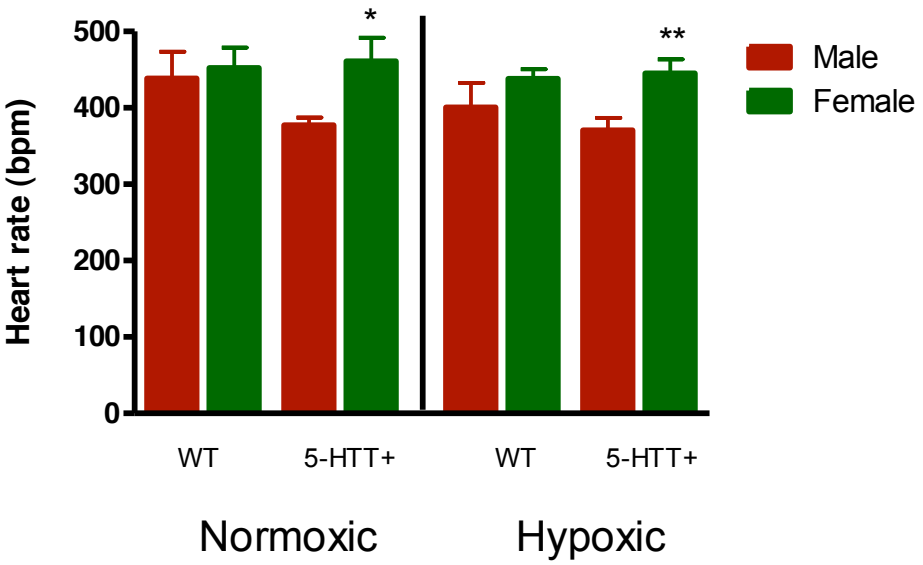


B

Figure 3.9 Comparison of male and female mSAP in WT and 5-HTT+ mice (A) and in BMPR2$^{+/-}$ and Cross TG mice (B) (n= 2 to 6 mice per group) under both normoxic and hypoxic conditions. Results expressed as mean ± SEM. * $p<0.05$ versus male using an unpaired two-tailed t-test.



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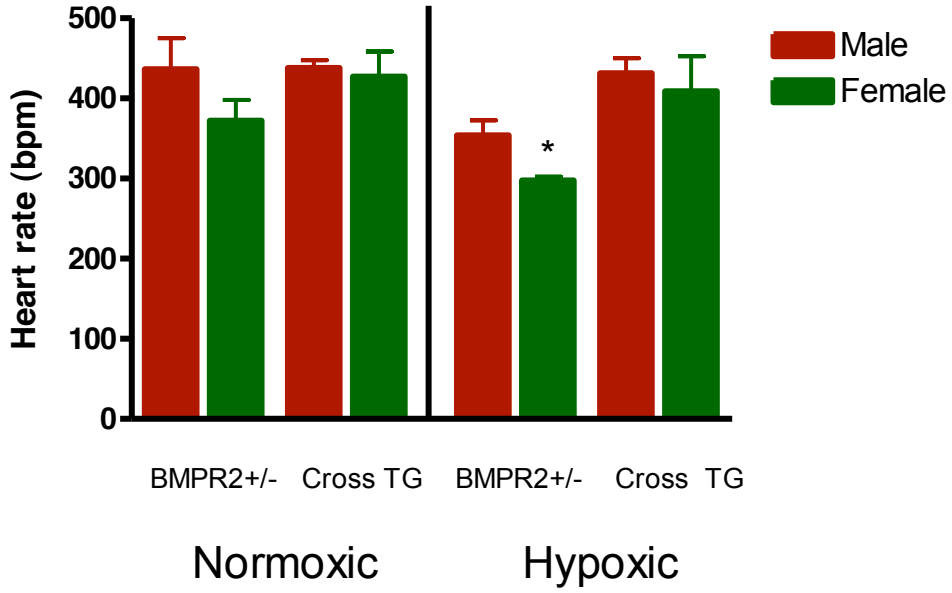
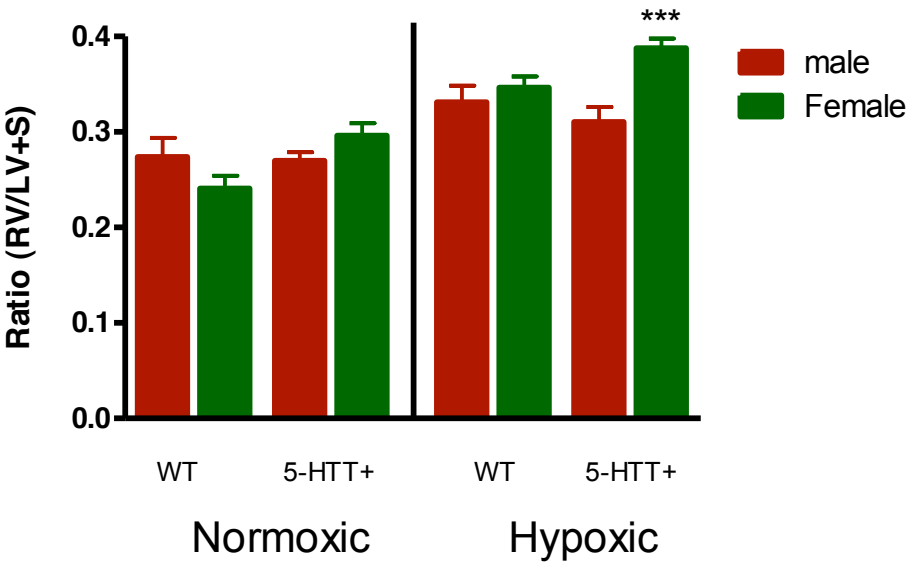


B

Figure 3.10 Comparison of male and female HR in WT and 5-HTT+ mice (A) and in BMPR2$^{+/-}$ and Cross TG mice (B) (n= 4 to 9 mice per group) under both normoxic and hypoxic conditions. Results expressed as mean ± SEM. * $p<0.05$, ** $p<0.01$ versus male using an unpaired two-tailed t-test.



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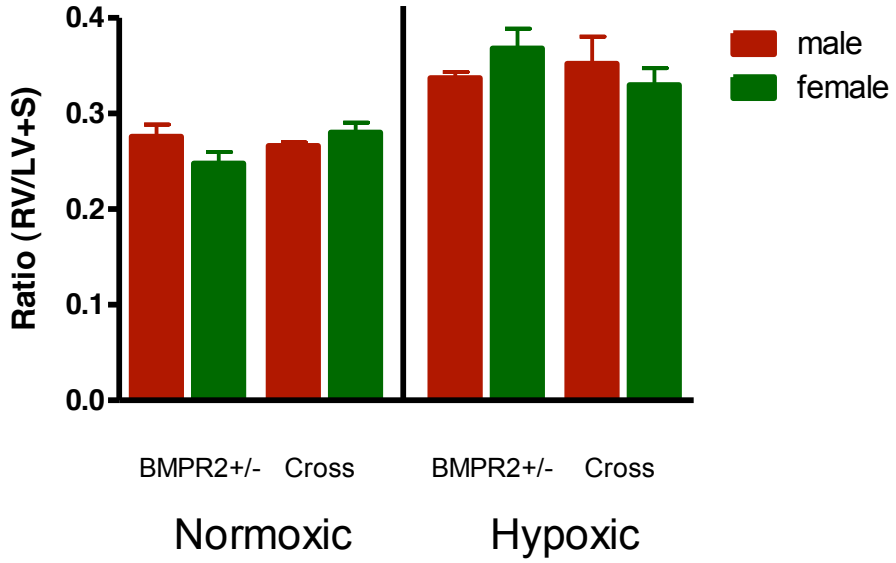
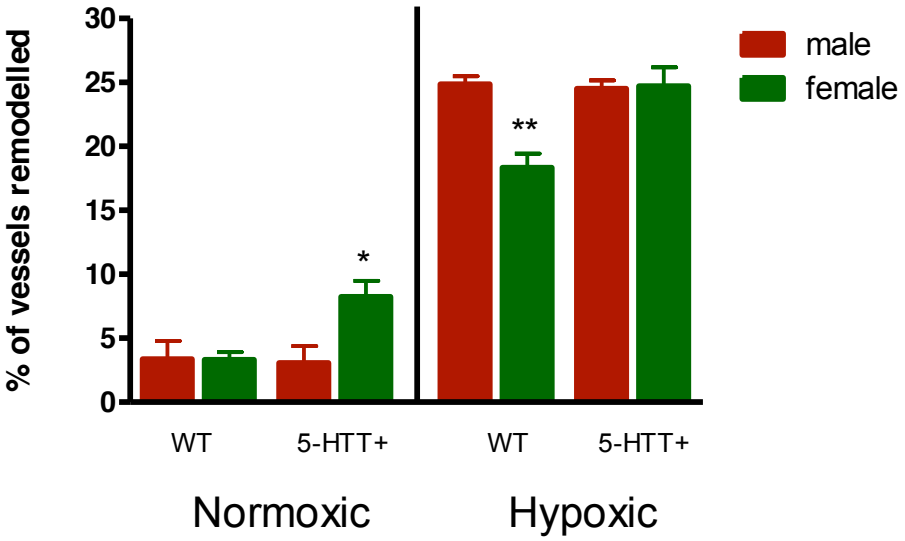
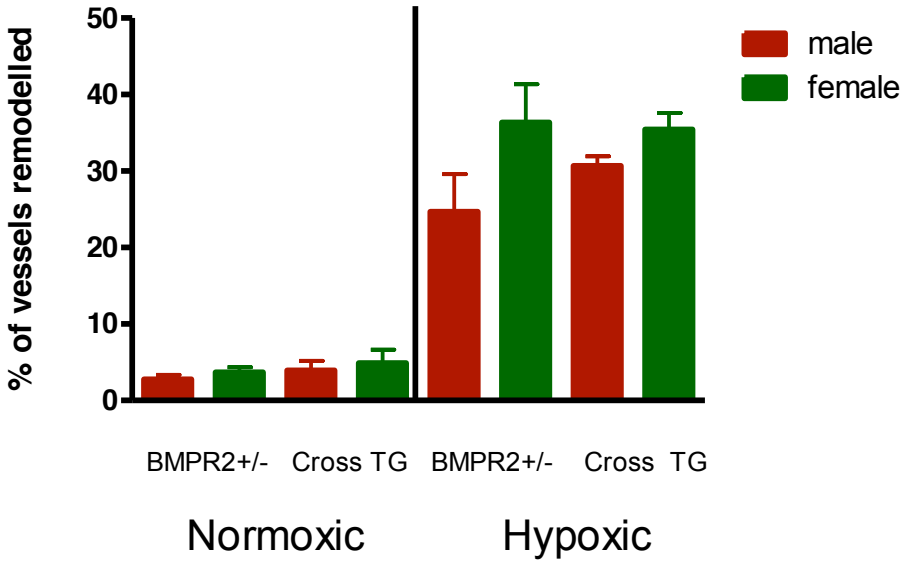


B

Figure 3.11 Comparison of male and female RVH in WT and 5-HTT+ mice (A) and in BMPR2$^{+/-}$ and Cross TG mice (B) (n= 4 to 19 mice per group) under both normoxic and hypoxic conditions. Results expressed as mean ± SEM. *** $p<0.001$ versus male using an unpaired two-tailed t-test.



A



B

Figure 3.12 Comparison of male and female remodelling in WT and 5-HTT+ mice (A) and in BMPR2$^{+/-}$ and Cross TG mice (B) (n= 4 mice per group) under both normoxic and hypoxic conditions. Results expressed as mean ± SEM. * $p<0.05$, ** $p<0.01$ versus male using an unpaired two-tailed t-test.

3.3 Discussion

There were two principle objectives in this series of experiments. The first was to monitor RVP *in vivo* in WT and TG animals followed by assessment of RVH and vascular remodelling post-mortem under normal room air conditions. The second was to determine the effect of hypoxia on these same parameters. Female 5-HTT+ mice were found to have elevated mRVP compared to WT mice under normoxia, a phenotype previously characterised in these mice (MacLean et al., 2004). This investigation, however, also showed that female 5-HTT+ mice have a greater number of remodelled vessels, the first time this phenotypic change has been observed in 5-HTT+ mice. Female 5-HTT+ mice also developed an exaggerated pulmonary hypertensive phenotype following hypoxia indicating an interaction between overexpression of the 5-HTT and a pulmonary hypertensive risk factor. These phenotypic changes in 5-HTT+ mice were unique to females with male mice showing similar results to WT. Interesting results were also obtained from $BMPR2^{+/-}$ animals. Both male and female normoxic $BMPR2^{+/-}$ mice were similar to WT in all parameters tested, however, both male and female $BMPR2^{+/-}$ mice were surprisingly less susceptible to hypoxic induced increases in mRVP. The remodelling results in female $BMPR2^{+/-}$ hypoxic animals were markedly different, however, with these animals displaying significantly more remodelling following hypoxia than mice with normal BMPR2 levels. This enhanced hypoxic remodelling is a novel finding in the $BMPR2^{+/-}$ mouse. Despite these modulating effects of BMPR2 deficiency and 5-HTT overexpression on the pulmonary phenotype of mice, no synergistic effect between these two genetic changes was observed in either male or female Cross TG mice that resulted in a more severe pulmonary hypertensive phenotype compared with either 5-HTT+ or $BMPR2^{+/-}$ mice.

It has been established that the proliferative effect of 5-HT on PASMCs is dependent on uptake via the 5-HTT (Lee et al., 1991; Eddahibi et al., 1999) and that 5-HTT levels are

increased in pulmonary arteries in FPAH/IPAH patients. The finding that 5-HTT levels are highest in vessels undergoing remodelling is convincing evidence for a central role of the 5-HTT in this critical component of the disease (Eddahibi et al., 2001). The increased vascular remodelling seen in 5-HTT+ normoxic mice is thought to occur following higher levels of 5-HT being taken up by PASMCs and subsequent activation of ROS and p-ERK1/2 which contributes to the mitogenic effect of 5-HT on PASMCs (Lee et al., 1999). The remodelling observed in female 5-HTT+ mice was relatively modest and is unlikely to make a significant contribution to the elevated RVP observed. The RVP in the female 5-HTT+ mice was not higher than the RVP reported by MacLean et al., 2004 in which no remodelling was found. The mechanisms that underlie the increased RVP in 5-HTT+ mice is unknown. It maybe that overexpression of the 5-HTT has as yet unknown effects on other vasoactive mediators that may contribute to increasing RVP.

It has been shown previously that female 5-HTT+ mice have an exaggerated response to hypoxia compared with WT mice. RVP, RVH and vascular remodelling are all higher in 5-HTT+ mice than in WT mice following hypoxia (MacLean et al., 2004) and the results presented here are broadly in agreement with these findings. Hypoxia induced an increase in RVH in all female groups with 5-HTT+ mice displaying a greater degree of RVH than WT or Cross TG mice. 5-HTT+ hypoxic mice also had more remodelling and a trend towards a higher mRVP compared with WT. None of these phenotypic changes observed in 5-HTT+ female mice under either normoxic or hypoxic conditions were observed in respective male mice. 5-HTT inhibitors attenuate vascular remodelling in hypoxic induced pulmonary hypertensive mice (Marcos et al., 2003) and mice lacking the 5-HTT (5-HTT$^{-/-}$ mice) also develop less severe hypoxic PAH than WT mice (Eddahibi et al., 2000). It would be logical to assume from these studies that perhaps hypoxia increases 5-HTT expression in the pulmonary vasculature but this has not been the case in several studies to date. 5-HTT transcription, receptor number and protein were decreased in both WT and 5-

HTT+ mice following hypoxia (MacLean et al., 2004) and 5-HT uptake is reduced in the lungs of WT hypoxic mice (Launay et al., 2002). Conflicting information has emerged from studies on hypoxic rats. Hypoxic rat PASMCs have increased expression of the 5-HTT that is associated with a greater proliferative response to 5-HT (Eddahibi et al., 1999) yet 5-HT uptake is reduced in the lungs of hypoxic rats (Jeffery et al., 2000). Significant experimental differences such as strain, age, sex and duration of hypoxia are likely to be important factors in explaining these observed differences.

Hypoxia has previously been shown to increase circulating 5-HT levels in mice (Callebert et al., 2006) and infusion of 5-HT exaggerates the hypoxic pulmonary hypertensive phenotype of rats (Eddahibi et al., 1997). Conversely, Tph1$^{-/-}$ mice, lacking peripheral 5-HT, display attenuated RVP and vascular remodelling following hypoxia compared with WT mice (Morecroft et al., 2007). It is clear from data presented here that overexpression of the 5-HTT is sufficient to induce elevated RVP and vascular remodelling in female mice. It maybe that hypoxia increases circulating 5-HT levels which results in a greater uptake of 5-HT into PASMCs activating vasoconstrictor and mitogenic pathways. The downregulation of the 5-HTT observed in hypoxic mice may be a compensatory mechanism for the rise in plasma [5-HT]. Overexpression of the 5-HTT may also affect other signalling pathways involved in inducing the pulmonary hypertensive phenotype. Mice that overexpress the 5-HTT in smooth muscle under the control of the SM22 promoter develop spontaneous PAH which worsens with age (Guignabert et al., 2006). Moreover, these mice also develop more severe PAH following both hypoxia and MCT. The SM22-5-HTT+ mouse was found to have reduced expression of Kv1.5 and Kv2.1 in the lung which would reduce Kv current promoting vasoconstriction, proliferation and a reduction in apoptosis. It is not known if Kv channel subunits are downregulated in PASMCs in the 5-HTT+ mouse in a similar way to the SM22-5-HTT+ mouse. If they are, they are likely to contribute to the pulmonary hypertensive phenotype and may partly

explain the enhanced response to hypoxia despite an apparent reduction in 5-HTT expression. Hypoxia reduces Kv1.5 and Kv2.1 expression (Archer et al., 1998a; Platoshyn et al., 2001) and it maybe be the Kv current is impaired to a greater degree in hypoxic 5-HTT+ mice compared with WT mice resulting in a greater degree of PAH. The study by Guignabert et al., 2006 also reveals that overexpression of the 5-HTT in smooth muscle alone is sufficient to induce PAH. This rules out changes in circulating 5-HT or bioavailability as a modulating factor in these mice.

Hypoxic 5-HTT+ female mice had higher mSAP than the other three groups; however, the pressure was still within the normal physiological range. The serotonin system is involved in controlling SAP at several levels. 5-HT acts neuronally to modulate the parasympathetic and sympathetic nervous systems thus influencing CO, HR and ventricular contractility. 5-HT also acts via 5-HT_1 and 5-HT_2 receptors on peripheral systemic arteries to alter vascular tone (Cote et al., 2004). Given that there was no difference between 5-HTT+ and WT mice under normoxia, it may be that hypoxia increases circulating 5-HT levels as previously described (Callebert et al., 2006) and through a central or peripheral site of action, acts to increase mSAP.

Both male and female normoxic $BMPR2^{+/-}$ mice were similar to WT in all parameters tested. There was no evidence of increased RVP, RVH or vascular remodelling. These results concur with earlier studies in the same $BMPR2^{+/-}$ mouse which found that the pulmonary vascular phenotype was similar to WT (Song et al., 2005; Long et al., 2006) but differ from results obtained by Beppu et al., 2004 whose $BMPR2^{+/-}$ mice had elevated PAP, PVR and an increased wall thickness of muscularised pulmonary arteries. SAP, like in this investigation was normal. The reasons for these discrepancies are unclear but are unlikely to be related to age as Song et al., 2005 compared RVP between WT and $BMPR2^{+/-}$ mice throughout the life cycle of the animals and the ages of mice utilised by Long et al., 2005 were comparable to those used by Beppu et al., 2004. Similar to this

investigation, Beppu et al., 2004 studied both male and female mice and found both genders displayed the altered pulmonary vascular phenotype and thus gender difference can be ruled out as a mitigating factor for the difference between this study and Beppu et al., 2004. The accumulated data for this particular $BMPR2^{+/-}$ mouse indicates that pulmonary vascular abnormalities are, at most, subtle. Given that the $BMPR2^{+/-}$ mouse is thought to mimic the haploinsufficiency seen in FPAH and that only a minority of human carriers of the BMPR2 mutation develop PAH, it is perhaps not surprising that the $BMPR2^{+/-}$ mouse does not display a pulmonary hypertensive phenotype under normal physiological conditions.

The $BMPR2^{+/-}$ mouse is, however, more susceptible to PAH following exposure to secondary risk factors such as 5-HT (Long et al., 2006) and the interaction of BMPR2 and the serotonergic system will be further investigated in subsequent chapters. The study in this chapter uncovered an important interaction between dysfunctional BMPR2 and hypoxic induced vascular remodelling. $BMPR2^{+/-}$ female mice were more sensitive to hypoxic induced remodelling, having more remodelled vessels than mice with normal BMPR2 levels. Similar to the 5-HTT+ mice, this phenotypic change was not observed in male BMPR2 hypoxic mice which had a similar level of remodelling to WT mice. Taken together, these results from the 5-HTT+ and $BMPR2^{+/-}$ mice reveal an important interaction between gender and genetic changes relevant to PAH. Unexpectedly, both male and female $BMPR2^{+/-}$ mice were more resistant to hypoxic induced increases in mRVP and mRVP was not significantly different to normoxic $BMPR2^{+/-}$ mice. Although female $BMPR2^{+/-}$ mice showed similar increases compared to WT, the power of the data is lower due to a lower n number and larger standard error which unfortunately is a weakness of the experiment. The mSAP and HR measurements, however, suggest an altered cardiovascular response in the $BMPR2^{+/-}$ mouse, at least in the females. Hypoxia decreased mSAP in $BMPR2^{+/-}$ female mice compared with their normoxic controls. HR was also

decreased in female BMPR2$^{+/-}$ mice following hypoxia to a level that was over 100 bpm slower than the other 3 genotypes. A decrease in mSAP has previously been shown in BMPR2$^{+/-}$ mice following hypoxia (Beppu et al., 2004). BMPs are involved in normal heart development (Chen et al., 2004) and the changes observed in mSAP and HR may relate to a cardiac phenotypic change, currently uncharacterised in BMPR2$^{+/-}$ mice, that interacts with hypoxia. The pulmonary results presented here contradict those presented by Beppu et al., 2004 who found that pulmonary haemodynamics were similar between WT and BMPR2$^{+/-}$ mice following hypoxia but that vascular remodelling as assessed by wall thickness was less in hypoxic BMPR2$^{+/-}$ mice than in WT. Long et al., 2006 also found that RVP and RVH were similar between WT and BMPR2$^{+/-}$ mice following hypoxia. It is unclear why the study by Beppu et al., 2004 found less vascular remodelling in BMPR2$^{+/-}$ animals following hypoxia which is in contrast to the greater degree of vascular remodelling seen in female BMPR2$^{+/-}$ mice in this thesis or to the levels similar to WT as seen in the males. The age range of the animals used by Beppu et al., 2004 was greater than that used in this study (2-5 months versus 5-6 months) and although the authors state that the pulmonary haemodynamic response was similar between male and female BMPR2$^{+/-}$ mice, it is unclear which gender was employed in the morphological examination of lung samples. Moreover, the duration of hypoxia differed between studies which may account for some of the discrepancies observed. Duration of hypoxia was found to be a factor in experiments using the BMPR2$^{+/-}$ mouse developed by Delot et al., 2003 (BMPR2$^{\Delta E2}$) which does have higher RVP and RVH following hypoxia but only after 5 weeks exposure and these mice do not have increased vascular remodelling. No differences were observed in RVP, RVH or remodelling between BMPR2$^{\Delta E2}$ and WT mice after 3 weeks hypoxia or under normoxia (Frank et al., 2008). There was also no difference in PVR between WT and BMPR2$^{+/-}$ mice (which may have indicted evidence of vascular remodelling) under hypoxia in the study by Long et al., 2006 but this study only used male

mice and no differences were noted in vascular remodelling between male $BMPR2^{+/-}$ and WT mice in this investigation.

PASMCs from $BMPR2^{+/-}$ and WT mice have similar levels of p-Smad1/5/8 (Beppu et al., 2004; Long et al., 2006) and both display similar elevated expression of p-Smad1/5/8 following hypoxia (Long et al., 2006). PASMCs from $BMPR2^{+/-}$ mice do, however, show less increase in p-Smad1/5/8 following incubation with BMP-2 than WT PASMCs (Beppu et al., 2004; Long et al., 2006) indicating impaired BMP signalling. BMP ligands have previously been shown to have anti-proliferative effects on human PASMCs (Zhang et al., 2003), a process dependent on activation of p-Smad1 (Yang et al., 2005). The elevated p-Smad1/5/8 seen in hypoxic murine PASMCs may act to try to counteract the proliferative response that hypoxia has on these cells. It is thought that hypoxia acts through numerous pathways to induce proliferation of PASMCs including G-protein mediated activation of ERK1/2 by mitogenic factors such as 5-HT and ET-1 released by PAECs and by growth factors and ROS released by PAFs (Stenmark et al., 2006). Hypoxia can also inhibit Kv channels present on PASMCs inducing vasoconstriction and promoting proliferation (Archer et al., 1998a; Platoshyn et al., 2001). As BMPR2 is decreased in all cells in the $BMPR2^{+/-}$ mouse, changes in BMP signalling in either the endothelium or smooth muscle layer of pulmonary arteries may facilitate hypoxic induced remodelling. Using a smooth muscle specific dominant negative BMPR2 (SM22-tet-$BMPR2^{delx4+}$) West et al., 2004 showed that mice expressing dominant negative BMPR2 in smooth muscle have increased RVP, RVH and vascular remodelling at 2 months old indicating that severe loss of functional BMPR2 in smooth muscle is sufficient to induce PAH. The level of muscularistion of pulmonary arteries was relatively modest in the SM22-tet-$BMPR2^{delx4+}$ mice and the increased RVP and RVH is likely to be related more to enhanced vasoconstriction than remodelling. A later study with these same TG mice showed that both males and females were equally susceptible to the pulmonary hypertensive phenotype

(Tada et al., 2007). From the results presented in this thesis, it can be determined that only female BMPR2$^{+/-}$ mice develop exaggerated remodelling following hypoxia and it may be that age is just as an important factor as gender. It may be that in mice of 2 months, vasoconstriction contributes more to the pulmonary hypertensive phenotype than remodelling but that mice that are 5-6 months old are more sensitive to vascular remodelling. If loss of BMPR2 in smooth muscle induces PAH in unchallenged mice but not in unchallenged BMPR2$^{+/-}$ mice, it may that other factors i.e. hypoxia in this case are required that further suppress BMPR2 signalling in PASMCs that lead to a loss of apoptosis and increased proliferation following stimulation by growth factors activated by hypoxia in the lung. Further studies using the SM22-tet-BMPR2^{delx4+} have shown that these animals have reduced whole lung Kv channel expression and that the elevated RVP could be brought back to control values by administration of Nifedipine, an L-type Ca^{2+} channel blocker. Administration of BMP-2 to human PASMCs increased Kv1.5 expression indicating regulation of Kv channels by BMP signalling (Young et al., 2006). If Kv channels were decreased in PASMCs in BMPR2$^{+/-}$ mice then further suppression of these channels by hypoxia may promote an increase in intracellular Ca^{2+} concentration promoting vasoconstriction and proliferation of these cells. The SM22-tet-BMPR2^{delx4+} mouse also has elevated levels of IL-6 and TGF- β and BMP-2 reduces both these cytokines in human PASMCs (Hagen et al., 2007). It is unknown if the BMPR2$^{+/-}$ mice used here have higher than normal lung expression of TGF-β or IL-6 similar to SM22-tet-BMPR2^{delx4+} mice. If they do, it could enhance the effect of hypoxia. Hypoxia increases both these agents (Stenmark et al., 2006) and both are implicated in the progression of PAH. IL-6 is increased in serum from PAH patients (Humbert et al., 1995) and administration of IL-6 induces a pulmonary hypertensive like phenotype in rats (Miyata et al., 2001).

Under normoxia, male Cross TG mice had similar mRVP to the other three groups. In

female mice, Cross TG animals had almost double the level of RVP compared to WT although this was not significant. This is possibly due to relatively low n numbers for the Cross TG mice due to the extreme difficulties in breeding these animals. There was no significant differences in mRVP between Cross TG mice and 5-HTT+ mice indicating that the presence of dysfunctional BMPR2 is not enhancing the predisposition towards enhanced mRVP in mice who are 5-HTT+. RVH and vascular remodelling were not present in Cross TG males. In female mice there was a trend towards an increase in RVH in Cross TG mice similar to that observed for 5-HTT+ mice. As for mRVP, the predominant factor for this increase is overexpression of the 5-HTT and the $BMPR2^{+/-}$ genotype confers no greater susceptibility. When the remodelling data is analysed, it appears that Cross TG mice have less remodelled vessels than 5-HTT+ mice and unlike the 5-HTT+ mice, Cross TG mice did not have significantly more remodelling than WT mice. It is unlikely, however, that the presence of the BMPR2 mutation is offering any protection against vascular remodelling especially considering the data for remodelling in $BMPR2^{+/-}$ female mice following hypoxia. Moreover, half the Cross TG mice (2 of 4) assessed for remodelling had evidence of remodelling similar to that seen for 5-HTT+ mice. Individual variations between animals may be the source of the differences between the 5-HTT+ and Cross TG groups.

Cross TG mice did not display an exaggerated pulmonary hypertensive phenotype to hypoxia relative to the single transgenics. In fact, the effect of hypoxia on RVH was significantly less in female cross TG mice compared with female 5-HTT+ mice. Overexpression of the 5-HTT did not enhance the vascular remodelling in hypoxic Cross TG females compared with $BMPR2^{+/-}$ single transgenic animals. The only indicator of any worsening of PAH was a trend towards an increase in the percentage of remodelled vessels in male hypoxic animals. There does not appear to be any significant cross talk between the 5-HTT and dysfunctional BMPR2 in the mice studied here, at least none that influence

the parameters analysed. Long et al., 2005 found that 5-HT could downregulate p-Smad1/5 in WT and $BMPR2^{+/-}$ PASMCs and increase p-ERK1/2 in PASMCs from $BMPR2^{+/-}$ mice. These effects are likely to contribute to the increased proliferation 5-HT had on $BMPR2^{+/-}$ PASMCs, an effect that was blocked by ketanserin but not by a 5-HTT antagonist. Thus, cross-talk between 5-HT and BMPR2 in the context of PASMC proliferation may be $5\text{-}HT_{2A}$ mediated and not 5-HTT mediated. There is currently little further data regarding the interactions between 5-HT and BMPR2 within the pulmonary vasculature and how a genetic change in one signalling system may affect the other. Urinary excretion of 5-HT is similar in the $BMPR2^{+/-}$ mouse utilised here compared with WT (Song et al., 2005) and mice that overexpress the 5-HTT in smooth muscle have normal expression levels of BMPR2, BMPR1A, BMPR1B, BMP-2 and BMP-4 (Guignabert et al., 2006). It has to be remembered also, that in human patients with PAH, both BMPR2 levels (in patients with mutations) and 5-HTT levels are lower and higher respectively than can be explained by genetic factors alone. More subtle changes in intracellular signalling molecules may occur via cross regulation between internalisation of 5-HT and BMPR2 pathways that don't by themselves uncover a pulmonary hypertensive phenotype. Evidence for this already exists in this thesis, in this chapter and in subsequent chapters related to the discovery that female mice are more prone to the pulmonary effects of the underlying genetic abnormalities. Some as yet unknown agent which differs between male and female mice alters the susceptibility of females to PAH relative to males perhaps through direct interactions with either the 5-HT or BMPR2 systems (see chapter 8 for more information on interactions between 5-HT, BMPR2 and gender).

As described in section 2.3.1, both sRVP and mRVP values were recorded. sRVP was, in general, more variable between experiments. This was partly related to inconsistent wave trace amplitudes recorded from animals in one experimental batch compared with animals in another which itself was related to experimental design and technical proficiency of the

procedure. mRVP, which should remain uniform regardless of wave amplitude is likely to be a better indicator for comparing RVP of different animals and will be solely used for interpreting pulmonary pressure data in this thesis.

To summarise, overexpression of the 5-HTT results in spontaneous elevation of mRVP and vascular remodelling. 5-HTT overexpression was also associated with a worsening of hypoxic PAH. $BMPR2^{+/-}$ mice are phenotypically normal in room air and were less susceptible to hypoxic induced rises in mRVP. Despite this, female $BMPR2^{+/-}$ mice did show elevated vascular remodelling following hypoxia compared with WT and 5-HTT+ mice, a result that highlights the important role BMPR2 has in modulating vascular integrity and how deficiency in BMPR2 can interact with an additional risk factor for PAH to promote vascular changes characteristic of the disease. This study also demonstrated through the use of the novel Cross TG mice that no synergistic effects were observed between BMPR2 deficiency and 5-HTT overexpression that resulted in a more pronounced pulmonary hypertensive phenotype. Perhaps the most surprising and key finding from this work was the discovery that only female TG mice express the exaggerated pulmonary hypertensive changes indicating an important interaction between gender and genetic predisposition to cause the symptoms of PAH.

Chapter 4

Dexfenfluramine dosing study in BMPR2$^{+/-}$ mice

4.1 Introduction

While the risk of PAH is increased in people exposed to Dfen (Abenhaim et al., 1996), it is not known why some people develop PAH following Dfen and others do not. Even a 23 fold increased risk of PAH in long-term Dfen users (Abenhaim et al., 1996) still represents an extremely low risk of developing the disease suggesting genetic predisposition plays an important part. Humbert et al., 2002 analysed fenfluramine users with PAH for BMPR2 mutations and found that 9% of patients carried BMPR2 mutations. As the authors acknowledge, the actual number of patients that may carry the BMPR2 mutation may be much higher due to incomplete screening of the BMPR2 gene. A BMPR2 mutation was also found in a patient that developed PAH following the ingestion of the appetite-suppressant amfepramone (Abramowicz et al., 2003). The need for genetic predisposition in Dfen associated PAH is supported by animal data where administration of Dfen has failed to induce PAH in mice (Launay et al., 2002) and rats (Mitani et al., 2002; Rochefort et al., 2006). The $BMPR2^{+/-}$ mouse used in this thesis has previously been shown to be more susceptible to PAH induced by inflammatory stress. The inflammatory response was induced by adenovirus mediated pulmonary overexpression of 5-LO (Song et al., 2005). Of particular interest to this investigation was the discovery by Long et al., 2006 that infusion of 5-HT for 2 weeks increased RVP in $BMPR2^{+/-}$ mice but not in WT mice, an effect that was exaggerated in hypoxia. It would, therefore, be beneficial to determine if $BMPR2^{+/-}$ mice are susceptible to Dfen induced PAH. In this chapter WT and $BMPR2^{+/-}$ mice will be administered Dfen and assessed for evidence of a pulmonary hypertensive phenotype. The results in the last chapter clearly demonstrated the importance of gender in determining the pulmonary hypertensive phenotype. To determine if the response to Dfen is affected by gender, both male and female mice will be studied.

4.2 Methods

Mice (male and female) were 2 months old at commencement of Dfen dosing and 3 months old at time of experimental analysis. Mice were dosed with dexfenfluramine hydrochloride (Sigma-Aldrich Ltd, Poole, UK) (5 mg/kg/day) dissolved in H_2O via oral gavage dosing for 28 days before analysis of pulmonary haemodynamics as described in section 2.3.

4.3 Results

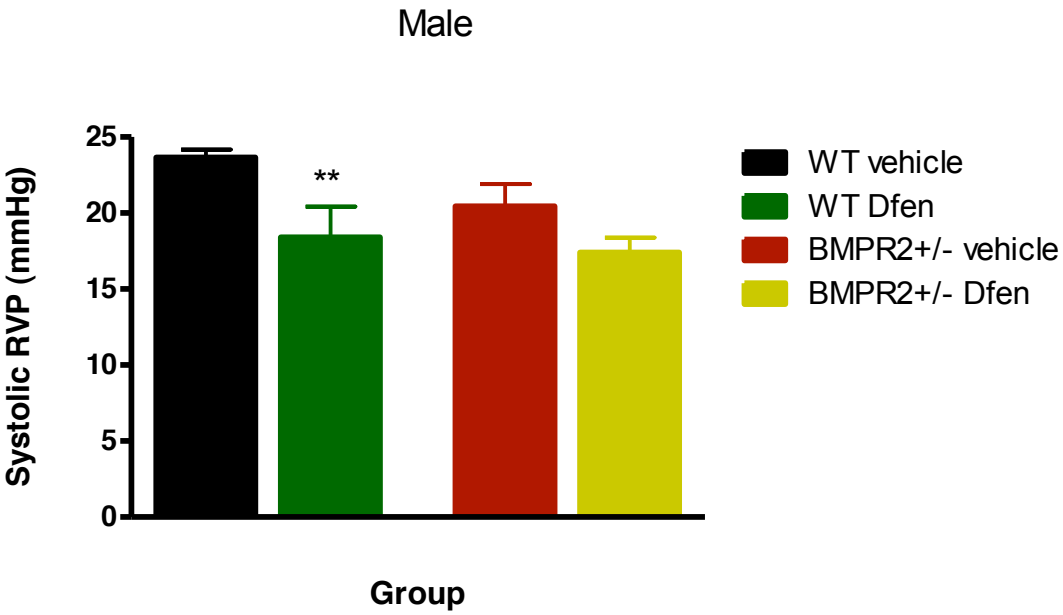
4.3.1 Pulmonary and systemic haemodynamics in male mice

sRVP and mRVP for WT and BMPR2$^{+/-}$ male mice dosed with Dfen or vehicle are presented in figure 4.1 Dfen caused a decrease in sRVP (vehicle: 23.7 ± 0.5 mmHg, n=19, Dfen: 18.4 ± 2.0 mmHg, n=9) and mRVP (vehicle: 12.7 ± 0.3 mmHg, n=19, Dfen: 10.8 ± 0.7 mmHg, n=9) in WT mice but had no effect on either sRVP or mRVP in BMPR2$^{+/-}$ mice. There were no differences noted between WT and BMPR2$^{+/-}$ mice.

mSAP was similar between the 4 groups (figure 4.2A). Dfen increased HR in BMPR2$^{+/-}$ mice compared with Dfen treated WT mice (WT: 383.9 ± 12.9 bpm, n=9, BMPR2$^{+/-}$: 428.3 ± 18.3 bpm, n=8) (figure 4.2B).

4.3.2 Pulmonary and systemic haemodynamics in female mice

sRVP and mRVP for WT and BMPR2$^{+/-}$ female mice dosed with Dfen or vehicle are presented in figure 4.3. Dfen increased sRVP (vehicle: 17.3 ± 1.5 mmHg, n=8, Dfen: 32.0 ± 0.5 mmHg, n=7) and mRVP (vehicle: 11.6 ± 0.9 mmHg, n=8, Dfen: 18.3 ± 1.1 mmHg, n=7) in WT mice. sRVP (vehicle: 17.5 ± 2.2 mmHg, n=7, Dfen: 27.9 ± 3.1 mmHg, n=6) and mRVP (vehicle: 12.7 ± 1.3 mmHg, n=7, Dfen: 20.2 ± 2.6 mmHg, n=6) were also increased in Dfen treated BMPR2$^{+/-}$ mice. There were no significant differences in sRVP or mRVP between WT and BMPR2$^{+/-}$ animals. There was insufficient data for a



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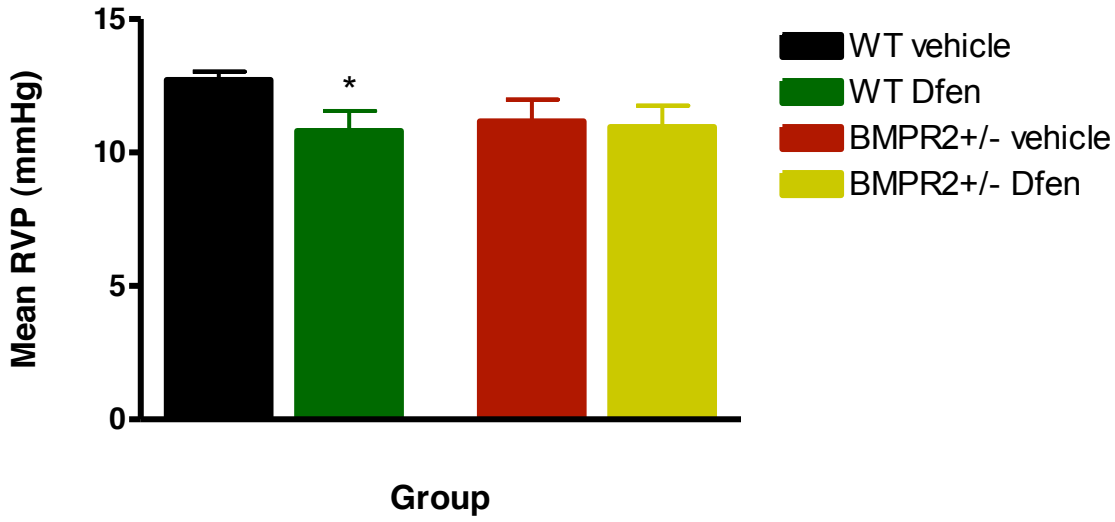
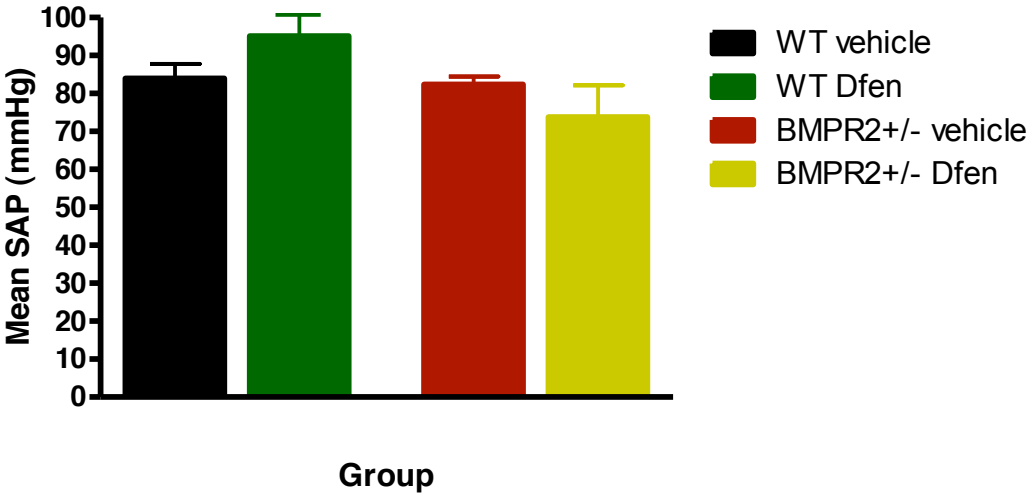


B

Figure 4.1 Effect of Dfen ingestion in WT and BMPR2$^{+/-}$ male mice (n = 7 to 19 mice per group) on sRVP (A) and mRVP (B). Results expressed as mean ± SEM. * $p<0.05$, ** $p<0.01$ versus WT vehicle using Bonferroni multiple comparison test.

Male



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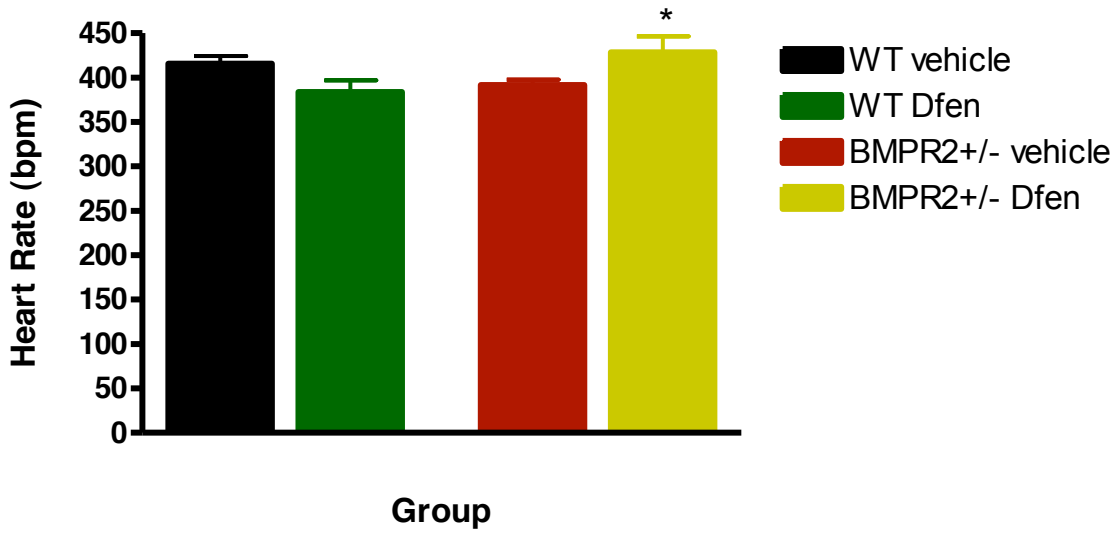
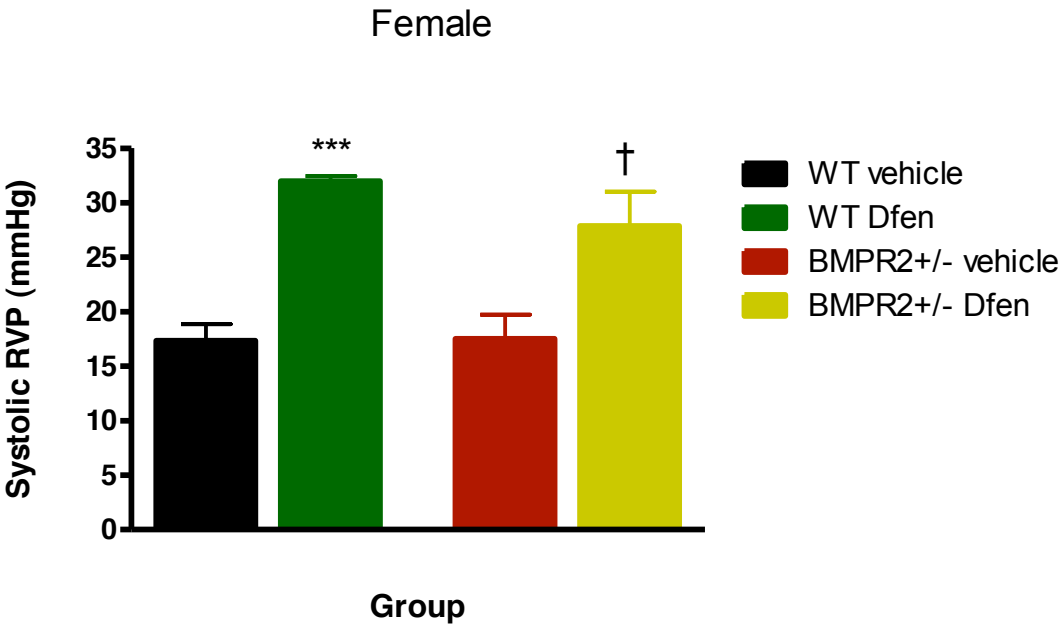


B

Figure 4.2 Effect of Dfen ingestion in WT and BMPR2$^{+/-}$ male mice (n = 3 to 19 mice per group) on mSAP (A) and HR (B). Results expressed as mean ± SEM. * $p<0.05$ versus WT Dfen using Bonferroni multiple comparison test.



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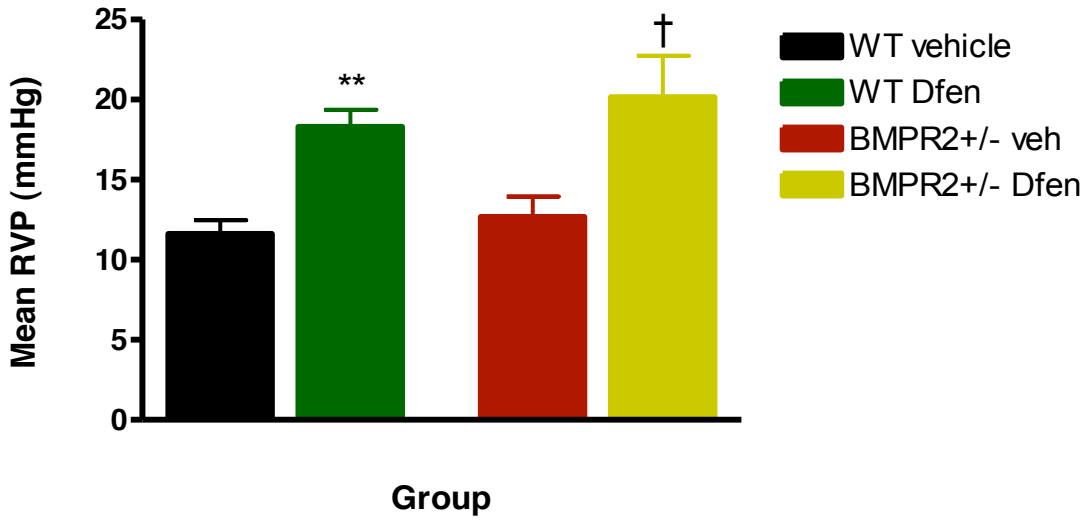


B

Figure 4.3 Effect of Dfen ingestion in WT and BMPR2$^{+/-}$ female mice (n = 6 to 8 mice per group) on sRVP (A) and mRVP (B). Results expressed as mean ± SEM. ** $p<0.01$, *** $p<0.01$ versus WT vehicle, † $p<0.01$ versus BMPR2$^{+/-}$ vehicle using Bonferroni multiple comparison test.

Female

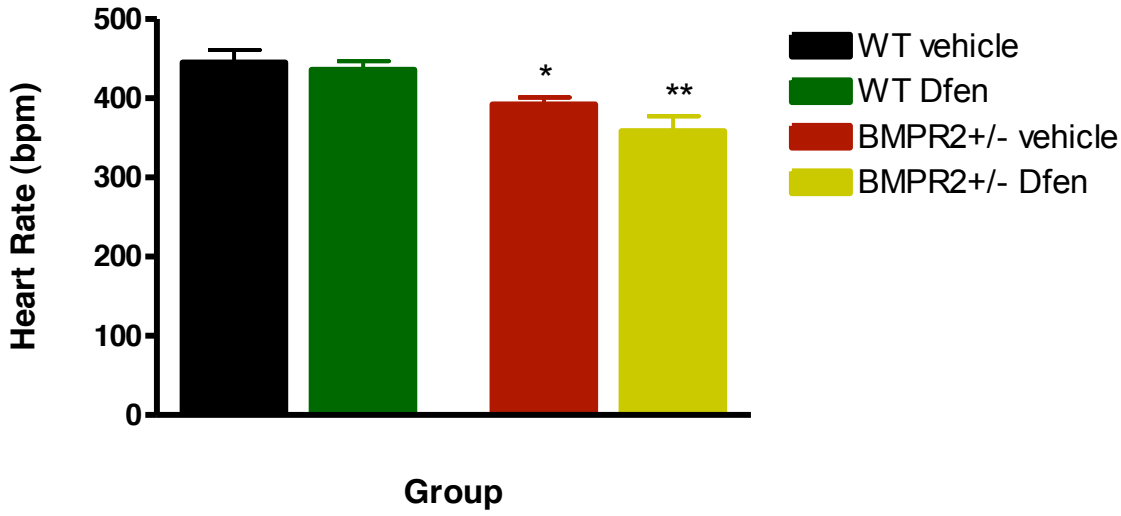


Figure 4.4 Effect of Dfen ingestion in WT and BMPR2$^{+/-}$ female mice (n = 6 to 8 mice per group) on HR. Results expressed as mean ± SEM. * $p<0.05$ versus WT vehicle, ** $p<0.01$ versus WT Dfen using Bonferroni multiple comparison test.

comparison of mSAP in female mice but HR was lower in BMPR2$^{+/-}$ mice, both for vehicle (WT: 445.0 ± 16.0 bpm, n=8, BMPR2$^{+/-}$: 392.1 ± 8.9 bpm, n=7) and Dfen (WT: 436.1 ± 10.8 bpm, n=7, BMPR2$^{+/-}$: 358.5 ± 18.7 bpm, n=6) treated animals (figure 4.4).

4.3.3 Right ventricular hypertrophy and vascular remodelling in male mice

Figure 4.5 illustrates RVH and vascular remodelling data for male mice. There were no differences observed for RVH between groups. BMPR2$^{+/-}$ mice dosed with Dfen had approximately twice the number of remodelled vessels compared to WT mice treated with Dfen and BMPR2$^{+/-}$ vehicle treated mice (WT Dfen: 3.0 ± 0.9%, n=4, BMPR2$^{+/-}$ vehicle: 3.1 ± 0.7%, n=4, BMPR2$^{+/-}$ Dfen: 6.0 ± 0.3%, n=4)

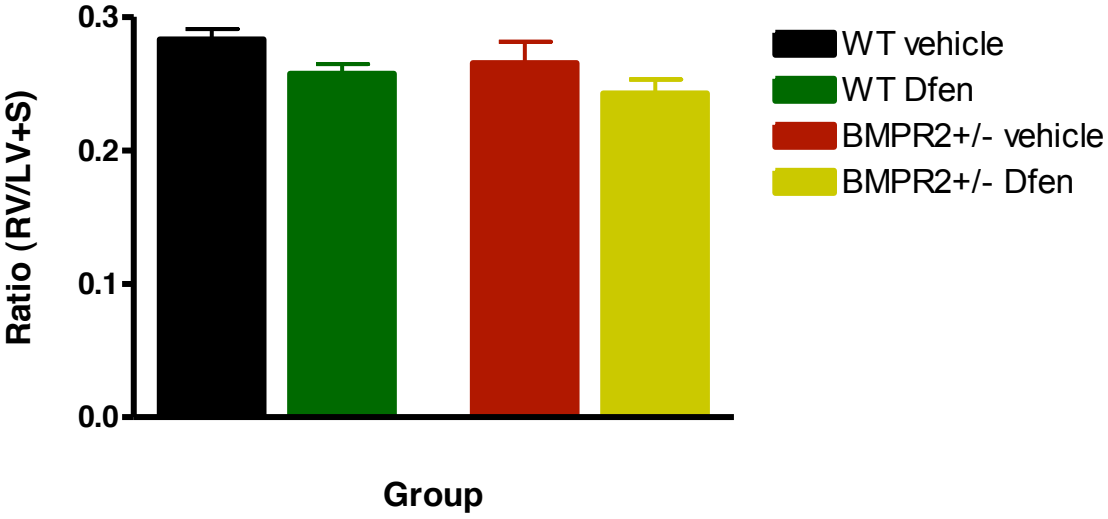
4.3.4 Right ventricular hypertrophy and vascular remodelling in female mice

Female RVH and remodelling data is shown in figure 4.6. RVH was not significantly different between groups. There was, however, a substantial increase in remodelling following Dfen in both WT (vehicle: 3.2 ± 0.3%, n=4, Dfen: 12.1 ± 1.2%, n=4,) and BMPR2$^{+/-}$ (vehicle: 4.0 ± 0.7%, n=4, Dfen: 12.5 ± 1.2, n=4) mice with similar levels between the two genotypes.

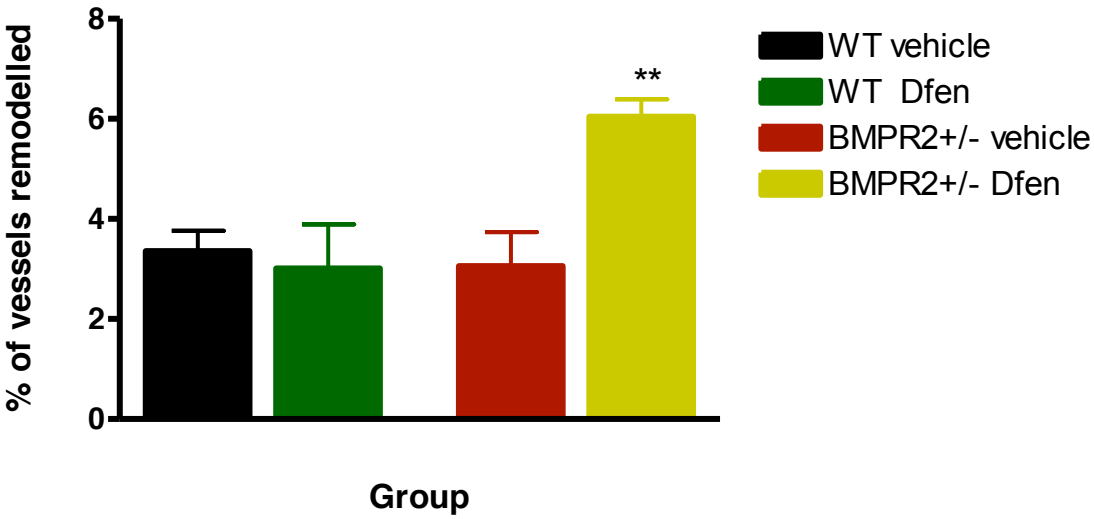
4.3.5 Gender differences

The preceding data was subjected to a gender analysis and is presented in figures 4.7 to 4.9. The most striking gender difference noted regarding RVP was the increase in sRVP elicited by Dfen in the females compared with the males (figure 4.7A). This occurred in both WT (male: 18.4 ± 2.0 mmHg, n=9, female: 32.0 ± 0.5 mmHg, n=7) and BMPR2$^{+/-}$ (male: 17.4 ± 1.0 mmHg, n=7, female: 27.9 ± 3.1 mmHg, n=6) mice. Similar differences also occurred for mean RVP (WT male: 10.8 ± 0.7 mmHg, n=9, WT female: 18.3 ± 1.1 mmHg, n=7, BMPR2$^{+/-}$ male: 11.0 ± 0.8 mmHg, n=7, BMPR2$^{+/-}$ female: 20.2 ± 2.6

Male

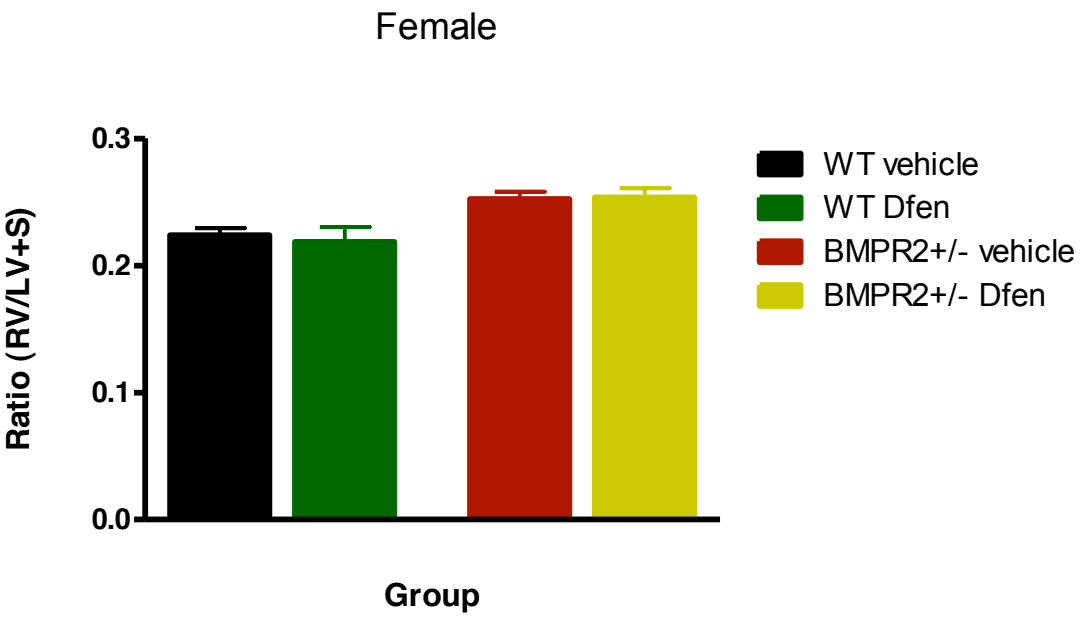


A



B

Figure 4.5 Effect of Dfen ingestion in WT and BMPR2$^{+/-}$ male mice (n = 4 to 18 mice per group) on RVH (A) and remodelling (B). Results expressed as mean ± SEM. ** $p<0.01$ versus WT Dfen and BMPR2$^{+/-}$ vehicle using Bonferroni multiple comparison test.



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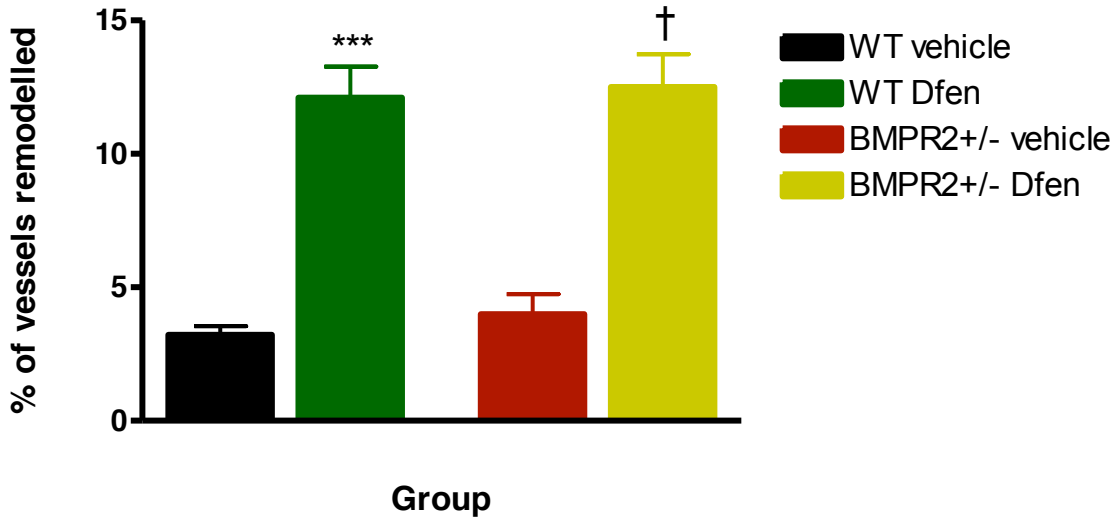


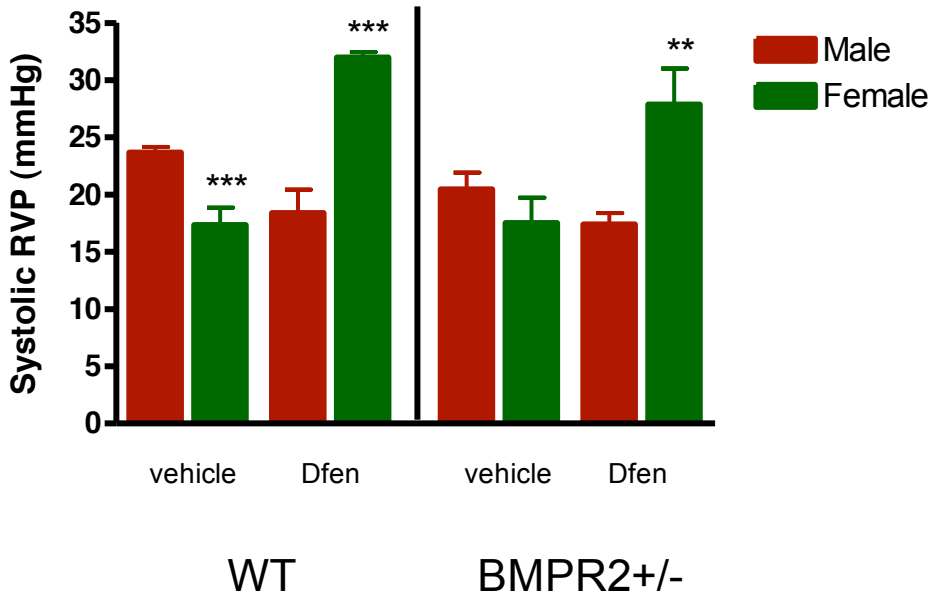
B

Figure 4.6 Effect of Dfen ingestion in WT and BMPR2$^{+/-}$ female mice (n = 4 to 13 mice per group) on RVH (A) and remodelling (B). Results expressed as mean ± SEM. *** $p<0.001$ versus WT vehicle, † $p<0.001$ versus BMPR2$^{+/-}$ vehicle using Bonferroni multiple comparison test.

mmHg, n=6) (figure 4.7B). In vehicle treated animals, male WT animals had higher sRVP than females (male: 23.7 ± 0.5 mmHg, n=19, female: 17.3 ± 1.5 mmHg, n=8). There was, however, no such difference for mean RVP.

There was insufficient data to do a male versus female comparison for mean SAP except for vehicle dosed BMPR2$^{+/-}$ mice where there was no significant difference (male: 82.4 ± 2.0 mmHg, n=5, female: 78.4 ± 7.4 mmHg, n=4). Variations in HR were noted between males and females treated with Dfen (figure 4.8). There was an increased HR in female WT mice compared with males (male: 383.9 ± 12.9 bpm, n=9, female: 436.1 ± 10.8 bpm, n=7) and a decreased HR in female BMPR2$^{+/-}$ mice compared with males (male: 428.3 ± 18.3 bpm, n=8, female: 358.5 ± 18.7 bpm, n=6).

RVH was higher in male WT mice in both vehicle treated (male: 0.28 ± 0.01, n=18, female: 0.22 ± 0.01, n=13) and Dfen treated (male: 0.26 ± 0.01, n=11, female: 0.21 ± 0.01, n=9) groups (figure 4.9A). The extent of RVH was similar in all four BMPR2$^{+/-}$ groups. Remodelling data is shown in figure 4.9. Dfen significantly increased the number of remodelled vessels in female WT mice compared to males (male: 3.0 ± 0.9%, n=4, female: 12.1 ± 1.2 %, n=4). There was also a marked increase in remodelling in female BMPR2$^{+/-}$ mice compared to males (male: 6.0 ± 0.3 %, n=4, female: 12.5 ± 1.2 %, n=4) (figure 4.9B).



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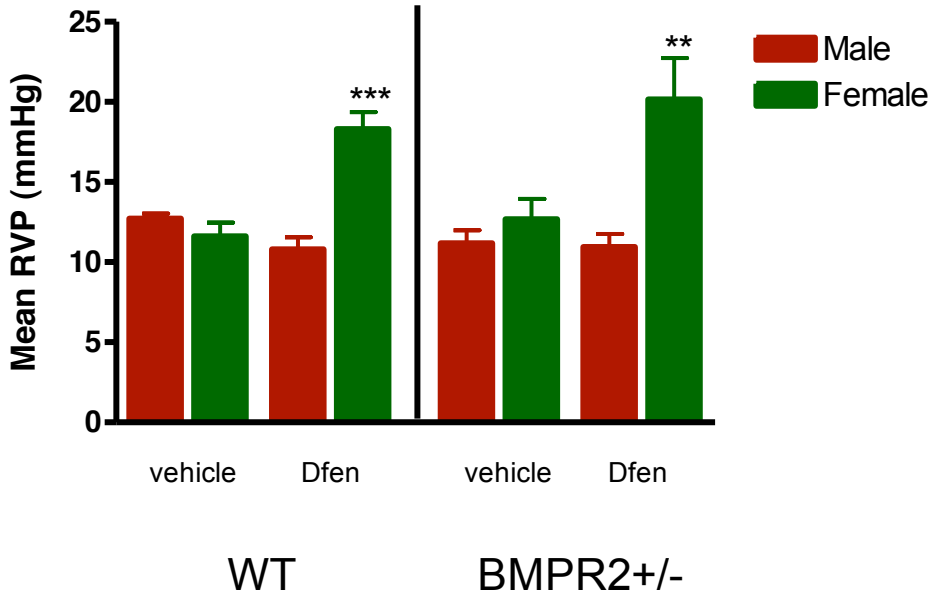


B

Figure 4.7 Comparison of male and female sRVP (A) and mRVP (B) in vehicle and Dfen treated WT and BMPR2$^{+/-}$ mice (n= 6 to 9 mice per group). Results expressed as mean ± SEM. ** $p<0.01$ versus male, *** $p<0.001$ versus male using an unpaired two-tailed t-test.

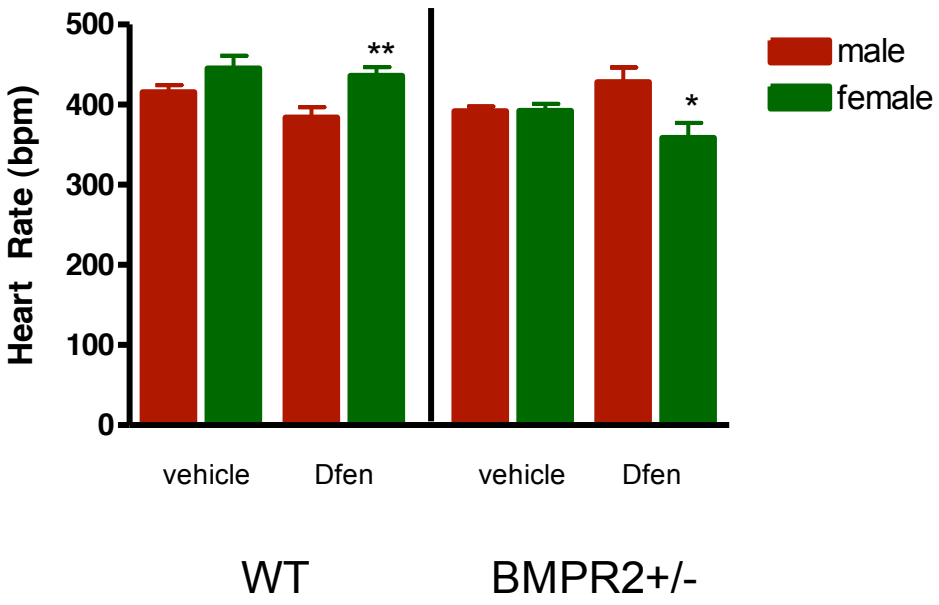
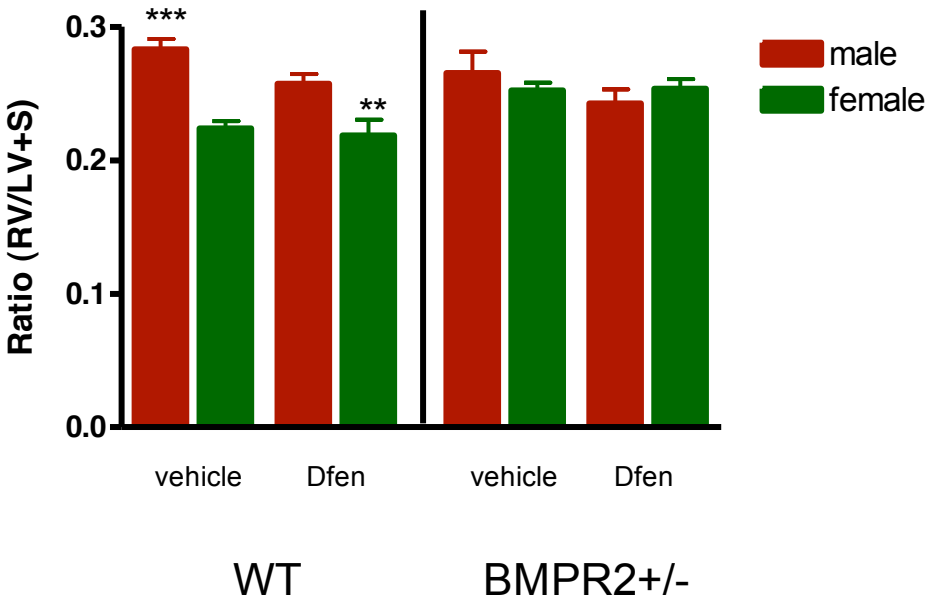


Figure 4.8 Comparison of male and female HR in vehicle and Dfen treated WT and BMPR2$^{+/-}$ mice (n = 6 to 19 mice per group). Results expressed as mean ± SEM. * $p<0.05$, ** $p<0.01$ versus male using an unpaired two-tailed t-test.



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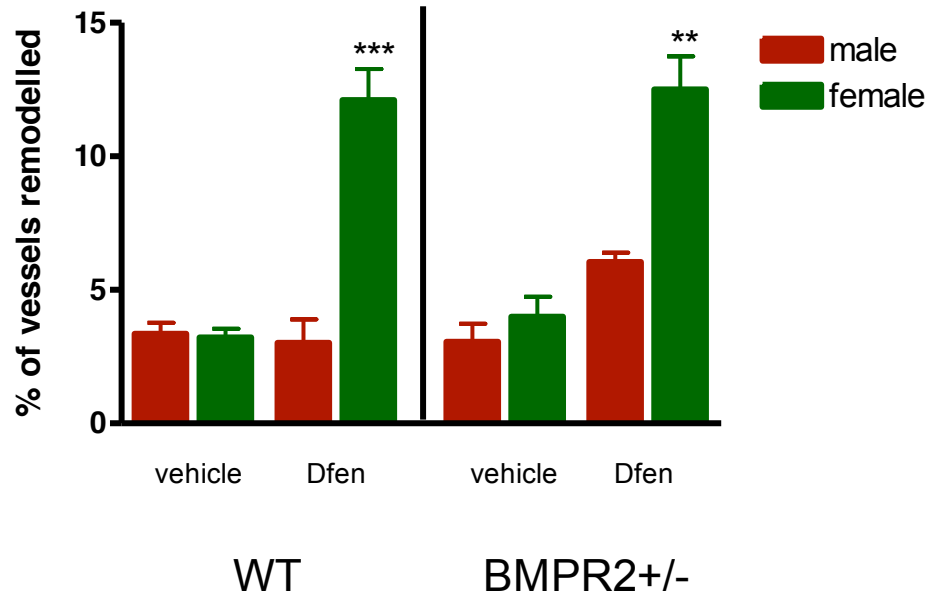


B

Figure 4.9 Comparison of male and female RVH (A) and remodelling (B) in vehicle and Dfen treated WT and BMPR2$^{+/-}$ mice (n=8 to 18 mice per group). Results expressed as mean ± SEM. ** p<0.01 versus male, *** p<0.001 versus male using an unpaired two-tailed t-test.

4.4 Discussion

This study shows Dfen to be a robust inducer of a pulmonary hypertensive phenotype in both WT and BMPR2$^{+/-}$ female mice. Results were similar between both genotypes indicating BMPR2 haploinsufficiency in this genetic model does not lead to a worsening of PAH in female mice. Neither WT nor BMPR2$^{+/-}$ male mice developed PAH following Dfen, indicating a critical role for female gender in Dfen induced PAH. Male BMPR2$^{+/-}$ mice did, however, show susceptibility, although at a lower level, to Dfen induced vascular remodelling.

Dfen induced PAH in female WT mice, increasing both mRVP and vascular remodelling but without causing RVH. Male WT mice were not susceptible to either of these effects and actually showed a decrease in mRVP following administration of Dfen. This decrease was very modest and perhaps partly related to a vasodilator response in the male vasculature acting to compensate the Dfen/5-HT induced vasoconstriction. The finding here, that male mice are resistant to Dfen induced pulmonary hypertension may help to explain why some previous animal studies that may have employed male rodents have failed to induce Dfen associated pulmonary hypertension. The Dfen data presented here indicates that female mice are more prone to a pulmonary hypertensive phenotype when challenged with a disease risk factor. There was no evidence at all in male WT mice for PAH. The results in chapter 3 showed that male WT mice did develop hypoxic induced PAH. Dfen appears to be a less potent inducer of PAH at the dose tested than hypoxia, with hypoxia causing a higher increase in mRVP and a greater degree of remodelling in female mice compared with Dfen. Male mice; therefore, may require a greater pulmonary challenge to overcome an inherent protective mechanism before a pulmonary hypertensive phenotype emerges.

No differences were noted between female WT and BMPR2$^{+/-}$ mice with both mRVP and vascular remodelling increased by a similar margin in both genotypes. Interestingly, male

BMPR2$^{+/-}$ mice did show a small but significant increase in the number of remodelled vessels following Dfen, something not observed in WT males. As outlined in the last chapter and shown by Beppu et al., 2004 there may be subtle vascular changes in BMPR2$^{+/-}$ mice that predispose these animals to an increase in remodelling. This is shown quite convincingly by the hypoxic female data and now also demonstrated in the Dfen treated BMPR2$^{+/-}$ males. The previous study by Long et al., 2006 that found a mild pulmonary hypertensive phenotype in BMPR2$^{+/-}$ mice infused with 5-HT utilised male mice, indicating that male BMPR2$^{+/-}$ mice are more susceptible to the mitogenic and vasoconstrictor effect of 5-HT which is thought to be the major mediator of Dfen induced PAH (see chapter 5 for further information on the mechanism of action of Dfen).

It is unclear if underlying mutations in BMPR2 increase the risk of developing PAH following anorexigen administration. Current evidence regarding human patients is inconclusive. BMPR2 mutations have been found in patients taking fenfluramines (Humbert et al., 2002). The duration of fenfluramine exposure was relatively short in patients with BMPR2 mutation, further indicating a facilitation of PAH through interaction with BMPR2 mutation and anorexigen use (Humbert et al., 2002). The majority of mutations recorded in the fenfluramine using patients do not, however, appear to be as detrimental on protein function as other mutations found in FPAH (Machado et al., 2006). Missense mutations in the ligand binding domain of BMPR2 in FPAH patients result in cysteine residue substitution with negative consequences for protein structure but cysteine residues are not affected by the missense mutations found in APAH patients. Some of the missense mutations discovered in APAH patients appear to occur in regions of the protein with no known functional significance. Similarly, of the three kinase domain mutations found, only 1 is proven to have a negative effect on kinase function (Machado et al., 2006). A mutation in the cytoplasmic domain with more functional significance (Machado et al., 2006) was found in a patient who had been exposed to amfepramone (a sympathomimetic

amphetamine-like anorexigen) and Abramowicz et al., 2003 suggest the time course of disease development and ingestion of amfepramone are a link that indicates the drug was the causative agent (Abramowicz et al., 2003). The fact that this is an isolated case makes any conclusion regarding a link between amfepramone and PAH impossible and it is far from certain that amfepramone was the cause of the disease. Moreover, in a large study performed in the USA, only fenfluramines were associated with the diagnosis of PAH. Other anorexigens, including amfepramone, were not associated with an increased risk of PAH (Rich et al., 2000). Proving a link between BMPR2 mutations and anorexigen use in humans is a difficult task and will require more accurate information on the distribution of BMPR2 mutations. Studies to date that have analysed APAH patients for BMPR2 mutations did not analyse the entire gene (Humbert et al., 2002) thus raising the possibility that the percentage of BMPR2 mutant carriers in this population may be greater. More detailed information on the functional consequences of the mutations that have been found in APAH patients and information on the varying penetrance of distinct BMPR2 mutations will all help to better characterise the interaction of BMPR2 mutations with other risk factors. It has been suggested that a pre-existing deficiency in pulmonary NO levels may act as a risk factor for Dfen APAH (Archer et al., 1998b). Inhibition of NO increases the pressor response to Dfen in rat lung (Weir et al., 1996) and Dfen users who develop PAH have been found to have lower levels of NO in the lung compared with control subjects (Archer et al., 1998b). This was found to be in contrast to patients with FPAH/IPAH who had increased lung expression of NO which is theorised to act as a counterbalance to the vasoconstriction observed in the diseased lung. The decreased levels of NO were found to persist for years after discontinuation of the anorectic agent perhaps indicating that the NO deficiency was independent of drug use and perhaps was an underlying genetic deficiency that on its own was of no clinical concern but interacted with Dfen to cause PAH (Archer et al., 1998b). This question can not be answered as NO levels were unknown prior to

disease onset but deserves more investigation. A further interesting discovery related to dysfunctional NO signalling in fenfluramine APAH comes from one fenfluramine user with PAH who carried a loss of function mutation in the 5-HT_{2B} receptor (Blanpain et al., 2003). This receptor induces NO in several cell lines through C-terminus dependent activation of eNOS and iNOS (Manivet et al., 2000) and mediates vasorelaxation in pig porcine arteries, a receptor that shares 95% homology with the human 5-HT_{2B} receptor (Glusa and Pertz, 2000). It could be speculated that this patient would have impaired 5-HT_{2B} mediated NO synthesis (assuming 5-HT_{2B} receptors were to mediate NO release in human pulmonary arteries) reducing the vascular protective effect of NO in PAH. The 5-HT_{2B} receptor has, however also been highlighted as playing a role in promoting vascular remodelling in animal models of the disease and partly mediating the effects of NDfen. The next chapter will discuss further the role of 5-HT receptors in fenfluramine APAH.

To conclude, this study reveals Dfen to be a robust inducer of PAH in female WT and $BMPR2^{+/-}$ mice. The pulmonary hypertensive effect of Dfen was completely negated in male WT mice while $BMPR2^{+/-}$ males did develop limited vascular remodelling but without elevated RVP. These results suggest female gender is a critical risk factor for development of Dfen APAH. Presence of BMPR2 mutation did not affect the degree of PAH in female mice but may pre-dispose the male vasculature to Dfen induced remodelling.

Chapter 5

Role of peripheral 5-HT in PAH

5.1 Introduction

The development of the Tph1$^{-/-}$ mouse offers a valuable genetic model for assessing the contribution of peripheral 5-HT in the development of PAH. As mentioned previously, levels of TPH1 in PAECs are increased in cells from IPAH patients compared to control subjects and this is associated with increased 5-HT release (Eddahibi et al., 2006). It has also recently been shown that hypoxic PAH is diminished in Tph1$^{-/-}$ mice compared with WT mice (Morecroft et al., 2007; Izikki et al., 2007).

As outlined previously, Dfen has complex effects on the serotonergic system. It is a 5-HT releaser (Zolkowska et al., 2006), a 5-HTT substrate (Rothman et al., 1999) and is converted to NDfen that induces vasoconstriction through activation of 5-HT$_{2A}$ receptors (Ni et al., 2005). It is unclear, however, whether Dfen induced PAH is caused by 5-HT release, through a direct vasoconstrictor/mitogenic action of Dfen or through NDfen induced activation of 5-HT receptors. It is possible that a combination of these factors coupled with an inhibition of K^+ channels contribute to Dfens mechanism of action. By utilising the Tph1$^{-/-}$ mouse, it should be possible to determine whether Dfen induces PAH through a direct 5-HT independent effect or whether peripheral 5-HT synthesis and release by Dfen is the mode of action. To answer this, female Tph1$^{-/-}$ mice were dosed with Dfen and pulmonary haemodynamics measured as in sections 2.3 and 4.2. The decision to use female mice was arrived at following the results obtained in the last chapter that showed only female mice develop Dfen associated PAH.

Morecroft et al., 2007 performed pharmacological experiments on pulmonary arteries from normoxic and hypoxic Tph1$^{-/-}$ mice. That study showed that 5-HT induced vasoconstriction is increased in Tph1$^{-/-}$ mice compared with WT. Unlike WT mice, where 5-HT induced vasoconstriction was enhanced following hypoxia, there was no difference in response to 5-HT between normoxic and hypoxic Tph1$^{-/-}$ mice. It would be useful to further determine pharmacological differences between WT and Tph1$^{-/-}$ mice and if

administration of Dfen affects the pharmacology of pulmonary arteries in either group.

To better understand any interaction that may exist between peripheral 5-HT, administration of Dfen and activity of 5-HT vasoconstrictor receptors, pulmonary arteries from WT and Tph1$^{-/-}$ female mice dosed with vehicle and Dfen were used to construct cumulative concentration-response curves (CCRCs) to 5-HT, Dfen and NDfen.

The final part of this study sought to investigate any cross-talk that might exist between 5-HT and BMPR2 signalling pathways. This involved western blot analysis of BMPR2 and p-Smad1/5/8 in tissue samples (IPAs + main pulmonary arteries) isolated from WT and Tph1$^{-/-}$ male mice. Both main pulmonary artery and IPA was utilised for analysis to ensure adequate protein expression for detection. Male mice were used due to availability of TG animals owing to the large number of female animals utilised in the *in vivo* study. Despite female mice being a necessity for studying the *in vivo* effects of Dfen in Tph1$^{-/-}$ mice, male mice were deemed sufficient for studying the interaction of 5-HT and BMPR2 signalling. This is derived from previous work studying the interaction of these two pathways in both whole lung and isolated PASMCs in BMPR2$^{+/-}$ and WT mice, a study that utilised only male mice (Long et al., 2006). Due to the important role p-ERK1/2 has in 5-HT mediated proliferation in PASMCs, it was also examined.

5.2 Methods

5.2.1 Animals

Mice (female) were 2 months old at commencement of Dfen dosing and 3 months old at time of experimental analysis. Western blotting experiments were performed on 2 month old male mice.

5.2.2 Myography

IPAs were isolated from mice from each of the four treatment groups and used for wire

myography experiments as described previously.

Following attainment of maximal contractile response to KCl as outlined in section 2.6.1.5, vessels were left to equilibrate for 30 minutes before CCRCs were performed for 5-HT (Sigma-Aldrich Ltd, Poole, UK), Dfen or NDfen (National Institute of Health, Maryland, USA). All drugs were dissolved in H_2O to give a solution of 10^{-2} M from which serial dilutions were created up to 10^{-6} M giving the range necessary for a 1 nM- 300 μM CCRC to be constructed. Each vessel segment was subjected to only one treatment.

5.3 Results

5.31 Dfen dosing study in Tph1$^{-/-}$ mice

Dfen dramatically increases both sRVP and mRVP in female WT mice (figure 5.1). In Tph1$^{-/-}$ female mice, however, Dfen has no effect on either sRVP (vehicle: 15.5 ± 1.0 mmHg, n=8, Dfen: 14.1 ± 1.0 mmHg, n=9) or mRVP (vehicle: 11.4 ± 0.9 mmHg, n=8, Dfen: 9.5 ± 1.0 mmHg, n=9).

Tph1$^{-/-}$ vehicle dosed mice had a lower HR compared with WT vehicle treated animals (WT: 445 ± 16 bpm, n=8, Tph1$^{-/-}$: 388.9 ± 21.7 bpm, n=8) (figure 5.2). There was no difference between the Dfen treated groups or between Dfen treated animals and respective controls.

RVH was similar between the four groups (figure 5.3A). Unlike the WT female mice that show substantial remodelling following Dfen, Tph1$^{-/-}$ mice dosed with Dfen have a similar level of remodelling to vehicle dosed mice (vehicle: 3.1 ± 0.4 %, n=4, Dfen: 3.6 ± 0.4 %, n=4) (figure 5.3B).

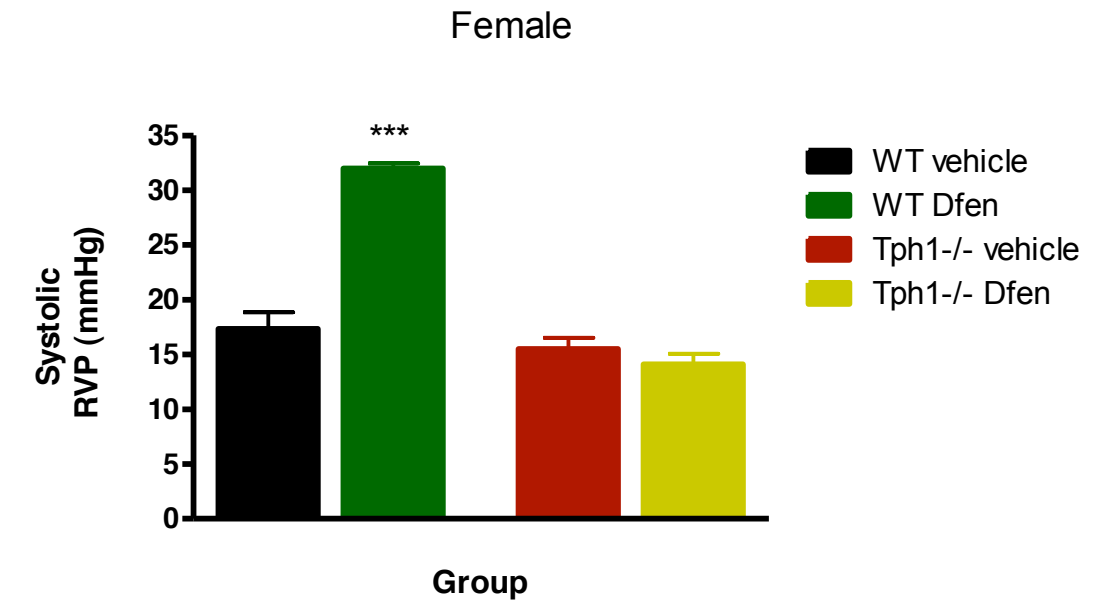
5.3.2 Assessment of contractile response to 5-HT, Dfen and NDfen in IPAs

Contractions in IPAs were measured relative to the contraction induced by 50 mM KCl. No significant differences were observed in KCl response between groups (WT veh: 2.2 ±

0.3 mN, n=6, WT Dfen: 2.3 ± 0.2 mN, n=6, Tph1$^{-/-}$ veh: 2.1 ± 0.3 mN, n=6, Tph1$^{-/-}$ Dfen: 1.9 ± 0.2 mN, n=6). CCRCs for 5-HT are presented in figure 5.4. The potency (pEC50) of 5-HT was greater in WT mice compared with Tph1$^{-/-}$ mice. This was true for the vehicle dosed group (WT: 7.7 ± 0.1, n=6, Tph1$^{-/-}$: 7.2 ± 0.0, n=6) and for the Dfen dosed group (WT: 7.7 ± 0.1, n=6, Tph1$^{-/-}$: 7.3 ± 0.1, n=6). There was no difference between Dfen dosed and vehicle dosed animals. Despite the potency of 5-HT being greater in the WT mice, Tph1$^{-/-}$ mice dosed with Dfen did show a greater maximal response (Emax) to 5-HT compared with the respective WT group (WT: 122.5 ± 4.8 % of the contraction induced by 50 mM KCl, n=6, Tph1$^{-/-}$: 138.0 ± 5.3 %, n=5). There was no such difference between vehicle dosed animals.

Results for the CCRCs to Dfen are presented in figure 5.5. A maximum response to Dfen was not obtained at the concentrations tested so neither pEC50 nor Emax could be calculated. No significant differences were found between groups at any of the concentrations used.

Results for the CCRCs to NDfen are presented in figure 5.6. pEC50 values were lower for mice dosed with Dfen than for vehicle dosed mice. This was true for both WT (vehicle: 6.1 ± 0.1, n=6, Dfen: 5.8 ± 0.1, n=6) and Tph1$^{-/-}$ (vehicle: 6.4 ± 0.0, n=6, Dfen: 6.0 ± 0.0, n=6) mice. Emax was significantly lower in WT mice dosed with Dfen compared with the vehicle dosed mice (vehicle: 103.0 ± 4.4 % of the contraction induced by 50 mM KCl, n=6, Dfen: 88.2 ± 5.0 %, n=6). Similar results were obtained for Tph1$^{-/-}$ mice (vehicle 109.1 ± 1.6 %, n=6, Dfen: 95.8 ± 3.2 %, n=6) however it did not reach significance. No significant differences were observed between WT and Tph1$^{-/-}$ mice in either treatment group.



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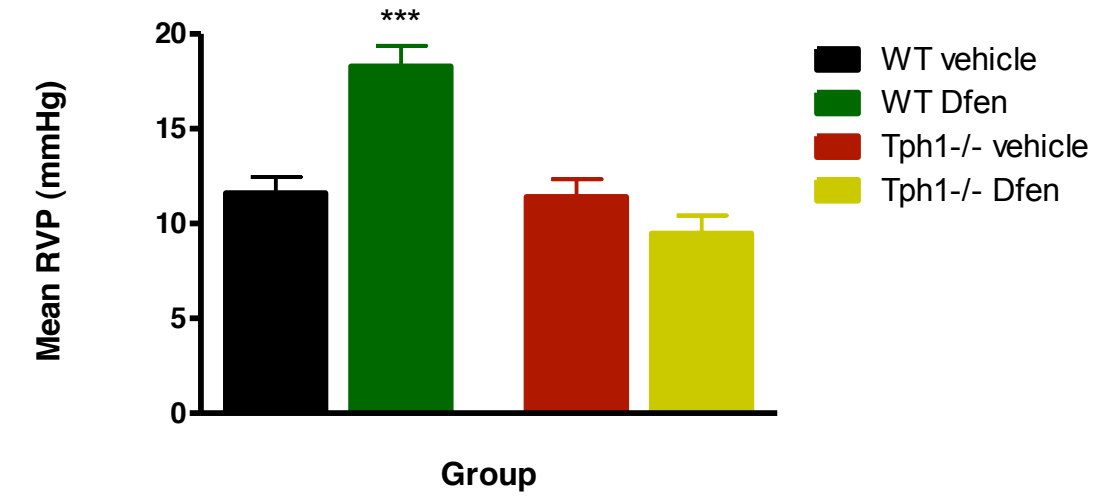


B

Figure 5.1 Effect of Dfen ingestion in WT and Tph1$^{-/-}$ female mice (n = 7 to 9 mice per group) on sRVP (A) and mRVP (B). Results expressed as mean ± SEM. *** $p<0.001$ versus WT vehicle and Tph1$^{-/-}$ Dfen using Bonferroni multiple comparison test.

Female

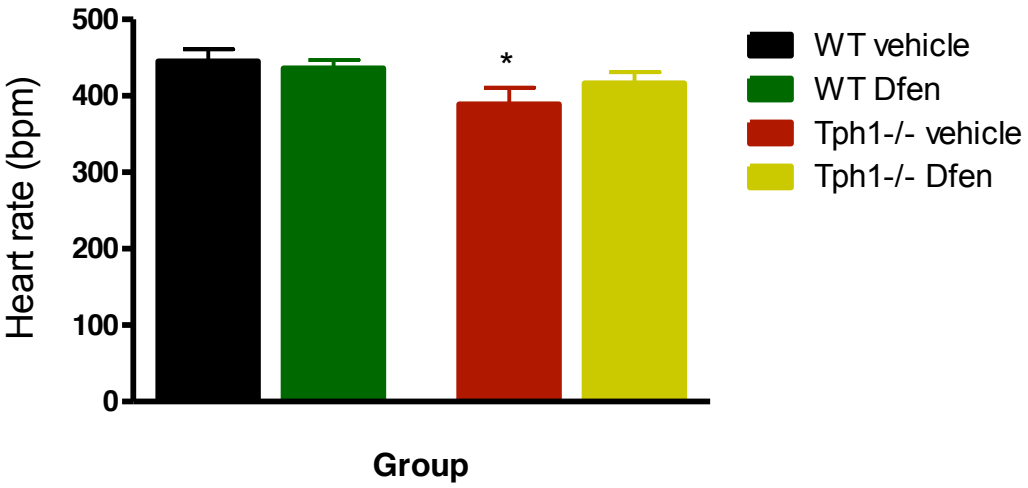
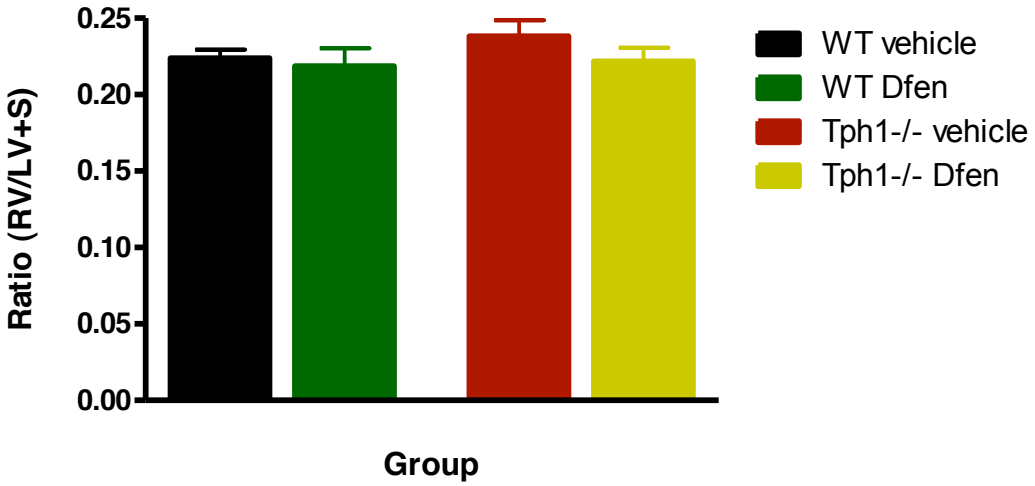


Figure 5.2 Effect of Dfen ingestion in WT and Tph1$^{-/-}$ female mice (n = 7 to 9 mice per group) on HR. Results expressed as mean ± SEM. * $p<0.05$ versus WT vehicle using Bonferroni multiple comparison test.

Female



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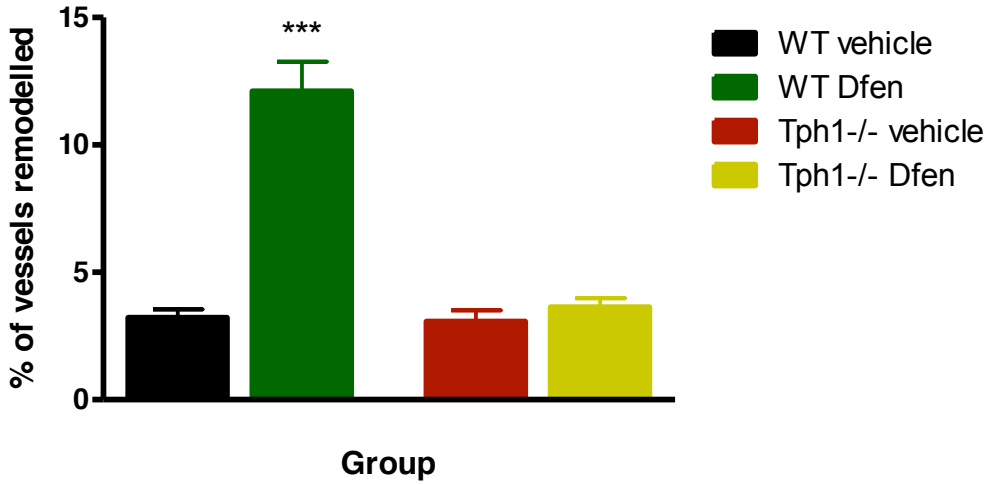


B

Figure 5.3 Effect of Dfen ingestion in WT and Tph1$^{-/-}$ female mice (n = 9 to 17 mice per group) on RVH (A) and remodelling (B). Results expressed as mean ± SEM. *** $p<0.001$ versus WT vehicle and Tph1$^{-/-}$ Dfen using Bonferroni multiple comparison test.

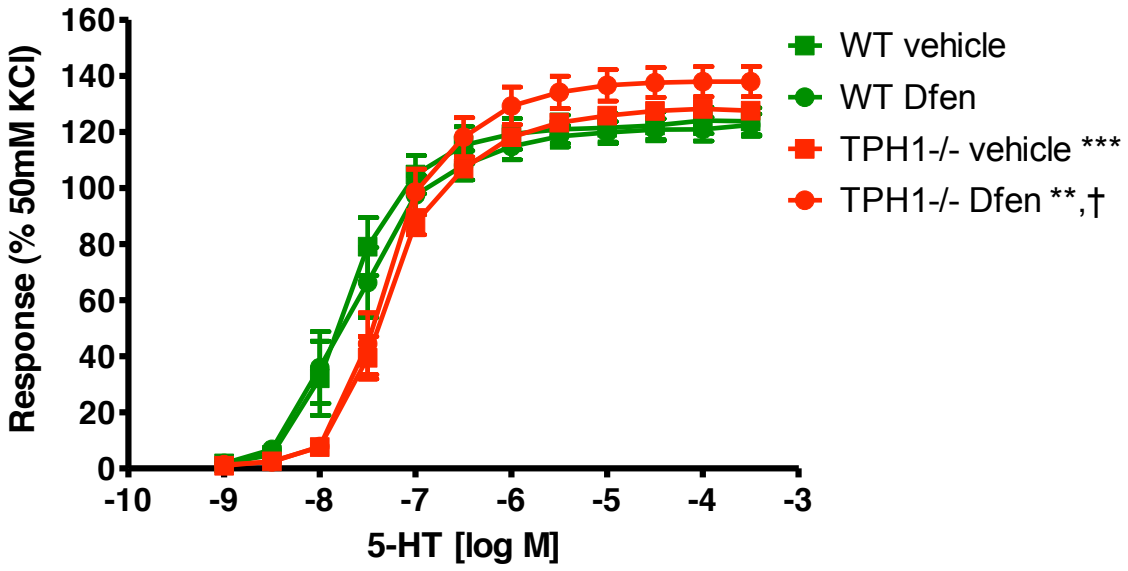


Figure 5.4 Effect of Tph1$^{-/-}$ gene knockout and Dfen ingestion on 5-HT induced contraction in female mouse IPAs (n = 6 mice per group). Results expressed as mean ± SEM. pEC50: ** $p<0.01$ versus WT Dfen, *** $p<0.001$ versus WT vehicle. Emax: † $p<0.05$ versus WT Dfen using Bonferroni multiple comparison test.

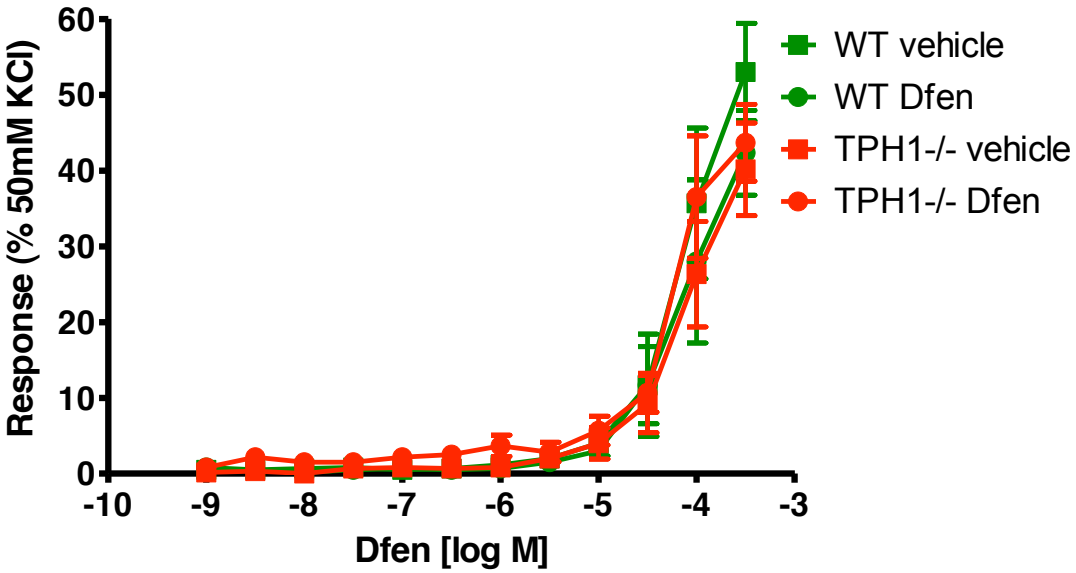


Figure 5.5 Effect of Tph1$^{-/-}$ gene knockout and Dfen ingestion on Dfen induced contraction in female mouse IPAs (n = 6 mice per group). Results expressed as mean ± SEM. Bonferroni multiple comparison test revealed no significant differences at any concentration tested.

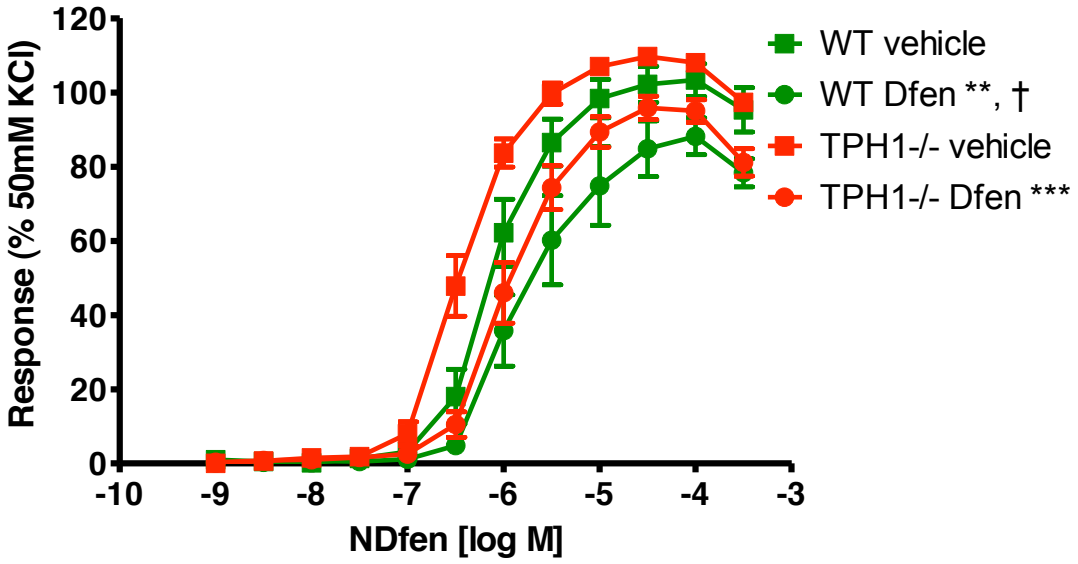
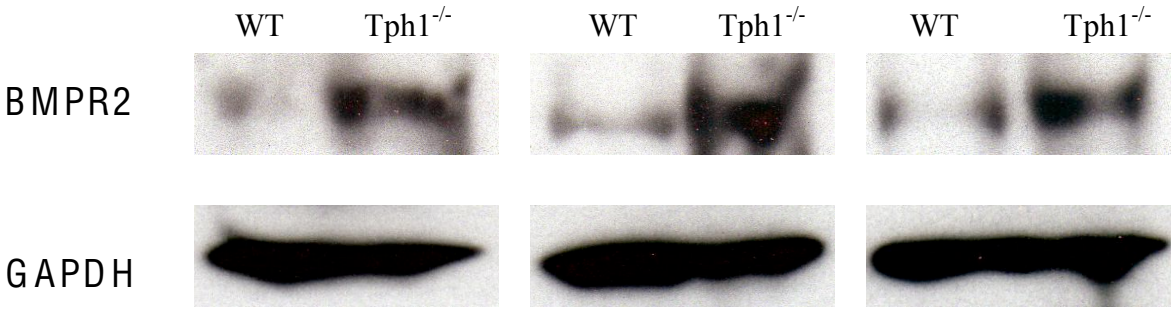


Figure 5.6 Effect of Tph1$^{-/-}$ gene knockout and Dfen ingestion on NDfen induced contraction in female mouse IPAs (n = 6 mice per group). Results expressed as mean ± SEM. pEC50: ** $p<0.01$ versus WT vehicle, *** $p<0.001$ versus Tph1$^{-/-}$ vehicle. Emax: † $p<0.05$ versus WT vehicle using Bonferroni multiple comparison test.

5.3.3 Influence of peripheral 5-HT on 5-HT and BMPR2 signalling proteins

$Tph1^{-/-}$ samples had visibly more BMPR2 protein expression than WT samples (figure 5.7). There was, however, no significant differences in the densitometric analysis between groups (figure 5.7). Data for p-Smad1/5/8 is presented in figure 5.8. While some experiments had visibly more p-Smad1/5/8 in $Tph1^{-/-}$ samples compared to WT, others showed no visible difference. This was reflected in the densitometry which yielded no significant differences between groups. Finally, p-ERK1/2 expression was assessed (figure 5.9). In the majority of experiments, p-ERK1/2 expression was visibly greater in WT mice compared with $Tph1^{-/-}$ mice. No significant differences emerged from the densitometry.



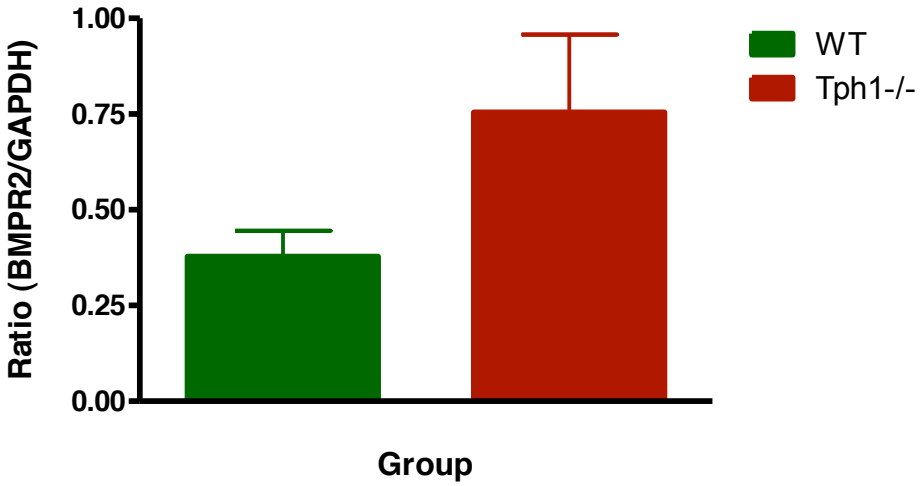


Figure 5.7 Representative autoradiograms (one from each of the three separate groups of tissue samples) and densitometric analysis of BMPR2 expression in WT and Tph1$^{-/-}$ male mouse IPAs + main pulmonary arteries (n=7). Results expressed as mean ± SEM. An unpaired two-tailed t-test revealed no significant difference between groups.

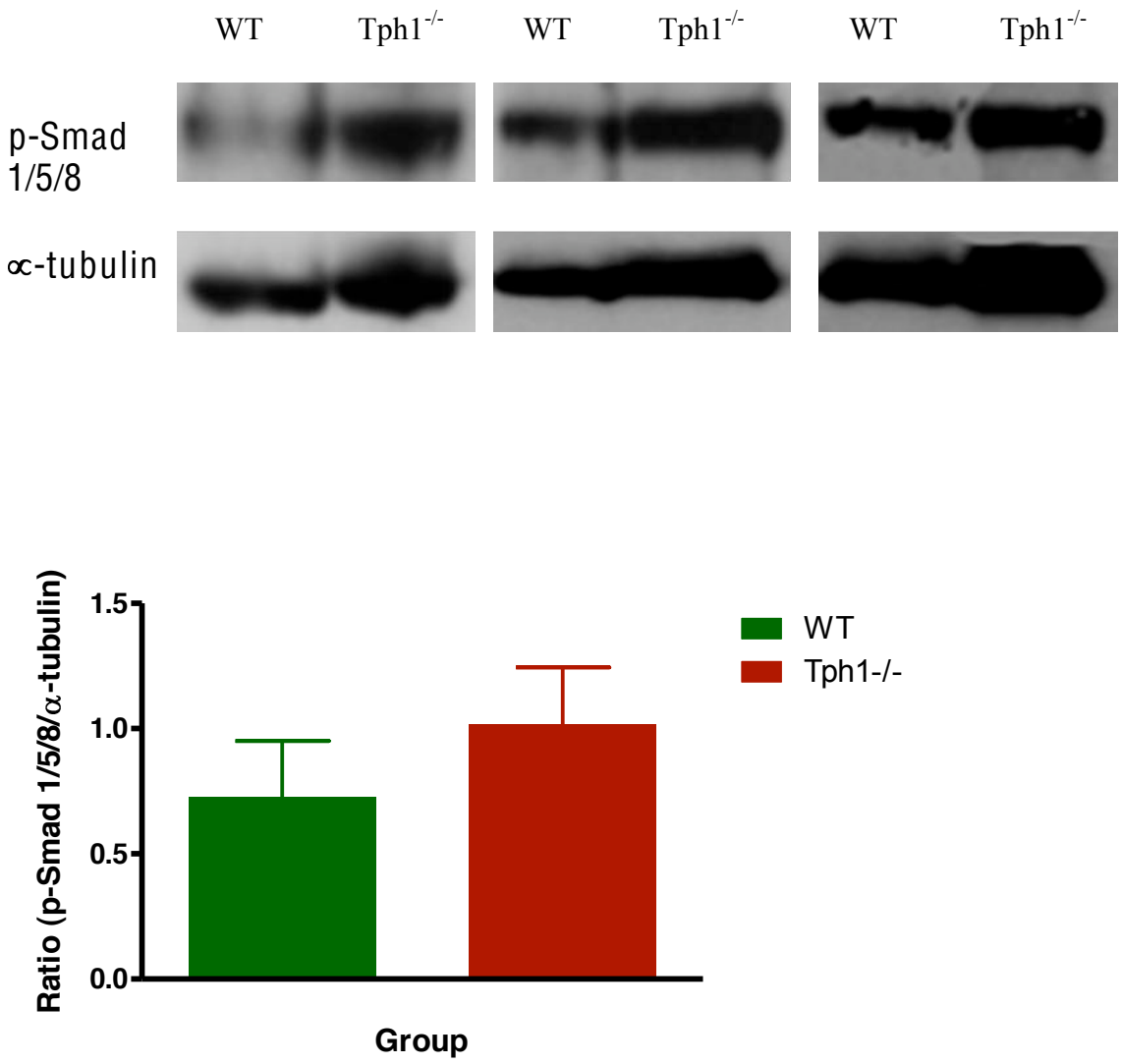


Figure 5.8 Representative autoradiograms (one from each of the three separate groups of tissue samples) and densitometric analysis of p-Smad1/5/8 expression in WT and Tph1$^{-/-}$ male mouse IPAs + main pulmonary arteries (n=8). Results expressed as mean ± SEM. An unpaired two-tailed t-test revealed no significant difference between groups.

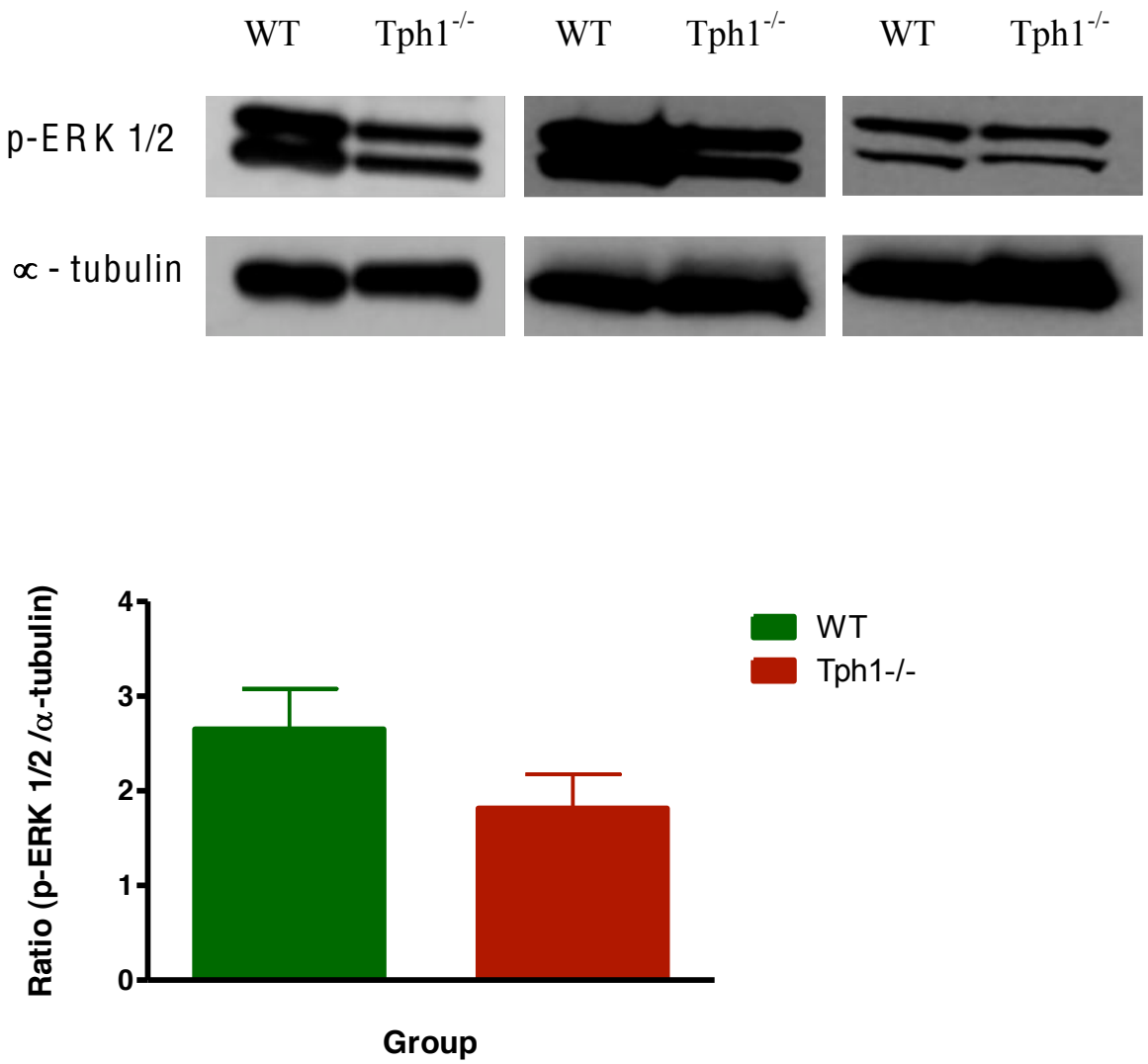


Figure 5.9 Representative autoradiograms (one from each of the three separate groups of tissue samples) and densitometric analysis of p-ERK 1/2 expression in WT and Tph1$^{-/-}$ male mouse IPAs + main pulmonary arteries (n=10). Results expressed as mean ± SEM. An unpaired two-tailed t-test revealed no significant difference between groups.

5.4 Discussion

This study has demonstrated a critical role for peripheral 5-HT synthesis in Dfen associated PAH with Tph1$^{-/-}$ mice completely protected against Dfen induced increases in RVP and vascular remodelling. This study also reveals previously uncharacterised pharmacological differences between WT and Tph1$^{-/-}$ mice and between mice that received Dfen and those that did not. Finally, the role of peripheral 5-HT on 5-HT and BMPR2 signalling molecules was examined. While no significant differences emerged, the results are suggestive of an inhibiting influence of 5-HT on BMPR2 which was found to be expressed more than two-fold higher in Tph1$^{-/-}$ mice compared with WT.

Dfen, as one of the few proven pharmaceutical risk factors for PAH is an extremely useful research tool for understanding the pathophysiology of the disease. As demonstrated in the previous chapter, Dfen induces a pulmonary hypertensive phenotype in female WT and BMPR2$^{+/-}$ mice. Understanding Dfens mechanism of action can help us gain insights into the underlying mechanisms of PAH. The fact that female Tph1$^{-/-}$ mice were resistant to the effects of Dfen on the pulmonary vasculature provides important data on how Dfen may be acting within the lung. It is clear from this study that Dfen requires the presence of peripheral 5-HT synthesis to exert its pulmonary hypertensive effect in mice (this data has been published in Dempsie et al., 2008). As a compound with multiple mechanisms of action, the ability of Dfen to release 5-HT from pulmonary vascular cells is determined to be a critical component of its action. Dfen is a potent 5-HTT substrate and 5-HTT substrates induce a much greater increase in extracellular 5-HT concentration compared with 5-HTT inhibitors (Rothman and Baumann, 2002). Moreover, the increase in plasma concentration of 5-HT induced by amphetamine analogues is proportional to their potency as 5-HTT substrates (Zolkowska et al., 2006). 5-HTT substrates release 5-HT by two mechanisms. The first involves carrier mediated exchange with 5-HT at the plasma membrane 5-HTT and the second involves interaction with the vesicular monoamine

transporter type 2 ($VMAT_2$) present on intracellular vesicles resulting in 5-HT being transported out of the vesicle and into the cytoplasm raising intracellular 5-HT levels (Partilla et al., 2006). This not just increases the available 5-HT in the cell for carrier mediated exchange out of the cell but also raises the possibility of increased activation of 5-HT dependent intracellular pathways. MDMA (3, 4-methylenedioxymethamphetamine or ecstasy), which has a mechanism of action very similar to the fenfluramines has also been shown to act on the $VMAT_2$ causing 5-HT release. The effect of MDMA was found to be dependent on an existing pool of vesicular 5-HT which MDMA released through a direct inhibition of $VMAT_2$ and a disruption of the transmembrane pH gradient that drives 5-HT uptake into the vesicle (Mlinar and Corradetti, 2003). Of these two mechanisms, a direct interaction with $VMAT_2$, not dissimilar to the carrier mediated exchange that occurs at the 5-HTT, is thought to be the most important factor in 5-HT vesicular release (Partilla et al., 2006). The resulting action of Dfen following i.v. administration in the rat is a large (19 fold at 1.0 mg/kg) increase in plasma 5-HT concentration reaching a peak concentration of 20 nM. This is above the threshold required to induce mitogenesis of human PASMCs (Zolkowska et al., 2006) and a sustained increase in plasma 5-HT concentration induced by Dfens role as a 5-HTT and $VMAT_2$ substrate may promote proliferation of PASMCs.

5-HTT substrates differ from 5-HTT inhibitors such as fluoxetine in two crucial ways. The 1st being the magnitude of increase in extracellular 5-HT. The 2nd being the uptake of compound into the cell and subsequent release of 5-HT from intracellular vesicles inducing a rise in 5-HT concentration in the cytoplasm. The combination of increased intracellular and extracellular 5-HT could be important in the development of vascular remodelling. This is a consequence of cross-talk between $5\text{-}HT_{1B/1D}$ and 5-HTT signalling. In human PASMCs activation of $5\text{-}HT_{1B}$ receptors results in phosphorylation of ERK1/2. The signal for nuclear translocation of p-ERK1/2 is dependent on 5-HT uptake by the 5-HTT, MAO

and ROS generation (Lawrie et al., 2005). As mentioned in section 1.2.3.1.3, this is in contrast to the methods by which p-ERK1/2 is nuclear translocated in bovine PASMCs. Both human and bovine PASMCs do, however, require both 5-$HT_{1B/1D}$ receptors and the 5-HTT for nuclear translocation of p-ERK1/2, both of which are likely to be activated by the elevated 5-HT induced by 5-HTT substrates such as Dfen. In the nucleus, p-ERK1/2 interacts with transcription factors such as GATA-4 (Suzuki et al., 2003) to upregulate gene transcription of proteins involved in PASMC proliferation such as S100A4/Mts1 (Lawrie et al., 2005). S100A4/Mts1 is upregulated in PASMCs in pulmonary hypertensive patients in arterial lesions with neointimal proliferation. Additionally, 5% of mice overexpressing S100A4/Mts1 display occlusive intimal lesions (Greenway et al., 2004).

The role of Dfen as a 5-HTT substrate appears a critical factor in Dfen induced PAH in mice but it may not be the only factor in its effect. The 5-HTT substrate *m*-chlorophenylpiperazine (mCPP) which is a greater 5-HT releaser *in vitro* than Dfen or its metabolites is not associated with PAH. mCPP also has activity at multiple 5-HT_2 receptors similar to NDfen. Unlike the fenfluramines, however, mCPP does not induce noradrenaline release and is not an amphetamine analogue (Rothman and Baumann, 2002). In addition to differences in ability to release noradrenaline, there are likely to be other pharmacological differences between non amphetamine and amphetamine 5-HTT substrates, perhaps including their regulation of unique Kv channel subunits and their effect on whole cell Kv current that determine their ability to induce PAH.

There are several unknown factors regarding the interaction of Dfen and the serotonin system in WT and $Tph1^{-/-}$ mice. The rate of uptake of Dfen into PASMCs in $Tph1^{-/-}$ mice compared to WT for example. This may be influenced significantly by intracellular 5-HT levels. The expression of the 5-HTT and 5-HT receptors may also differ between mice with normal and mice with no or minimal peripheral 5-HT although evidence shows that the function of the 5-HTT is similar in WT and $Tph1^{-/-}$ mice (Ni et al., 2005). Little is also

known regarding the effects of chronic Dfen treatment on the expression of 5-HT receptors. Most studies assessing the effects of Dfen on the serotonergic system have focussed on extracellular 5-HT levels following acute administration. Performing CCRCs to 5-HT, Dfen and NDfen on WT and Tph1$^{-/-}$ mice treated chronically with and without Dfen as performed in this investigation has provided some evidence of altered serotonergic function. Interestingly, the potency of 5-HT was reduced in Tph1$^{-/-}$ mice irrespective of treatment with Dfen. This is in contrast to experiments performed by Morecroft et al., 2007 who found an increased contractile response to 5-HT in IPAs from Tph1$^{-/-}$ mice. The animals used in that investigation were 5-6 months old (personal communication, Ian Morecroft) whereas the animals used here were 3 months at the time of being culled which may explain this difference. An enhanced response to 5-HT might be expected if vasoconstrictor 5-HT receptors were upregulated in response to a lack of extracellular 5-HT. A decreased response to 5-HT in Tph1$^{-/-}$ mice may be due to a component of vasoconstriction in WT mice being due to 5-HT release or higher expression of either vasoconstrictor receptors or intracellular signalling molecules associated with vasoconstriction, a process that would be absent in Tph1$^{-/-}$ mice. A downregulation of 5-HT receptors in Tph1$^{-/-}$ mice is also not inconceivable. In relation to the 5-HTT, 5-HT promotes stability in transporter expression and a loss of 5-HT results in downregulation of the transporter in HEK239 cells (Ni and Watts, 2006). Similar processes may occur in relation to the 5-HT$_{1B}$ or 5-HT$_{2A}$ receptors in mice.

Consistent with its low affinity for 5-HT receptors (Rothman and Baumann, 2002), Dfen induced vasoconstriction only at high concentrations (>10 μM). The vasoconstrictor response to Dfen is only partly mediated by 5-HT$_2$ receptors and a direct action on K^+ channels is also likely to be an important mediator of Dfen induced vasoconstriction (Belohlavkova et al., 2001). No differences were observed in the response to Dfen in any of the 4 groups indicating the dominant factor underlying Dfen induced vasoconstriction is

similar between groups. This could be expression and activity of Kv channels or even a direct effect of Dfen inside the cell (Lee et al., 2001). At 10 μM Dfen is likely to be transported inside the cell and accumulate to a high level where it may activate 5-HT signalling pathways such as ROCK promoting vasoconstriction. Dfen has been shown to mimic 5-HT in fibroblasts by activating mitogenic compounds such as ERK1/2 (Lee et al., 2001) and this may also occur in relation to vasoconstrictor signalling molecules. If a direct action of Dfen in the cell was to be the major factor in Dfen induced vasoconstriction it would mean that the 5-HTT is similarly expressed in WT and $Tph1^{-/-}$ mice, is unaffected by chronic dosing with Dfen and that Dfen uptake by the 5-HTT is not influenced by intracellular 5-HT. A combination of an intracellular action and a contribution from either $5\text{-}HT_{1B}$ or $5\text{-}HT_{2A}$ receptors that owing to the low affinity of Dfen at these receptors only induce vasoconstriction at high concentrations may mediate the vasoconstriction induced by Dfen (see next chapter for more on Dfen mechanism of action).

The arterial contraction induced by NDfen, unlike the responses to 5-HT and Dfen was influenced by chronic administration of Dfen. The potency of NDfen was lower in both WT and $Tph1^{-/-}$ mice dosed with Dfen and the Emax of NDfen was lower in WT mice dosed with Dfen compared with controls. NDfen is a potent $5\text{-}HT_{2B}$ and $5\text{-}HT_{2C}$ agonist and has moderate potency at $5\text{-}HT_{2A}$ receptors (Rothman and Baumann, 2002). NDfen stimulation of $5\text{-}HT_{2C}$ receptors most likely contributes to its anorectic properties (Curzon et al., 1997; Vickers et al., 1999) and activation of $5\text{-}HT_{2B}$ receptors may contribute to vascular remodelling in mice but without influencing vasoconstriction (Launay et al., 2002). A direct action at $5\text{-}HT_{2A}$ receptors is the most likely explanation for NDfen induced vasoconstriction and this has been shown to underlie NDfen induced vasoconstriction in mouse aorta, an effect independent of endogenous 5-HT release (Ni et al., 2005). This is similar to results presented here where no significant differences in

NDfen contractile response between WT and Tph1$^{-/-}$ mice were found. Combining the pharmacological results for 5-HT, Dfen and NDfen leads to some interesting conclusions regarding 5-HT receptor expression between WT and Tph1$^{-/-}$ mice and in Dfen and non-Dfen treated mice. 5-HT response was reduced in Tph1$^{-/-}$ mice whereas the response to NDfen was not and indeed, an observable trend towards an enhanced response to NDfen was noted in the Tph1$^{-/-}$ animals. We can conclude from this that 5-HT and NDfen are exerting their vasoconstrictor response through different mechanisms. This may be 5-HT$_{1B}$ receptors mediating 5-HT and 5-HT$_{2A}$ receptors mediating NDfen induced vasoconstriction. The 5-HT$_{1B}$ receptor is involved in hypoxic induced vasoconstriction, vascular remodelling and RVH in mice (Keegan et al., 2001) and may be decreased in young adult Tph1$^{-/-}$ mice. Assuming the 5-HT$_{1B}$ receptor is mediating the vasoconstriction to 5-HT, it is unaffected by chronic administration of Dfen. This is in contrast to the 5-HT$_{2A}$ receptor, thought to underlie the NDfen vasoconstriction. Mice dosed with Dfen may have decreased expression of 5-HT$_{2A}$ receptors. This downregulation occurred in Tph1$^{-/-}$ mice as well as WT indicating a direct action by Dfen or NDfen in downregulating the 5-HT$_{2A}$ receptor perhaps as a compensatory mechanism to the potent continuous agonist action of NDfen over the 28 day treatment at this receptor.

The Tph1$^{-/-}$ mouse can further be utilised to examine the role that peripheral 5-HT has on expression of signalling molecules relevant to PAH. In this chapter, pulmonary arteries from Tph1$^{-/-}$ mice were examined for protein expression of BMPR2 and p-Smad1/5/8 and compared with WT so that the influence of 5-HT on these key regulators of vascular function could be assessed. An influence of 5-HT on BMPR2 expression may also be relevant to Dfen induced PAH. The Tph1$^{-/-}$ mouse has provided important data on the role of peripheral 5-HT in Dfen associated PAH and BMPR2 mutations are found in some Dfen users who develop PAH (Humbert et al., 2002). A discovery of altered BMPR2 expression between WT and Tph1$^{-/-}$ mice may indicate cross-talk between 5-HT and BMPR2 to

support the hypothesis that enhanced serotonergic function interacts with BMPR2 deficiency in PAH. The mitogenic mediator p-ERK1/2 is activated by 5-HT and Dfen (Lee et al., 1999; Lee et al., 2001) and by analysing expression of p-ERK1/2 in pulmonary arteries from Tph1$^{-/-}$ mice, it can be determined if 5-HT has a basal effect on maintaining p-ERK1/2 level. As described previously, Long et al., 2006 uncovered evidence of cross-talk between 5-HT and BMPR2 signalling in male PASMCs including 5-HT depressing BMP-2 induced phosphorylation of Smad1/5 and an increase in 5-HT induced ERK1/2 activation in BMPR2$^{+/-}$ PASMCs compared with WT PASMCs, an effect thought to mediate 5-HT induced proliferation of these cells. Although no significant differences were observed in this study between WT and Tph1$^{-/-}$ animals, there is a strong suggestion of an influence of 5-HT on BMPR2. Expression of BMPR2 in Tph1$^{-/-}$ pulmonary arteries was almost double the level seen in WT arteries although a large variation was seen in results within the Tph1$^{-/-}$ group. A large standard error was also associated with both p-Smad 1/5/8 results and those results suggest no notable differences in expression between groups. A trend towards decreased p-ERK1/2 expression was noted in Tph1$^{-/-}$ mice and this was visible on some of the blots. A decrease in p-ERK1/2 in Tph1$^{-/-}$ pulmonary arteries would not be surprising given it is activated by 5-HT. Activation of p-ERK1/2 may also participate in 5-HT induced negative regulation of the BMPR2 pathway. p-ERK1/2 is a negative regulator of Smad-1. It binds to a region of the Smad-1 protein which it phosphorylates causing a form of Smad-1 incapable of nuclear translocation (Massague, 2003). An increase in p-ERK1/2 induced by 5-HT would therefore suppress the p-Smad1/5/8 pathway. The BMPR2 receptor is expressed at a lower level in the lungs of PAH patients than genetics would predict. There was visibly more BMPR2 expression in pulmonary arteries from Tph1$^{-/-}$ mice compared with WT mice perhaps indicating peripheral 5-HT also acts to control expression of mature BMPR2 and that elevated 5-HT acting via the 5-HTT or 5-HT$_{2A}$ receptors suppresses BMPR2 and p-Smad1/5/8 in PAH

patients leading to a proliferative phenotype.

The discovery that Dfen requires peripheral 5-HT to exert its pulmonary hypertensive effect supports the evidence in favour of the 'serotonin hypothesis' of PAH. This is further supported by other recent data looking at peripheral 5-HT synthesis in pulmonary hypertension both in humans and in animals. The finding that hypoxia increases Tph1 expression in rabbit lung 3 fold (Pan et al., 2006) and hypoxic PAH is attenuated in $Tph1^{-/-}$ mice (Morecroft et al., 2007; Izikki et al., 2007) coupled with human data indicating TPH1 is upregulated in PASMCS in IPAH patients (Eddahibi et al., 2006) all favour a role for increased peripheral 5-HT in PAH.

Chapter 6

Investigation of pharmacological response to dexfenfluramine and nordexfenfluramine

6.1 Introduction

Dfen has previously been shown to be a pulmonary vasoconstrictor (Belohlavkova et al., 2001), an effect attributed to either 5-HT release, a direct action on 5-HT receptors or through inhibition of K^+ channels. Dfen is known to be a partial or full agonist at many different 5-HT receptors but shows only low affinity for the vasoconstrictor 5-HT_{1B} and 5-HT_{2A} receptors (Fitzgerald et al., 2000; Rothman et al., 2000). As determined in the previous chapter, Dfen is a poor vasoconstrictor in WT and $Tph1^{-/-}$ mice, inducing vasoconstriction only at concentrations >10 μM. To discover whether Dfen induced vasoconstriction may be increased by the genetic changes relevant to PAH, IPAs from WT, 5-HTT+, $BMPR2^{+/-}$ and Cross TG mice were all exposed to CCRCs to Dfen. Experiments were performed in IPAs from male and female mice so a gender comparison could be performed.

As already mentioned, Dfen undergoes metabolism to NDfen, a metabolite with twice the plasma half life of Dfen (Caccia et al., 1985). NDfen is thought to induce vasoconstriction through activation of 5-HT_{2A} receptors (Ni et al., 2005) and NDfen has a significantly higher affinity for this receptor than Dfen (Fitzgerald et al., 2000; Rothman et al., 2000). It is, therefore, important to also test the pulmonary vasoconstrictor response to this metabolite and this was performed in the same manner as for Dfen. There were, however, insufficient TG animals available for a gender comparison to NDfen or to use just one gender so both male and female mice were used in this experiment (see discussion for further information on gender comparison experiments).

6.2 Methods

6.2.1 Animals

All mice were age 5-6 months

6.2.2 Myography

IPAs were isolated from mice from each of the four genotypes and used for wire myography experiments as described previously.

Following attainment of maximal contractile response to KCl as outlined in section 2.6.1.5, vessels were left to equilibrate for 30 minutes before CCRCs were performed for Dfen or NDfen. All drugs were dissolved in H_2O to give a solution of 10^{-2} M from which serial dilutions were created up to 10^{-6} M giving the range necessary for a 1 nM- 300 μM CCRC to be constructed. Each vessel segment was subjected to only one treatment.

6.3 Results

Contractions in IPAs were measured relative to the contraction induced by 50 mM KCl. No significant differences were observed in KCl response between genotypes or gender (WT male: 3.7 ± 0.3 mN, n=6, WT female: 3.0 ± 0.3 mN, n=6, 5-HTT+ male: 3.4 ± 0.5 mN, n=6, 5-HTT+ female: 2.7 ± 0.3 mN, n=6, $BMPR2^{+/-}$ male: 3.4 ± 0.6 mN, n=6, $BMPR2^{+/-}$ female: 3.0 ± 0.2 mN, n=6, Cross TG male: 2.8 ± 0.3 mN, n=6, 3.0 ± 0.2 mN, n=6).

6.3.1 Dfen

CCRCs were performed for Dfen in male mice (figure 6.1) and female mice (figure 6.2). Results did not allow calculation of pEC50 or Emax so each concentration tested was statistically compared between groups. There were no significant differences between genotypes at any concentration tested for male mice (figure 6.1). In female mice, Dfen

exerted a significantly greater contraction in Cross TG mice at 1 μM (WT: 2.0 ± 1.2% of the contraction induced by 50 mM KCl, n=5, 5-HTT+: 3.7 ± 2.1%, n=7, $BMPR2^{+/-}$: 1.3 ± 0.9%, n=6, Cross TG: 33.5 ± 11.5%, n=4), 3 μM (WT: 2.8 ± 1.7%, n=5, 5-HTT+: 7.1 ± 5.0%, n=7, $BMPR2^{+/-}$: 1.8 ± 1.2%, n=4, Cross TG: 50.5 ± 11.1%, n=4), 10 μM (WT: 5.0 ± 1.9%, n=5, 5-HTT+: 9.0 ± 3.4%, n=7, $BMPR2^{+/-}$: 3.3 ± 1.6%, n=6, Cross TG: 48.8 ± 8.8%, n=4), and 30 μM (WT: 19.6 ± 9.9%, n=5, 5-HTT+: 9.7 ± 1.9%, n=7, $BMPR2^{+/-}$: 6.5 ± 3.4%, n=6, Cross TG: 35.8 ± 7.0%, n=6) compared to the other three genotypes (figure 6.2). Figures 6.3 and 6.4 show a male versus female comparison for each genotype. There were no differences between male and female WT mice at any concentration used (figure 6.3A). Dfen exerted a greater contraction in IPAs in male 5-HTT+ mice compared to female 5-HTT+ mice at 10 μM (male: 37.8 ± 10.6% of the contraction induced by 50 mM KCl, n=6, female: 9.0 ± 3.4%, n=7), 30 μM (male: 38.8 ± 8.8%, n=6, female: 9.7 ± 1.9%, n=7) and 100 μM (male: 31.8 ± 8.9%, n=6, female: 9.0 ± 3.4%, n=7) (figure 6.3B). The only notable difference between genders in $BMPR2^{+/-}$ mice was at 100 μM with male IPAs having the greatest contractile response (male: 45.0 ± 7.2%, n=4, female: 19.7 ± 6.6%, n=6) (figure 6.4A). In relation to the Cross TG mice, female IPAs were more sensitive to Dfen than male IPAs at 1 μM (male: 2.8 ± 1.7%, n=5, female: 33.5 ± 11.4%, n=4) and 3 μM (male: 13.2 ± 8.1%, n=5, female: 50.5 ± 11.1%, n=4) (figure 6.4B).

6.3.2 NDfen

CCRCs were performed for NDfen and the data is presented in figure 6.5. NDfen was significantly more potent in all TG mice compared to WT (pEC50; WT: 5.7 ± 0.1, n=6, 5-HTT+: 6.0 ± 0.1, n=6, $BMPR2^{+/-}$: 6.1 ± 0.0, n=6, Cross TG: 6.1 ± 0.0, n=6). $BMPR2^{+/-}$ mice displayed a higher Emax to NDfen compared with WT and Cross TG (WT: 82.2 ± 5.8% of the contraction induced by 50 mM KCl, n=6, $BMPR2^{+/-}$: 106.2 ± 2.4%, n=6, Cross TG: 86.8 ± 1.6%, n=6) groups.

Male

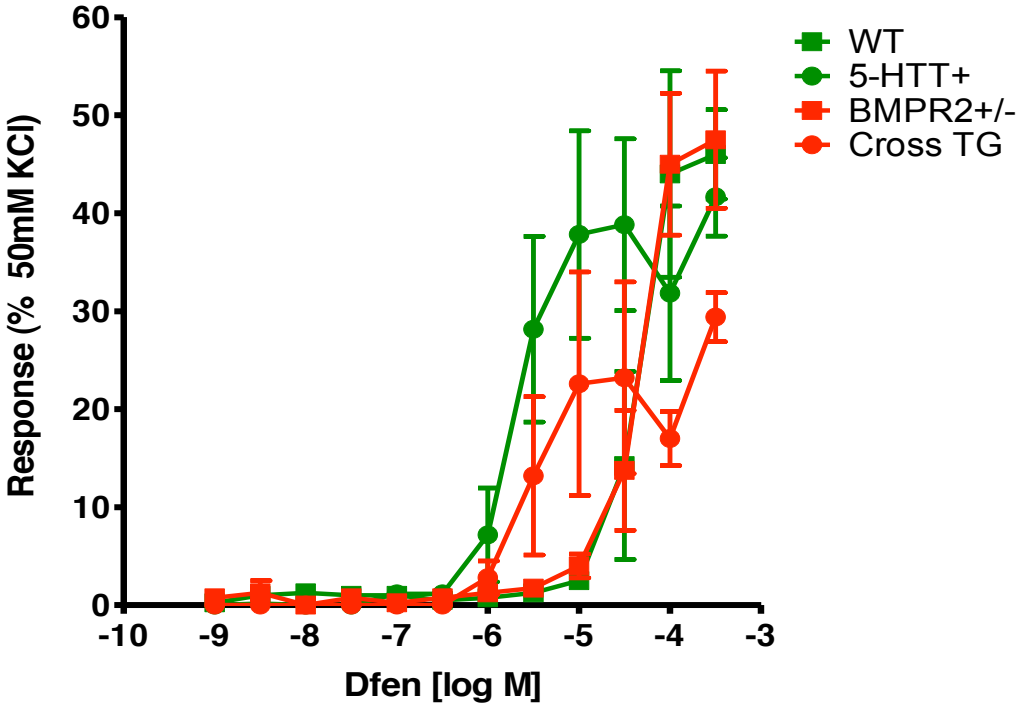


Figure 6.1 Cumulative concentration-response curves to Dfen in IPAs from WT, 5-HTT+, $BMPR2^{+/-}$ and Cross TG male mice (n = 4 to 6 mice per group). Results expressed as mean ± SEM. Newman-Keuls multiple comparison test revealed no significant differences between groups at any concentration tested.

Female

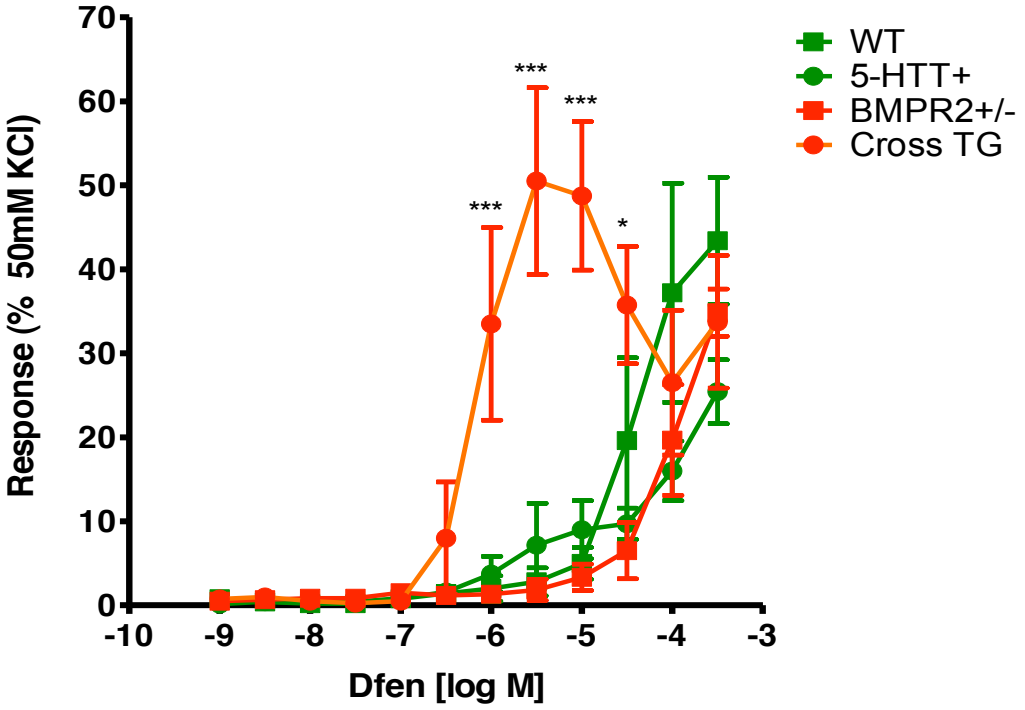
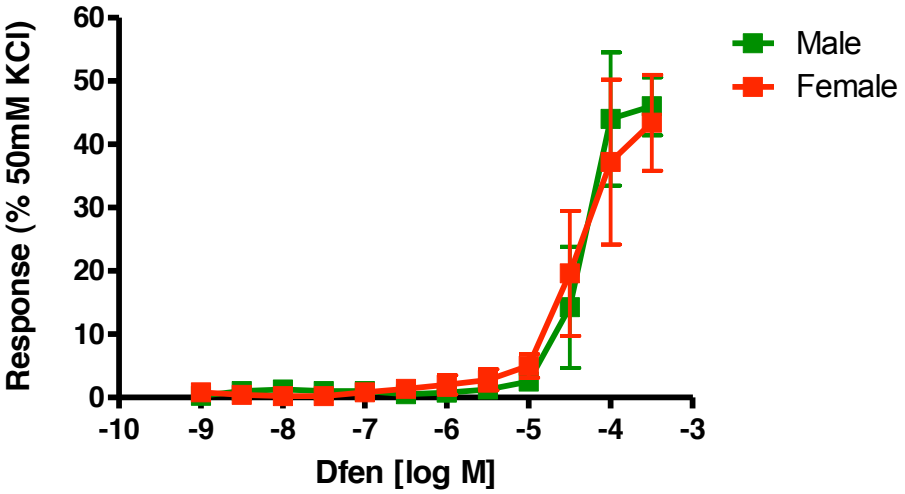


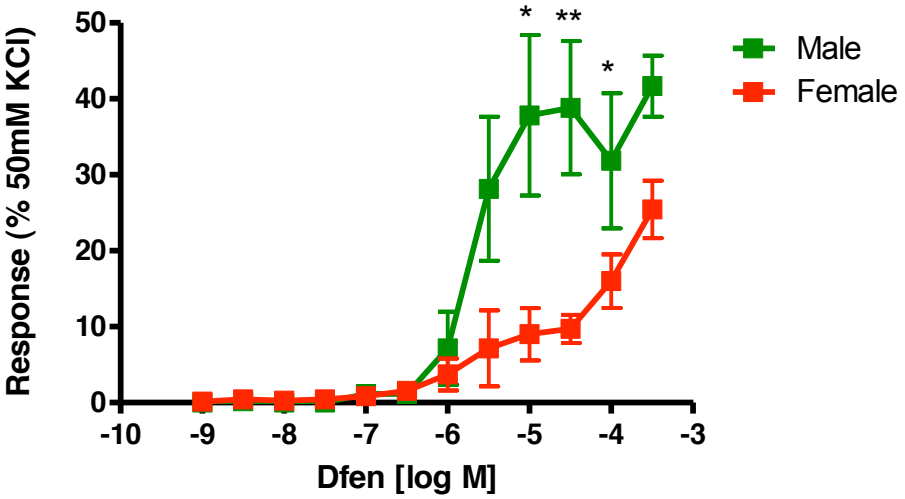
Figure 6.2 Cumulative concentration-response curves to Dfen in IPAs from WT, 5-HTT+, BMPR2$^{+/-}$ and Cross TG female mice (n = 4 to 7 mice per group). Results expressed as mean ± SEM. * $p<0.05$, *** $p<0.001$ versus corresponding concentration in WT, 5-HTT+ and BMPR2$^{+/-}$ mice using Newman-Keuls multiple comparison test.

WT



A

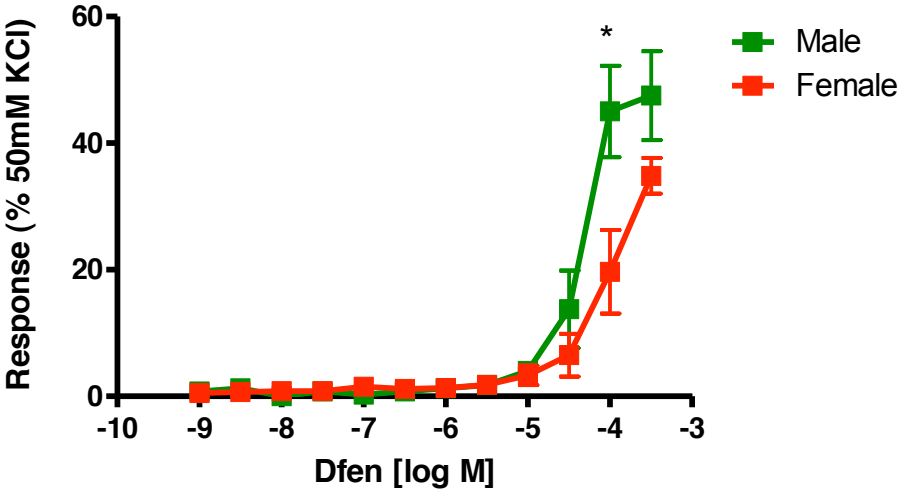
5-HTT+



B

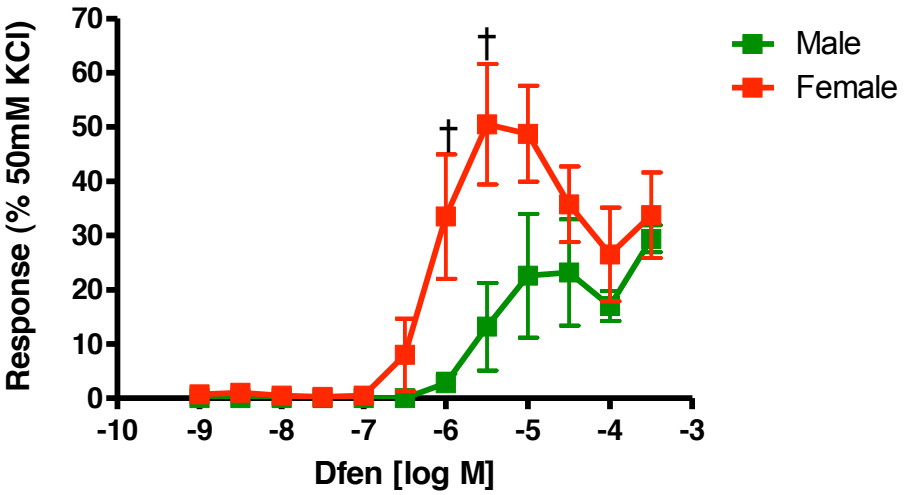
Figure 6.3 Comparison of male and female cumulative concentration-response curves to Dfen in IPAs from WT (A) and 5-HTT+ (B) mice (n = 4 to 7 mice per group). Results expressed as mean ± SEM. * $p<0.05$, ** $p<0.01$ versus corresponding concentration in female mice using an unpaired two-tailed t-test.

BMPR2$^{+/-}$



A

Cross TG



B

Figure 6.4 Comparison of male and female cumulative concentration-response curves to Dfen in IPAs from BMPR2$^{+/-}$ (A) and Cross TG (B) mice (n = 4 to 6 mice per group). Results expressed as mean ± SEM. * $p<0.05$ versus corresponding concentration in female mice, † $p<0.05$ versus corresponding concentration in male mice using an unpaired two-tailed t-test.

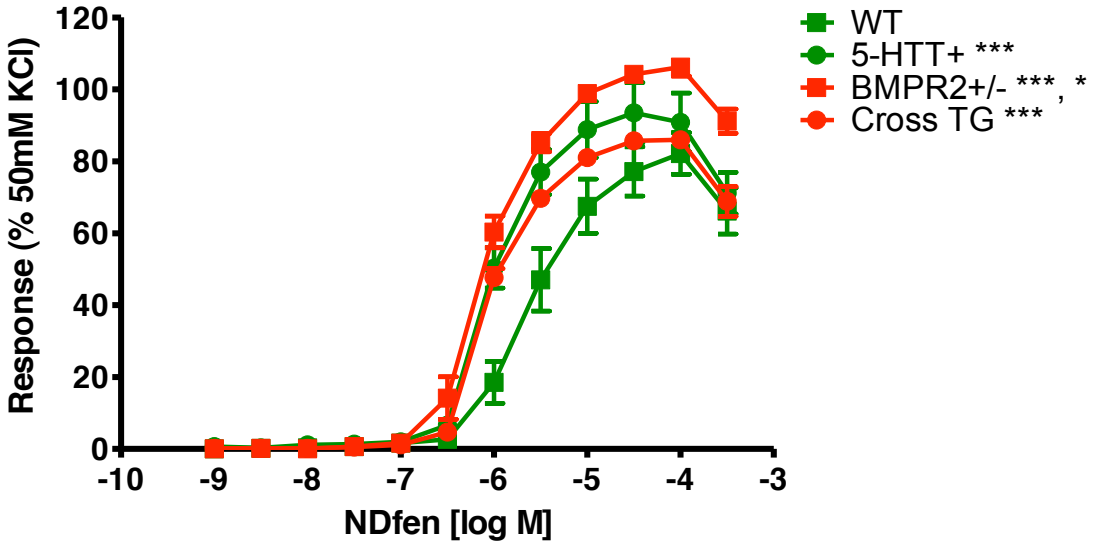


Figure 6.5 Cumulative concentration-response curves to NDfen in IPAs from WT, 5-HTT+, BMPR2$^{+/-}$ and Cross TG male/female mice (n = 6 mice per group). Results expressed as mean ± SEM. pEC50: *** $p<0.001$ versus WT, Emax: * $p<0.05$ versus WT and Cross TG using Newman-Keuls multiple comparison test.

6.4 Discussion

Several interesting observations emerged from this series of experiments involving the role of gender and how the genetics of the mouse influenced which gender was more sensitive to Dfen. Perhaps the most interesting and important results came in the Cross TG mice where females were more sensitive to Dfen induced vasoconstriction than males. In the female Cross TG mice, Dfen induced vasoconstriction at a lower concentration than any other group (>30% of KCl at 1 μM). The maximum vasoconstriction recorded in the Cross TG females was over 50% of KCl response measured at 3μM Dfen. This was 40% higher than in males at the same concentration and the highest recorded contractile response to Dfen measured across all experiments. Results in the Cross TG mice were in contrast to results in the 5-HTT+ mice where Dfen induced greater vasoconstriction in male mice at several concentrations tested compared with females. There was a slight male gender influence on Dfen in the $BMPR2^{+/-}$ mice also with a greater response to Dfen at 100 μM. No differences, however, were noted in Dfen response between WT male and females where the response was similar to that observed in the 2-3 month old WT females in chapter 5.

The 5-HTT, unsurprisingly, is a major determinant in these experiments to the response of Dfen. Although no significant differences were noted between genotypes in the male group due to the large variability in Dfen response and low n numbers there is a clear trend towards enhanced vasoconstriction to Dfen in the 5-HTT+ and Cross TG mice compared with WT and $BMPR2^{+/-}$ mice at lower concentrations. Interestingly, the 5-HTT+ females were unremarkable in their response to Dfen compared with WT. It was only when dysfunctional BMPR2 was combined with overexpressed 5-HTT that the enhanced sensitivity to Dfen emerged. These results suggest that in males, dysfunctional BMPR2 may protect against the 5-HTT mediated enhanced response to Dfen whereas in females dysfunctional BMPR2 enhances the Dfen response. The result in the Cross TG mice is

particularly interesting as significant vasoconstriction occurred to Dfen at clinically relevant plasma concentrations. The optimum plasma concentration for Dfen to induce weight loss is ~ 1 μM. Cross TG females were the only group to show significant vasoconstriction at this concentration. Assuming human pulmonary arteries were to respond to Dfen in a similar way, then females with a BMPR2 mutation and overexpression of the 5-HTT would have pulmonary vasoconstriction following administration of Dfen. Dfen induced a biphasic CCRC in mice overexpressing the 5-HTT, an effect most likely related to multiple sites of action. As this biphasic response was only observed in mice overexpressing the 5-HTT, a heightened interaction between Dfen and the 5-HTT would seem to cause this effect. A combination of a 5-HTT inhibitor and 5-HT receptor antagonists would be useful in the presence of Dfen to better understand the pharmacology of Dfen induced vasoconstriction. One hypothesis for the biphasic response is that the initial enhanced vasoconstriction observed in 5-HTT+ mice is due to either an intracellular effect of Dfen on vasoconstrictor pathways or an action on Kv channels that are perhaps already dysfunctional in 5-HTT+ mice. The secondary vasoconstriction could be the result of a direct effect of Dfen on vasoconstrictor 5-HT receptors that is only attainable at high concentrations.

It was during the course of the Dfen pharmacology experiments in this chapter that clear differences emerged in results obtained in male and female mice. These were the first of many experiments outlined in this thesis to look at the influence of gender as a modifying factor. The availability of TG animals meant that direct gender analysis experiments could not be performed for every experiment and priority was given to the *in vivo* experiments in preceding chapters. For this reason, a gender comparison was not performed for NDfen in these sets of experiments and a mixture of male and female mice, determined by availability of animals, was used. A direct male versus female comparison for NDfen in WT mice can be found in the set of experiments looking at the influence of sex hormones

on pulmonary vasoconstriction in chapter 8.

The potency of NDfen was greater in all TG groups with Emax also increased in $BMPR2^{+/-}$ mice. Interestingly, the Emax of NDfen in $BMPR2^{+/-}$ mice was significantly greater than Cross TG mice. As discussed in the preceding chapter, NDfen induced vasoconstriction is thought to occur via the 5-HT_{2A} receptor (Ni et al., 2005). Ni et al., 2005 showed that in isolated aorta, NDfen induces vasoconstriction via 5-HT_{2A} receptors and that vasoconstriction was independent of any 5-HTT activity. The increase in Emax in $BMPR2^{+/-}$ mice may indicate an increase in 5-HT_{2A} receptor number which supports pharmacological experiments on IPAs from $BMPR2^{+/-}$ mice conducted by Long et al., 2006 that demonstrated an increased contractile response to the 5-HT_{2A} agonist, α–methly-5-HT, compared to WT mice. Given the important role 5-HT_{2A} receptors have in vasoconstriction and proliferation of PASMCs, any increase in activity or receptor number in pulmonary arteries would enhance serotonergic signalling. Despite NDfen induced aortic vasoconstriction being independent of 5-HTT activity, this does not necessarily apply to pulmonary arteries and a direct intracellular action of NDfen on serotonergic vasoconstrictor pathways or Kv channels may also contribute to the enhanced vasoconstriction observed in the TG animals.

The most important finding in this chapter was the genetic synergy between overexpression of the 5-HTT and $BMPR2^{+/-}$ that increased the contractile response to Dfen in Cross TG mice. This effect only occurred in female mice supporting female gender as a risk factor for Dfen induced vasoconstriction when combined with genetic pre-disposition. The other key finding was that all TG groups demonstrated enhanced vasoconstriction to NDfen, possibly indicating enhanced 5-HT_{2A} receptor activity in IPAs from these mice.

Chapter 7

Investigation of pharmacological response to 5-HT, BMP-2 and linopirdine

7.1 Introduction

5-HT acting via 5-HT_{2A} and/or 5-$HT_{1B/1D}$ receptors is a pulmonary vasoconstrictor, however, the plasma concentration of 5-HT that would be required to induce vasoconstriction is higher than physiological 5-HT plasma concentrations (Zolkowska et al., 2006). As described in section 1.2.3.1.2, it is still a matter of debate whether the plasma concentration of 5-HT is a good indicator of any potential pathological role for 5-HT in the disease progression. Moreover, plasma [5-HT] may not reflect local 5-HT concentrations in the lung with PAECs synthesising and releasing 5-HT locally at a rate which increases in IPAH (Eddahibi et al., 2006). It may also be the case that other factors act to enhance 5-HT induced vasoconstriction in PAH. Important support for this theory comes from the finding that pulmonary arteries from $BMPR2^{+/-}$ mice show a greater maximum contractile response to 5-HT than corresponding vessels from WT mice (Long et al., 2006). Dysfunctional BMPR2 may, therefore, enhance 5-HT receptors or signalling molecules associated with vasoconstriction. In this chapter, the contractile response to 5-HT and possible influencing factors upon that vasoconstriction are investigated. In the first part of this study, the contractile response to 5-HT was assessed in WT, 5-HTT+, $BMPR2^{+/-}$ and Cross TG mice. To further elucidate any interaction that may exist between BMPR2 and 5-HT induced vasoconstriction, the effect of BMP-2 on the contractile response to 5-HT was assessed in the four genotypes.

As outlined in the introduction Kv channel currents on PASMCs regulate vasoconstriction in addition to proliferation and apoptosis. While members of the Kv1-6, Kv8-9 group such as Kv1.5 and Kv2.1 have been studied most extensively in relation to PASMC K^+ current and PAH, the Kv7.1-7.5 (KCNQ) group have recently been found to contribute to the resting V_m in PASMC and inhibition of these channels results in pulmonary vasoconstriction (Joshi et al., 2006). Changes in expression of the 5-HTT and BMPR2 can directly influence Kv channel subunit expression. Both Kv1.5 and Kv2.1 are decreased in

the lungs of mice that overexpress 5-HTT in smooth muscle (Guignabert et al., 2006) and mice that lack BMPR2 in smooth muscle have decreased expression of Kv1.1, Kv1.5 and Kv4.3 (Young et al., 2006). It is not known if KCNQ channel current is affected by genetic changes in the 5-HTT or BMPR2. To help determine this, CCRCs were performed to the KCNQ channel blocker linopirdine in WT, 5-HTT+, $BMPR2^{+/-}$ and Cross TG mice to look for any differences in KCNQ mediated vasoconstriction between genotypes.

Activation of 5-HT vasoconstrictor receptors results in G protein mediated changes in ion channel function (Varghese et al., 2006) but the role of Kv channels in 5-HT induced vasoconstriction remains unclear. To assess the role of KCNQ channels in 5-HT induced vasoconstriction, CCRCs to 5-HT were performed in WT, 5-HTT+, $BMPR2^{+/-}$ and Cross TG mice in the presence and absence of linopirdine.

7.2 Methods

7.2.1 Animals

All mice were age 5-6 months. As mentioned in chapter 6, the restricted number of TG animals meant that groups containing both male and female mice had to be used in the conduct of the following experiments.

7.2.2 Myography

IPAs were isolated from mice from each of the four genotypes and used for wire myography experiments as described previously.

Following attainment of maximal contractile response to KCl as outlined in section 2.6.1.5, vessels were left to equilibrate for 30 minutes. For BMP-2 (R & D Sytsems, Abingdon, UK) experiments, BMP-2 (2.34×10^{-6} M) or H_2O vehicle was added following equilibration and left for 3 hours before CCRCs to 5-HT were performed. To assess the contractile response to linopirdine (Tocris Bioscience, Bristol, UK) CCRCs were

performed for linopirdine. To determine the effect of linopirdine on 5-HT induced vasoconstriction, linopirdine (10^{-7} M) or H_2O vehicle was added to vessels following equilibration and left for 15 minutes before CCRCs to 5-HT were performed. For 5-HT and linopirdine CCRCs, drugs were dissolved in H_2O to give a solution of 10^{-2} M from which serial dilutions were created up to 10^{-6} M giving the range necessary for a 1 nM- 300 μM CCRC to be constructed. Each vessel segment was subjected to only one treatment.

7.3 Results

Contractions in IPAs were measured relative to the contraction induced by 50 mM KCl. As stated in section 6.3, no significant differences were observed in KCl response between 5-6 month WT, 5-HTT+, $BMPR2^{+/-}$ and Cross TG mice or between genders.

7.3.1 Effect of 2.34 x 10^{-6} M BMP-2 on CCRC to 5-HT

CCRCs to 5-HT were performed in WT and TG mice following BMP-2 (2.34 x 10^{-6} M) or vehicle (figure 7.1). Both groups of mice overexpressing the 5-HTT+ had lower pEC50 values for 5-HT than mice with normal 5-HTT expression. This was true for both vehicle treated animals (WT: 7.3 ± 0.1, n=6, 5-HTT+: 6.0 ± 0.1, n=6, $BMPR2^{+/-}$: 7.6 ± 0.1, n=6, Cross TG: 6.0 ± 0.1, n=6) and for BMP-2 treated animals (WT: 7.2 ± 0.1, n=6, 5-HTT+: 6.0 ± 0.1, n=5, $BMPR2^{+/-}$: 7.2 ± 0.3, n=6, Cross TG: 6.0 ± 0.1, n=6). The presence of the BMPR2 mutation did not affect the potency of 5-HT in either $BMPR2^{+/-}$ mice or in Cross TG mice. Only the potency of 5-HT was affected by overexpression of the 5-HTT with Emax values not significantly different between groups. The *Hill coefficient* differed significantly ($p<0.05$) between the BMP-2 treated $BMPR2^{+/-}$ group and the BMP-2 treated Cross TG group ($BMPR2^{+/-}$: 0.5 ± 0.2, n=6, Cross TG: 1.2 ± 0.2, n=6). BMP-2 did not alter the pEC50 or maximal response of 5-HT in any of the four genotypes (figure 7.1).

7.3.2 CCRC to linopirdine

CCRCs to linopirdine are presented in figure 7.2. CCRCs did not follow a classical sigmoidal shape and the Hill equation was not applied to the data. pEC50 and Emax values, therefore, were not determined so each concentration tested was statistically compared between groups. Linopirdine induced vasoconstriction was greater in WT mice compared with all three TG groups. At 300 nM, linopirdine induced vasoconstriction in WT mice was significantly greater than in $BMPR2^{+/-}$ mice (WT: 12.7 ± 6.5% of the contraction induced by 50 mM KCl, n=6, $BMPR2^{+/-}$: 0.75 ± 0.3%, n=8). The contractile response was greater in WT mice at 1 μM (WT: 51.0 ± 14.9%, n=6, 5-HTT+: 1.5 ± 0.5%, n=6, $BMPR2^{+/-}$: 0.75 ± 0.31%, n=8, Cross TG: 1.7 ± 1.3%, n=6), 3 μM (WT: 63.2 ± 17.0%, n=6, 5-HTT+: 7.7 ± 5.5%, n=6, $BMPR2^{+/-}$: 9.4 ± 5.9%, n=8, Cross TG: 15.2 ± 9.6%, n=6), 10 μM (WT: 75.5 ± 13.3%, n=6, 5-HTT+: 21.5 ± 10.5%, n=6, $BMPR2^{+/-}$: 20.4 ± 8.9%, n=8, Cross TG: 29.2 ± 17.7%, n=6) and 30 μM (WT: 77.3 ± 13.7%, n=6, 5-HTT+: 33.8 ± 9.1%, n=6, $BMPR2^{+/-}$: 32.3 ± 5.5%, n=8, Cross TG: 43.0 ± 16.1%, n=6) compared with the three TG groups. Vasoconstriction to linopirdine was also greater in WT mice compared with $BMPR2^{+/-}$ mice at the highest concentrations of 100 μM (WT: 69.8 ± 12.3%, n=6, $BMPR2^{+/-}$: 20.4 ± 4.6%, n=8) and 300 μM (WT: 33.2 ± 5.1%, n=6, $BMPR2^{+/-}$: 6.6 ± 2.2%, n=8).

7.3.3 Effect of 10^{-7}M linopirdine on CCRC to 5-HT

CCRCs to 5-HT were performed in WT and TG mice following linopirdine (10^{-7}M) or vehicle (figure 7.3). The potency of 5-HT was greater in $BMPR2^{+/-}$ mice compared with the other three groups. This was true for vehicle treated animals (pEC50; WT: 6.6 ± 0.1, n=6, 5-HTT+: 5.6 ± 0.1, n=6, $BMPR2^{+/-}$: 7.3 ± 0.0, n=6, Cross TG: 5.7 ± 0.0, n=6) and for linopirdine treated animals (pEC50; WT: 6.7 ± 0.1, n=6, 5-HTT+: 6.1 ± 0.0, n=6, $BMPR2^{+/-}$: 7.7 ± 0.3, n=6, Cross TG: 5.8 ± 0.1, n=6). There were also significant

differences in pEC50 values between WT and 5-HTT+ and between WT and Cross TG mice, with 5-HT being more potent in WT mice. This was independent of linopirdine treatment in Cross TG mice but only in the vehicle treated 5-HTT+ animals was 5-HT less potent than the corresponding WT group. No differences were observed between 5-HTT+ and Cross TG animals. The maximum response to 5-HT also differed between groups. Emax values for 5-HT were higher in $BMPR2^{+/-}$ vehicle treated animals compared with WT and 5-HTT+ vehicle treated animals (WT: 95.3 ± 8.0% of the contraction induced by 50 mM KCl, n=6, 5-HTT+: 97.3 ± 4.5%, n=6, $BMPR2^{+/-}$: 117.3 ± 4.6%, n=6). In the linopirdine treated groups, the only significant difference observed in Emax values was between 5-HTT+ and $BMPR2^{+/-}$ mice with $BMPR2^{+/-}$ mice having the higher Emax (5-HTT+: 97.7 ± 4.9%, n=6, $BMPR2^{+/-}$: 120.2 ± 4.7%, n=6). A significant difference in the *Hill coefficient* ($p<0.01$) was observed between $BMPR2^{+/-}$ vehicle treated mice and Cross TG vehicle treated mice ($BMPR2^{+/-}$: 0.6 ± 0.2, n=6, Cross TG: 1.5 ± 0.2, n=6). Linopirdine did not significantly affect either pEC50 or the Emax of 5-HT in any of the four groups.

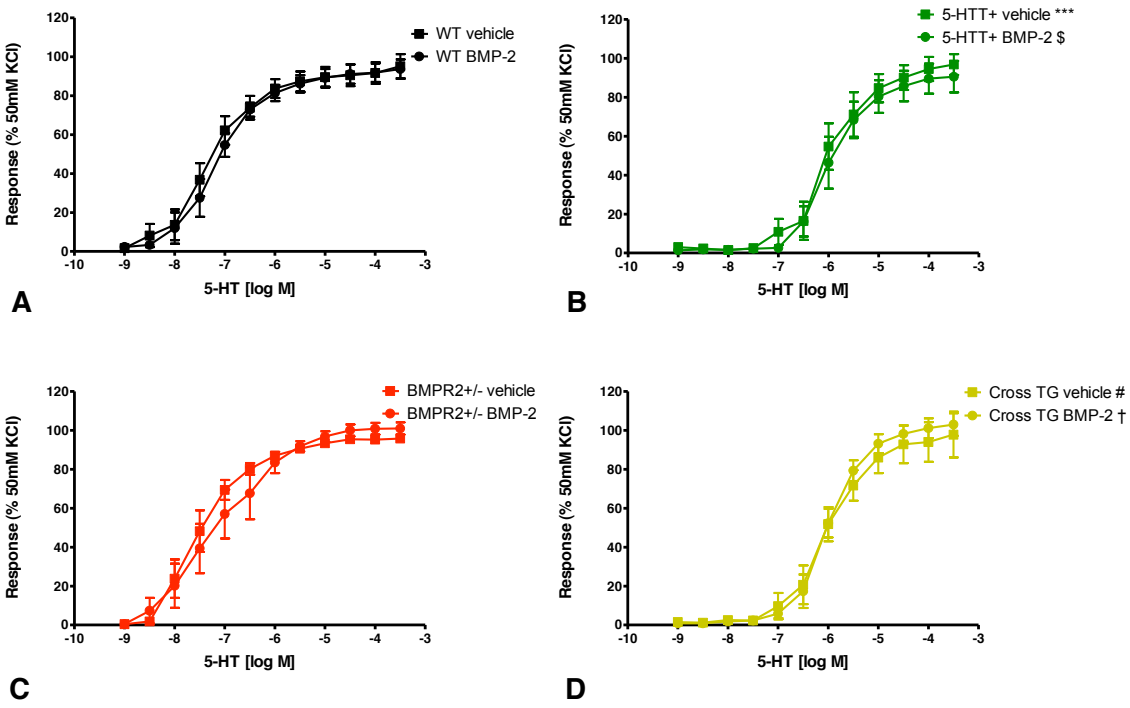


Figure 7.1 Cumulative concentration-response curves to 5-HT following BMP-2 (2.34 x 10^{-6}M) or vehicle in IPAs from WT (A), 5-HTT+ (B), BMPR2$^{+/-}$ (C) and Cross TG (D) mice (n= 5 to 6 mice per group). Results expressed as mean ± SEM. pEC50: *** $p<0.001$ versus WT vehicle and BMPR2$^{+/-}$ vehicle, $ $p<0.001$ versus WT BMP-2 and BMPR2$^{+/-}$ BMP-2, # $p<.0001$ versus WT vehicle and BMPR2$^{+/-}$ vehicle, † $p<0.001$ versus WT BMP-2 and BMPR2$^{+/-}$ BMP2 using Bonferroni multiple comparison test.

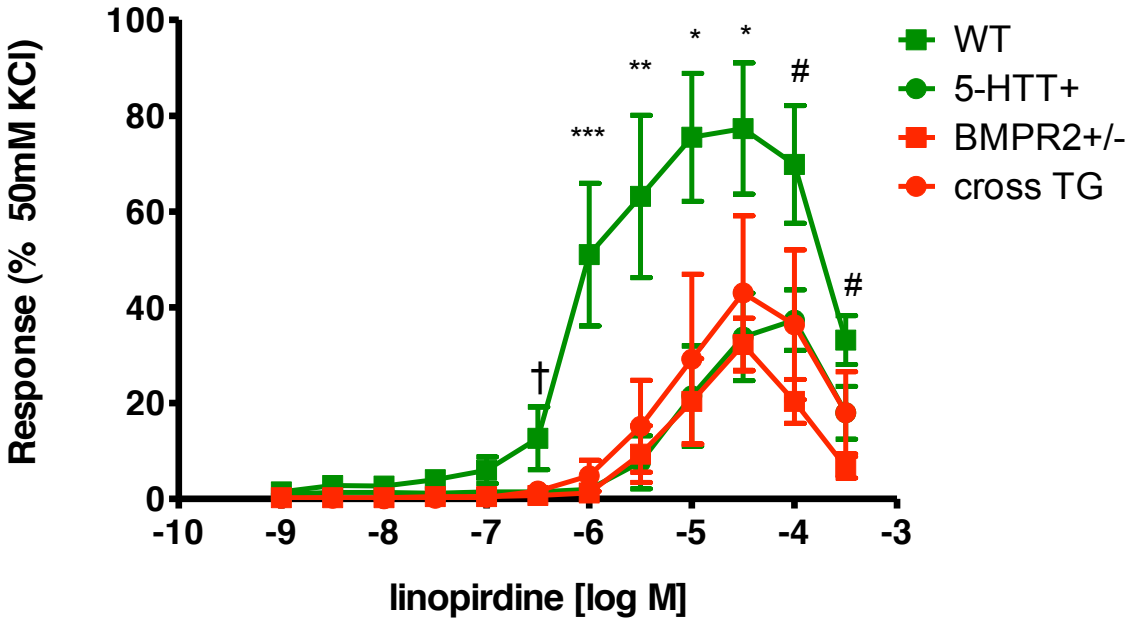


Figure 7.2 Cumulative concentration-response curves to linopirdine in IPAs from WT, 5-HTT+, BMPR2$^{+/-}$ and Cross TG mice (n = 6 to 8 mice per group). Results expressed as mean ± SEM. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ versus corresponding concentration in 5-HTT+, BMPR2$^{+/-}$ and Cross TG mice. † $p<0.05$, # $p<0.01$ versus corresponding concentration in BMPR2$^{+/-}$ mice using Newman-Keuls multiple comparison test.

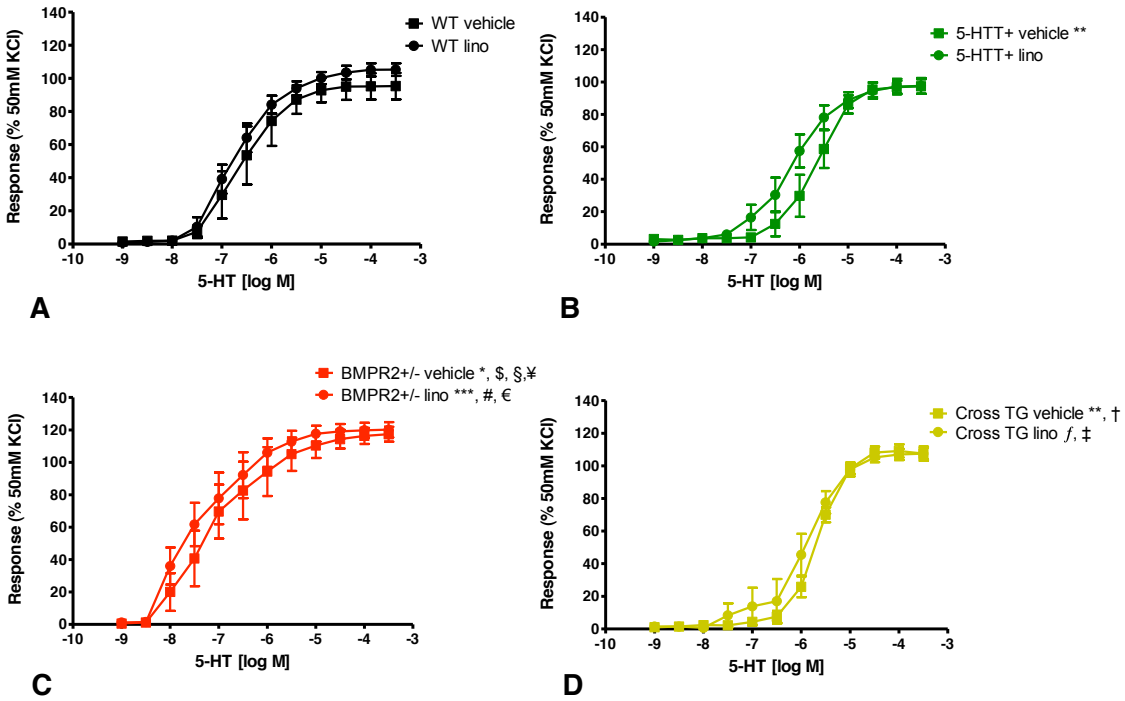


Figure 7.3 Cumulative concentration-response curves to 5-HT following linopirdine (10^{-7}M) or vehicle in IPAs from WT (A), 5-HTT+ (B), BMPR2$^{+/-}$ (C) and Cross TG (D) mice (n= 6 mice per group). Results expressed as mean ± SEM. pEC50: * $p<0.05$, ** $p<0.01$ versus WT vehicle, *ƒ* $p<0.01$, *** $p<0.001$ versus WT lino, $, $p<0.001$ versus 5-HTT+ vehicle, # $p<0.001$ versus 5-HTT+ lino, † $p<0.001$ versus BMPR2$^{+/-}$ vehicle, ‡ $p<0.001$ versus BMPR2$^{+/-}$ lino. Emax: § $p<0.01$ versus WT vehicle, ¥ $p<0.05$ versus 5-HTT+ vehicle, € $p<0.01$ versus 5-HTT+ lino using Bonferroni multiple comparison test.

7.4 Discussion

Overexpression of the 5-HTT had a dramatic effect on 5-HT induced vasoconstriction in both 5-HTT+ and Cross TG mice. The decreased potency of 5-HT in mice overexpressing the 5-HTT can be reversed by the 5-HTT inhibitor citalopram (Dempsie et al., 2008) indicating the decreased potency of 5-HT is due to overexpression of the 5-HTT. The most likely explanation for this effect is a greater uptake of 5-HT into the cell removing 5-HT available for activation of 5-HT_{2A} and 5-HT_{1B} receptors. Interestingly, the pEC50 and Emax of 5-HT was increased in $BMPR2^{+/-}$ IPAs compared with WT mice. This observation, however, only occurred in the set of experiments involving linopirdine (figure 7.3) and not in the experiments involving BMP-2 (figure 7.1). The ratio of male to female mice used for the WT and $BMPR2^{+/-}$ analysis was similar between the two sets of experiments so gender difference can be ruled out as a mitigating factor for the discrepancy. An inspection of figures 7.1 and 7.3 reveals a similar response to 5-HT in the $BMPR2^{+/-}$ mice in both the linopirdine and BMP-2 experiments and that it is, in fact, the response to 5-HT in WT mice that differs between experiments with a greater contractile response to 5-HT in the BMP-2 control experiments. The reason for this discrepancy is not known. The only difference in experimental protocol was a longer period of time during which the vessel was in the Krebs solution on the myograph in the BMP-2 experiments (owing to the longer incubation of BMP-2 and consequently H_2O vehicle). This is unlikely, however, to make a significant difference to 5-HT induced vasoconstriction, especially in WT but not in $BMPR2^{+/-}$ animals. The result is most likely due to batch differences in the WT mice due to natural variations in pulmonary artery phenotype within a population. The enhanced vasoconstrictor effect of 5-HT in $BMPR2^{+/-}$ IPAs confirm the results obtained by Long et al., 2006 who found an increased maximum response to 5-HT and 5-HT_{2A} agonist α–methly-5-HT in IPAs from $BMPR2^{+/-}$ mice. Long et al., 2006 did not find an increase in potency to 5-HT or α–methly-5-HT but did see a leftward shift in the CCRC to

the 5-HT_1 agonist 5-CT in $BMPR2^{+/-}$ mice. Both the pEC50 and Emax of 5-HT were increased in IPAs from $BMPR2^{+/-}$ vessels in this investigation and likely reflects an involvement of both 5-HT_{2A} and 5-HT_{1B} receptors and blockade of either of these receptors attenuates the 5-HT response in $BMPR2^{+/-}$ IPAs (Long et al., 2006). A change in the Hill coefficient was observed between $BMPR2^{+/-}$ and Cross TG mice. The *Hill coefficient* is a measure of the nature of ligand binding to receptor(s) complexes. The change in *Hill coefficient* indicates different binding characteristics for 5-HT between the two genotypes, perhaps reflecting a change in the respective utilisation of 5-HT_{1B} and 5-HT_{2A} receptors or a change in receptor activation following 5-HT binding, which may be secondary to overexpression of the 5-HTT.

Linopirdine, a selective KCNQ channel inhibitor induced vasoconstriction in WT mice reaching a maximum contraction at 30 μM before inducing vasorelaxation at higher concentrations. Vasoconstriction to linopirdine was significantly less in all TG groups at 1 – 30 μM with additional differences noted between WT and $BMPR2^{+/-}$ groups at 300 nM, 100 μM and 300 μM. No significant differences were noted between TG groups. The vasoconstriction induced by linopirdine in WT mice is independent of endothelial and neuronal derived factors (Joshi et al., 2006) and is via a direct action on KCNQ channels on PASMCs, an effect attributable to membrane depolarisation and voltage-gated Ca^{2+} entry (Joshi et al., 2009). The effect of linopirdine on systemic vessels appears to be significantly less although results have been inconsistent in studies to date. Linopirdine and XE991 induced vasoconstriction was found to be minimal or completely absent in systemic vessels such as carotid and mesenteric arteries in studies by Joshi et al., 2006 and Joshi et al., 2009. This data agrees with results by Yeung et al., 2007 who found that XE991 induced minimal contraction in femoral and carotid arteries and no contraction in mesenteric arteries in the absence of pre-tone. Interestingly, in the presence of pre-tone XE991 did induce contraction of mesenteric arteries (Yeung et al., 2007). Contradictory

results were demonstrated by Mackie et al., 2008 who found that linopirdine did induce vasoconstriction in mesenteric arteries on a pressure myograph. The maximum vasoconstriction induced by linopirdine in rat mesenteric arteries was ~ 35% of KCl response (Mackie et al., 2008) opposed to the near 100% of KCl response induced by linopirdine in rat pulmonary arteries (Joshi et al., 2009).

The enhanced vasoconstriction to linopirdine in pulmonary arteries over systemic arteries may be may be attributable to the KCNQ4 subunit which is expressed at a higher level than other KCNQ channel subunits in pulmonary arteries (Joshi et al., 2009) and is expressed more than 10 fold higher in pulmonary arteries compared with mesenteric arteries (Joshi et al., 2009). The results by Joshi et al., 2006 and confirmed here indicate that KCNQ channels are open at rest and are a major contributor to the resting membrane potential in murine PASMCs.

One possibility for the decreased response to linopirdine in TG mice is that PASMCs in these animals are hyperpolarised relative to WT PASMCs and therefore a greater number of KCNQ channels must be blocked to depolarise the cell membrane to a level sufficient to induce activation of VDCC. Alternatively, KCNQ channel subunit expression or activity may be lower in the TG animals compared with WT reducing the effect of linopirdine. This may or may not be associated with electrophysiological changes. BMP-2 increases whole-cell K^+ current in human PASMCs and divergently regulates expression of many Kv subunits, altering expression of certain subunits >10 fold (Young et al., 2006; Fantozzi et al., 2006). Loss of BMPR2 may result in a decrease in whole-cell K^+ current and changes in KCNQ channel expression. The increase in whole-cell K^+ current induced by BMP-2 would result in a shift to an apoptotic, anti-mitogenic and pro-relaxant cellular phenotype and thus, loss of BMPR2 would have the opposite effects. The molecular mechanism by which BMPR2 interacts with Kv channel subunits is unknown but may involve the presence or absence of SMAD and c-myc binding sites in the promoter regions of Kv

channel genes. This could account for the divergent effects BMP-2 has on various Kv channel genes (Fantozzi et al., 2006). Overexpression of the 5-HTT+ was sufficient to reduce linopirdine induced vasoconstriction at 1 - 30 μM compared with WT. Overexpression of the 5-HTT in smooth muscle is sufficient to reduce Kv1.5 and Kv2.1 in the lungs of mice (Guignabert et al., 2006) and thus it is conceivable that other Kv channel subunits such as KCNQ may also be downregulated by 5-HTT overexpression. Results in the Cross TG mice were similar to both 5-HTT+ and $BMPR2^{+/-}$ groups indicating KCNQ channel function/expression is not additively altered by 5-HTT overexpression and BMPR2 downregulation.

The evidence in this thesis that linopirdine induces vasoconstriction of mouse IPAs supports previous work indicating that linopirdine not only contracts isolated pulmonary arteries but also increases PAP *in vivo* (Joshi et al., 2009). Moreover, the discovery that KCNQ channel activators can induce relaxation of pulmonary arteries (Joshi et al., 2009) provides further evidence that KCNQ channels are involved in regulating pulmonary artery tone. Due to this important role for KCNQ channels in the pulmonary circulation, experiments were performed to assess how linopirdine would affect the vasoconstrictor response to 5-HT in WT and TG mice. Linopirdine did not alter the 5-HT response in any group which would indicate that 5-HT induced vasoconstriction is not dependent on the state of KCNQ channels. The relative contribution of voltage-dependent and voltage-independent pathways that mediate 5-HT induced vasoconstriction is not known in detail and conflicting evidence in this area of research has emerged (Varghese et al., 2006). For example, 5-HT inhibits Kv current and depolarises the cell membrane in rat PASMCs and Ltk^- cells expressing cloned human Kv1.5, effects attenuated by several compounds including ketanserin and PKC inhibitors (Cogolludo et al., 2006). Moreover, 5-HT induced vasoconstriction of rat pulmonary arteries is also inhibited by ketanserin and PKC inhibitors in addition to L-type Ca^{2+} channel blockers (Cogolludo et al., 2006). 5-HT partly

induces vasoconstriction of canine pulmonary arteries through L-type Ca^{2+} channel activation, an effect also mediated by 5-HT_{2A} receptors (Wilson et al., 2005). In contrast to these studies, Guibert et al., 2004 found that 5-HT_{2A} mediated vasoconstriction of rat pulmonary arteries was independent of voltage-gated Ca^{2+} channels. Factors other than inhibition of Kv current could account for 5-HT induced vasoconstriction in the murine IPAs studied in this investigation such as Ca^{2+} release from intracellular stores and Ca^{2+} sensitisation via ROCK (Rodat-Despoix et al., 2008). The vasoconstrictor pathways activated by 5-HT may also depend on the relative expression of 5-HT_{1B} and 5-HT_{2A} receptors. 5-HT induced vasoconstriction in rat pulmonary arteries can be inhibited by 5-HT_{1B} and 5-HT_{2A} antagonists but 5-HT induced inhibition of Kv currents is only prevented by 5-HT_{2A} antagonists (Cogolluda et al., 2006). It maybe that Kv channels are more important in 5-HT induced vasoconstriction in arteries with a higher concentration of 5-HT_{2A} receptors although this may be species specific.

Chapter 8

Gender as a modifying factor in PAH

8.1 Introduction

The increased risk of PAH in women of child bearing age suggests that circulating female sex hormones may underlie the increased risk. Experiments utilising both the MCT and hypoxic models, however, have generally shown that female mice are more resistant to developing PAH than males and that female sex hormones attenuate the development of both hypoxic and MCT PAH through vasodilator and anti-mitogenic effects.

The effect of sex hormones on vascular tone appears dependent on species, gender, vascular origin of artery and whether the artery has intact endothelium. Estradiol relaxes isolated perfused rat tail arteries pre-contracted with noradrenaline in female rats but not in males and this effect is dependent on an intact endothelium (McNeill et al., 1996). This may indicate that estradiol modulates adrenergic effects *in vivo* in female rats. Estradiol is also a vasodilator of human coronary arteries where it also acts via an endothelium dependent process (Gilligan et al., 1994). The endothelium dependent effect of estrogens is thought to occur either through the rapid release of NO via a non genomic estrogen receptor (ER) mediated process (Chen et al., 1999; Stefano et al., 2000) or via a non ER cell surface receptor involving stereospecific interactions with an unknown receptor at the cell surface that induces vasodilation independent of NO and prostaglandins (Teoh et al., 2000a; Shaw et al., 2000). Alternatively, estradiol can induce vasodilation in endothelium denudated vessels (Sudhir et al., 1995; Chester et al., 1995), a process governed by K^+ and Ca^{2+} channels (Sudhir et al., 1995). The discrepancy in these results most likely involves differences in tissue preparation, vascular origin of tissue, sex of animal and species. Estradiol can also act as a vasoconstrictor enhancing the vasoconstriction of several common vasoconstrictors (Vargas et al., 1995). Whether estrogens act as vasoconstrictors or vasodilators may be regional specific, for example, in the rabbit, estradiol relaxes pre-constricted coronary and ear arteries but increases the contractile force of pre-constricted pulmonary and thoracic arteries. The vasoconstrictor effect of estradiol was attenuated by

indomethacin indicating a role for prostaglandins in this process (Opgaard et al., 2002). Estradiol also modulates both HPV and ET-1. For example, estradiol reduces HPV in sheep (Sylvester et al., 1985) and rats (Lahm et al., 2007). Estradiol also reduces lung levels of ET-1 and prevents hypoxia induced increases in ET-1 by preventing the action of HIF-1α (Earley and Resta, 2002). Estradiol undergoes metabolism via several enzyme pathways to form non-estrogenic metabolites (Dubey et al., 2004). As mentioned in section 1.2.3.2.4, one of these metabolites, 2-ME, has demonstrated to be protective in animal models of PAH. The vasoactive role of 2-ME in systemic and pulmonary arteries has yet to be determined in detail but sufficient evidence exists that it may be as important or more important than estradiol in modulating vasoactive compounds. 2-ME is a more potent inhibitor of ET-1 (Dubey et al., 2001) and a more potent inducer of prostacyclin synthesis (Seeger et al., 1999) compared with estradiol in endothelial cells.

Testosterone has also been studied for its vasoactive properties in different vessel types including pulmonary and coronary arteries from several species (Littleton-Kearney and Hurn, 2004). While there is data supporting a vasodilator role for testosterone (Honda et al., 1999; English et al., 2001; Ding and Stallone, 2001), a process thought to involve activation of NO or a direct action on ion channels present on smooth muscle cells (Littleton-Kearney and Hurn, 2004), there is also opposing data favouring a vasoconstrictor role for testosterone (Quan et al., 1999; Teoh et al., 2000a; Teoh et al., 2000b), a process possibly related to an inhibition of prostacyclin or inhibition of Kv channels (Littleton-Kearney and Hurn, 2004).

The concentration of sex hormones required to induce vasodilation or vasoconstriction in isolated tissue preparations are usually within the pharmacological range, however, HPV is reduced in extralobar pulmonary arteries from rats during the proestrus menstrual phase (Lahm et al., 2007) indicating that small changes in circulating estrogen levels can alter the vascular response to a vasoconstrictor stimulus. Additionally, physiological concentrations

(1-30 nM) of estradiol and testosterone can influence 5-HT and ET-1 induced vasoconstriction in porcine coronary arteries (Teoh et al., 2000a).

The experimental evidence just outlined offers the possibility for interaction of sex hormones with potential mediators of PAH. There have been relatively few studies examining the role of gender in animal models of PAH and the effect that male hormones such as testosterone and female hormones such as estradiol and 2-ME have on pulmonary arteries and on PAECs and PASMCs. Many animal studies in PAH have used only male gender for experimental procedures or mixed gender populations that do not discriminate between males and females. Indeed, several of the *in vitro* pharmacology experiments in this thesis used mixed gender groups of animals due to the limited number of TG animals available. It was during these *in vitro* experiments that clear gender differences emerged in the response to vasoconstrictors that subsequently resulted in specific male versus female comparisons being performed for the hypoxic studies in chapter 3 and the Dfen *in vivo* and *in vitro* pharmacology work in chapters 4 and 6. The results of these experiments coupled with the importance of female gender as a modifying risk factor for PAH warranted further experimental analysis. In this chapter, the vasoconstrictor/vasodilator properties of both male and female sex hormones and their effect on 5-HT vasoconstriction were analysed in pulmonary arteries of both male and female mice. To further determine any modulating effect estradiol might have on serotonergic induced vasoconstriction relevant to PAH, CCRCs were also performed to NDfen in the presence and absence of estradiol. Male and female mouse lungs were also analysed for any differences in 5-HT and BMPR2 signalling molecules.

8.2 Methods

8.2.1 Animals

Animals were age 2-3 months

8.2.2 Myography

IPAs were isolated from male and female WT mice and used for wire myography experiments as described previously.

Following attainment of maximal contractile response to KCl as outlined in section 2.6.1.5, vessels were left to equilibrate for 30 minutes.

To assess the vasoconstrictor properties of sex hormones, CCRCs to estradiol (Sigma-Aldrich Ltd, Poole, UK), 2-ME (Sigma-Aldrich Ltd, Poole, UK), testosterone (Sigma-Aldrich Ltd, Poole, UK) and vehicle were performed in IPAs from male and female mice. To assess the vasodilator properties of sex hormones CCRCs were performed in IPAs from male and female mice pre-constricted with phenylephrine (a concentration in the range 10^{-8} – 10^{-7} M dissolved in H_2O that induced vasoconstriction equal to 70% of 50 mM KCl). All hormones were dissolved in ethanol to give a solution of 10^{-3} M from which serial dilutions were created in H_2O up to 10^{-8} M giving the range necessary for a 10 pM – 10 μM CCRC to be constructed.

Following the final concentration of hormone (10^{-5} M) added during the experiments assessing vasoconstriction, vessels were left for 20 minutes before a CCRC to 5-HT was performed to assess the effects of sex hormones on 5-HT induced vasoconstriction.

Separate experiments were performed to assess the effect of a physiological concentration (1 nM) of estradiol and testosterone on 5-HT. Hormones or ethanol vehicle were incubated for 20 minutes before a CCRC to 5-HT was constructed.

Finally, 1 nM of estradiol or ethanol vehicle was incubated for 20 minutes prior to CCRCs for NDfen being performed in IPAs from male and female mice.

5-HT and NDfen were dissolved in H_2O to give a solution of 10^{-2} M from which serial dilutions were created up to 10^{-6} M giving the range necessary for a 1 nM- 300 μM CCRC to be constructed

As stated above, CCRCs to 5-HT following 10^{-5} M of estradiol/2-ME/testosterone were performed on vessels following the initial CCRC to each hormone. For all other experiments vessels were subjected to only one treatment.

8.3 Results

Contractions in IPAs were measured relative to the contraction induced by 50 mM KCl. No significant differences were observed in KCl response between genders (male: 2.5 ± 0.3 mN, n=6, female: 2.4 ± 0.2 mN, n=6).

8.3.1 Assessment of contractile response to estradiol, 2-ME and testosterone in male and female IPAs

CCRCs to estradiol, 2-ME and testosterone are presented in figure 8.1. No vasoconstriction was observed up to 10^{-5} M for any hormone tested in either male or female mice.

8.3.2 Assessment of vasodilator response to estradiol, 2-ME and testosterone in male and female IPAs

Figure 8.2A shows CCRCs for each hormone in IPAs from male mice pre-constricted with phenylephrine. All three hormones displayed significant vasodilator effects at concentrations equal to or greater than 0.1 μM in male mice and this data is presented in table 8.1A. The only significant difference noted between hormones in the relaxation response was at 10 μM where estradiol induced a greater degree of relaxation ($p<0.05$) compared with 2-ME (figure 8.2A). In female IPAs (figure 8.2B) testosterone induced a significant vasodilator effect at 3 μM and above with both female hormones exerting a

significant vasodilator effect from 10 μM (table 8.1B). No significant differences were observed between hormones. The only gender differences to emerge were for estradiol. The vasodilation induced by estradiol was greater in male mice ($p<0.05$) compared with female mice at 100 nM (male: -6.9 ± 2.9% of phenylephrine induced pre-constriction, n=6, female: 2.8 ± 0.7%, n=6), 300 nM (male: -6.1 ± 2.4%, n=6, female: -0.2 ± 0.8%, n=6), 1 μM (male: -10.2 ± 2.6%, n=6, female: -0.7 ± 2.1%, n=6) and 10 μM (male: -38.6 ± 4.7%, n=6, female: -24.3 ± 2.8%, n=6).

8.3.3 Influence of a high concentration (10^{-5} M) of estradiol, 2-ME and testosterone on 5-HT induced vasoconstriction.

Following the CCRCs to each hormone, an additional CCRC to 5-HT was performed in the presence of 10^{-5} M estradiol, 2-ME and testosterone (figure 8.3). In male mice (figure 8.3A), the pEC50 of 5-HT was decreased ($p<0.05$) in estradiol treated vessels compared with vehicle (vehicle: 7.5 ± 0.1, n=7, estradiol: 7.1 ± 0.1, n=6) and there was a significant difference ($p<0.05$) in the pEC50 between the estradiol and testosterone treatment groups with the greater pEC50 being in animals that had been treated with testosterone (estradiol: 7.1 ± 0.1, n=6, testosterone: 7.4 ± 0.1, n=6). Hormones affected the maximal response to 5-HT to a much greater degree than pEC50 with each hormone significantly ($p<0.001$) reducing the Emax of 5-HT compared with vehicle (vehicle: 124.0 ± 4.2% of the contraction induced by 50 mM KCl, n=7, estradiol: 93.3 ± 5.9%, n=6, 2-ME: 98.3 ± 6.1%, n=6, testosterone: 92.0 ± 7.5%, n=6). There were no differences, however, in the maximal response to 5-HT between the estradiol, 2-ME or testosterone groups

In female IPAs, testosterone increased ($p<0.01$) the pEC50 of 5-HT compared with estradiol and 2-ME (estradiol: 7.2 ± 0.1, n=6, 2-ME: 7.2 ± 0.1, n=7, testosterone: 7.6 ± 0.1, n=6) but did not differ significantly from the vehicle group. The only significant difference in Emax was between the testosterone and estradiol groups with testosterone

increasing ($p<0.05$) the Emax of 5-HT compared to estradiol (estradiol: 93.5 ± 5.2%, n=6, testosterone: 120.2 ± 6.9%, n=6) (figure 8.3B).

Data was analysed to determine any differences between 5-HT response in male and female IPAs. No differences were found in pEC50 values but Emax values did differ significantly ($p<0.05$) in the testosterone groups (male: 92.0 ± 7.5%, n=6, female: 120.2 ± 6.9%, n=6).

8.3.4 Influence of a low concentration (10^{-9} M) of estradiol and testosterone on 5-HT induced vasoconstriction

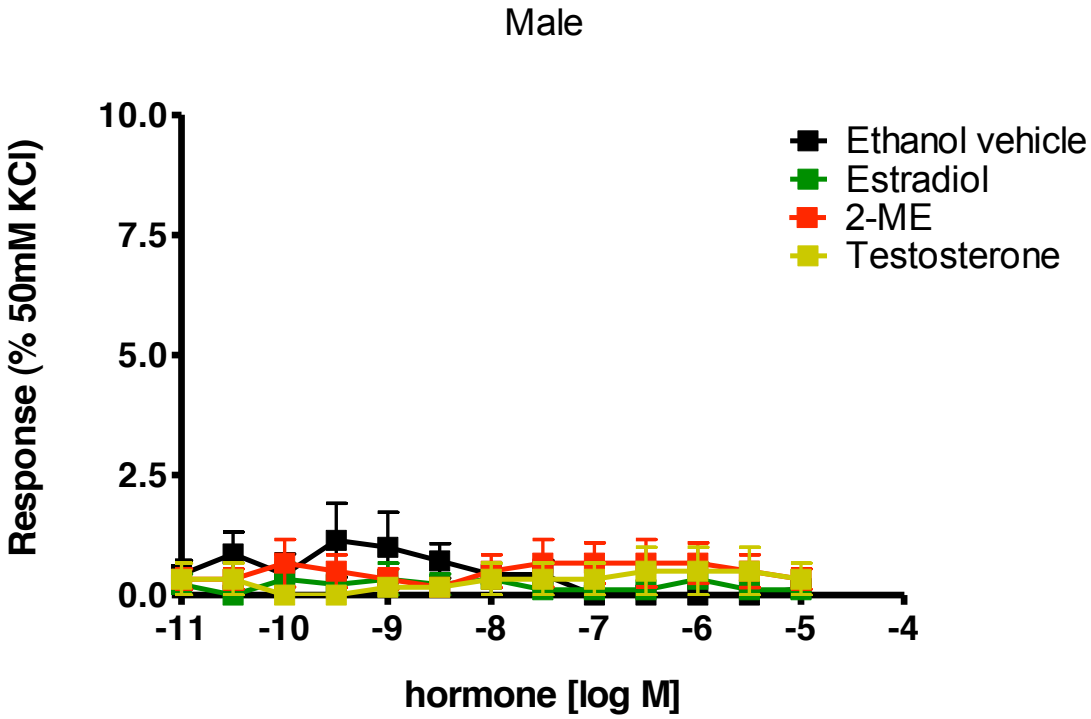
The effect of a physiological (10^{-9} M) concentration of estradiol and testosterone was tested on the vasoconstrictor effect of 5-HT in male and female IPAs (figure 8.4). No differences were observed in pEC50 or Emax in 5-HT response following estradiol or testosterone in either male or female mice. There were also no differences between male and female groups for respective hormones or vehicle.

8.3.5 Influence of a low concentration (10^{-9} M) of estradiol on NDfen induced vasoconstriction

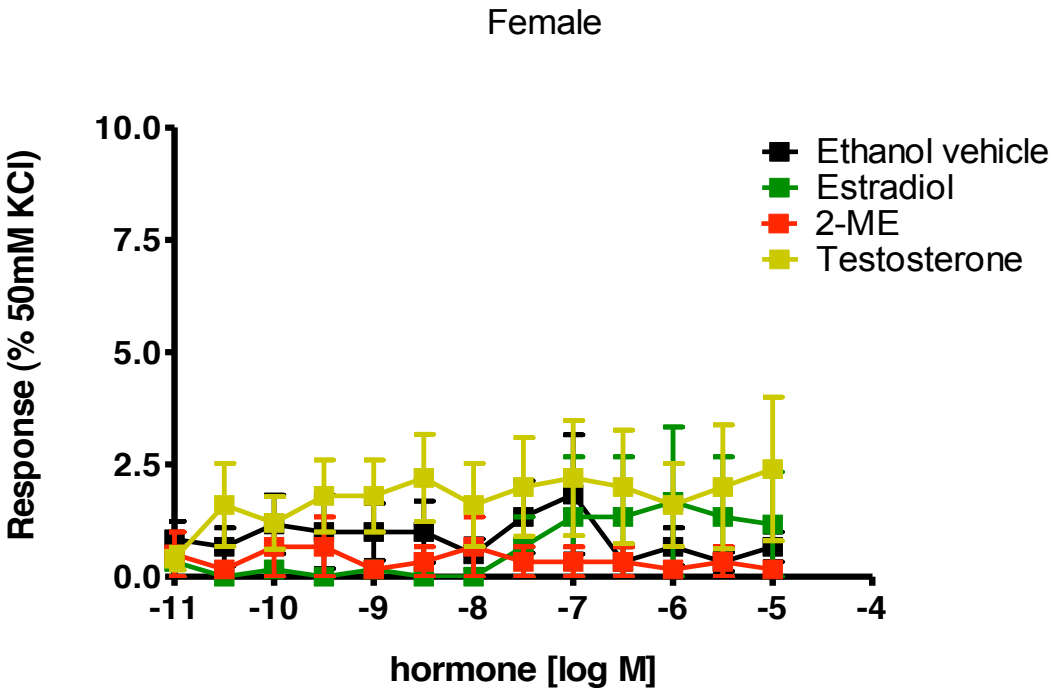
To determine whether a physiological concentration of estradiol could influence the vasoconstriction induced by NDfen, CCRCs to NDfen were performed in the presence of 10^{-9} M estradiol in male and female IPAs (figure 8.5). NDfen was significantly more potent in IPAs from male mice in both vehicle (pEC50; male: 6.1 ± 0.1, n=7, female: 5.5 ± 0.1, n=7) and estradiol (pEC50; male: 6.0 ± 0.0, n=6, female: 5.3 ± 0.1, n=6) treated groups with maximal response unaffected. Estradiol had no effect on vasoconstriction in either gender.

8.3.6 Expression of 5-HT and BMPR2 signalling molecules in male and female mouse lung.

Expression of p-Smad1/5/8 (figure 8.6), p-p38 MAPK (figure 8.7) and p-ERK1/2 (figure 8.8) were analysed by western blotting in male and female whole mouse lung. p-Smad1/5/8 and p-p38 MAPK expression did not differ between genders but p-ERK1/2 was expressed more strongly in female mouse lung.



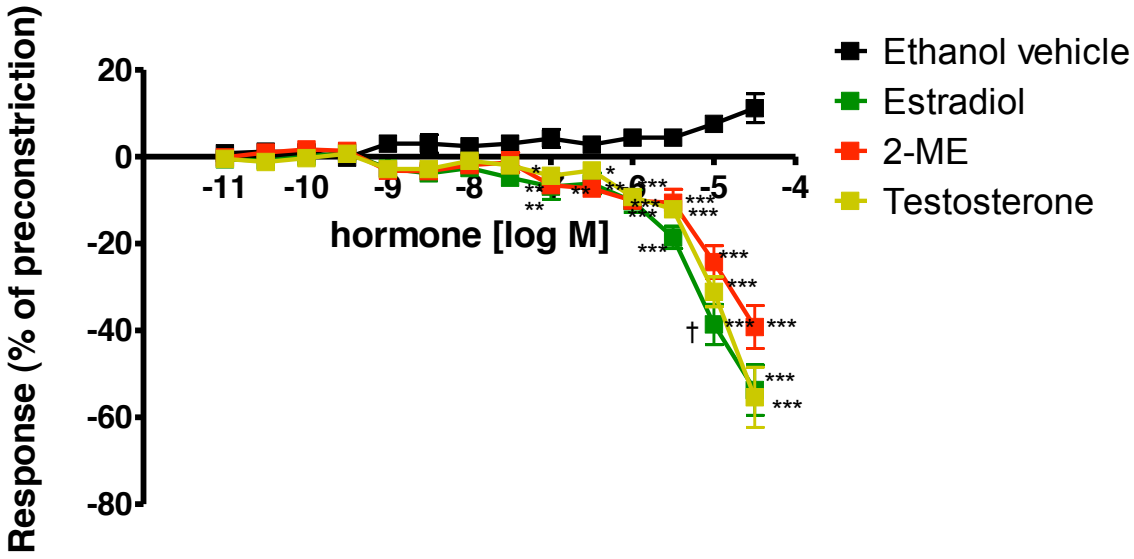
A



B

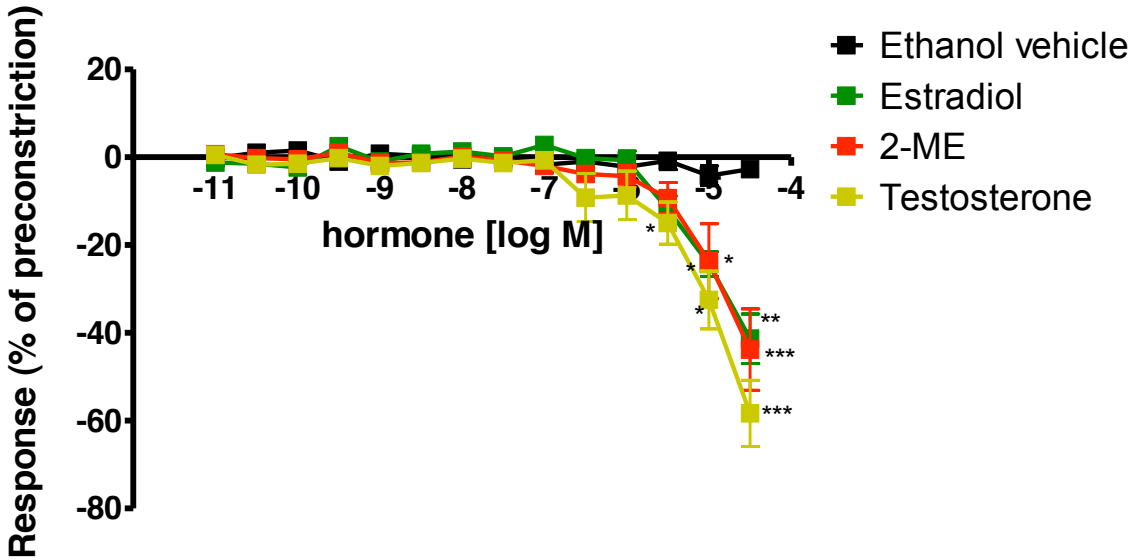
Figure 8.1 Cumulative concentration-response curves to estradiol, 2-ME, testosterone and ethanol vehicle in IPAs from male (A) and female (B) WT mice (n=6-9 mice per group). Results expressed as mean ± SEM. Newman-Keuls multiple comparison test revealed no significant differences between groups at any concentration tested.

Male



A

Female



B

Figure 8.2 Cumulative concentration-response curves to estradiol, 2-ME, testosterone and ethanol vehicle in IPAs (pre-constricted with phenylephrine) from male (A) and female (B) WT mice (n=6 mice per group). Results expressed as mean ± SEM (Results corrected to account for ethanol vehicle response). * $p<0.05$, ** $p<0.01$, *** $p<0.001$ versus corresponding concentration for ethanol vehicle. † $p<0.05$ versus 2-ME using Newman-Keuls multiple comparison test.

Treatment	100 nM	300 nM	1 μM	3 μM
vehicle	4.2 ± 2.1	2.8 ± 1.2	4.4 ± 1.3	4.4 ± 1.4
estradiol	-6.9 ± 2.9 **	-6.1 ± 2.4 **	-10.2 ± 2.6 ***	-18.6 ± 2.6 ***
2-ME	-6.5 ± 1.8 **	-7.3 ± 1.6 **	-10.1 ± 1.9 ***	-10.6 ± 3.1 ***
testosterone	-4.4 ± 1.0 *	-3.1 ± 1.2 *	-9.2 ± 0.9 ***	-12.1 ± 1.7 ***

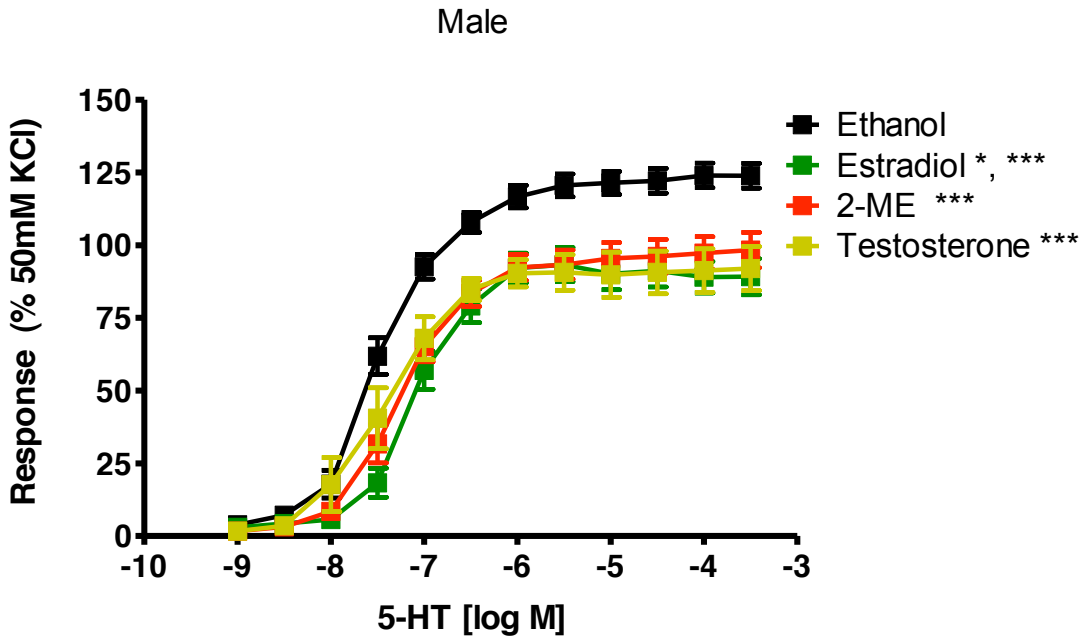
Treatment	10 μM	30 μM		
vehicle	7.6 ± 1.9	11.2 ± 3.3		
estradiol	-38.6 ± 4.7 ***,†	-53.7 ± 5.8 ***		
2-ME	-24.3 ± 3.8 ***	-39.2 ± 4.9 ***		
testosterone	-31.1 ± 3.5 ***	-55.4 ± 6.9 ***		

A

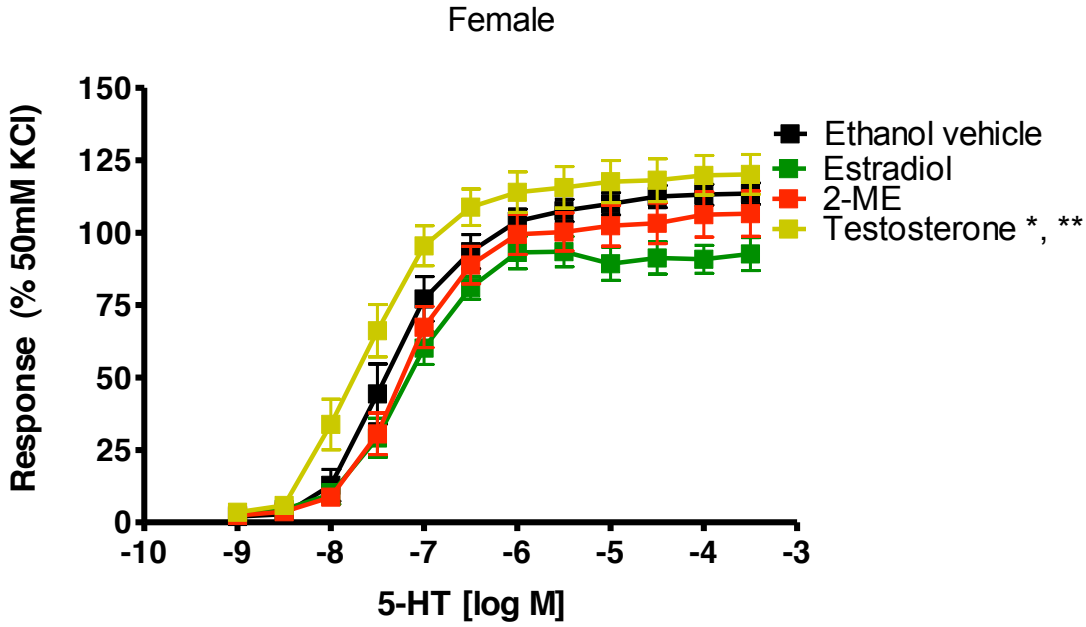
Treatment	3 μM	10 μM	30 μM
vehicle	-0.8 ± 2.0	-4.2 ± 2.2	-2.7 ± 1.2
estradiol	-11.8 ± 3.0	-24.3 ± 2.8 *	-41.2 ± 5.7 **
2-ME	-9.3 ± 3.6	-23.7 ± 8.5 *	-43.8 ± 9.3 ***
testosterone	-15.0 ± 4.8 *	-32.5 ± 6.6 *	-58.3 ± 7.6 ***

B

Table 8.1 Significant vasodilator effect of estradiol, 2-ME and testosterone in vessels pre-constricted with phenylephrine in male (A) and female (B) IPAs (n=6 mice per group). Results expressed as mean (% of pre-constriction) ± SEM (Results corrected to account for ethanol vehicle response). * $p<0.05$, ** $p<0.01$, *** $p<0.001$ versus corresponding vehicle value. † $p<0.05$ versus 2-ME using Newman-Keuls multiple comparison test.

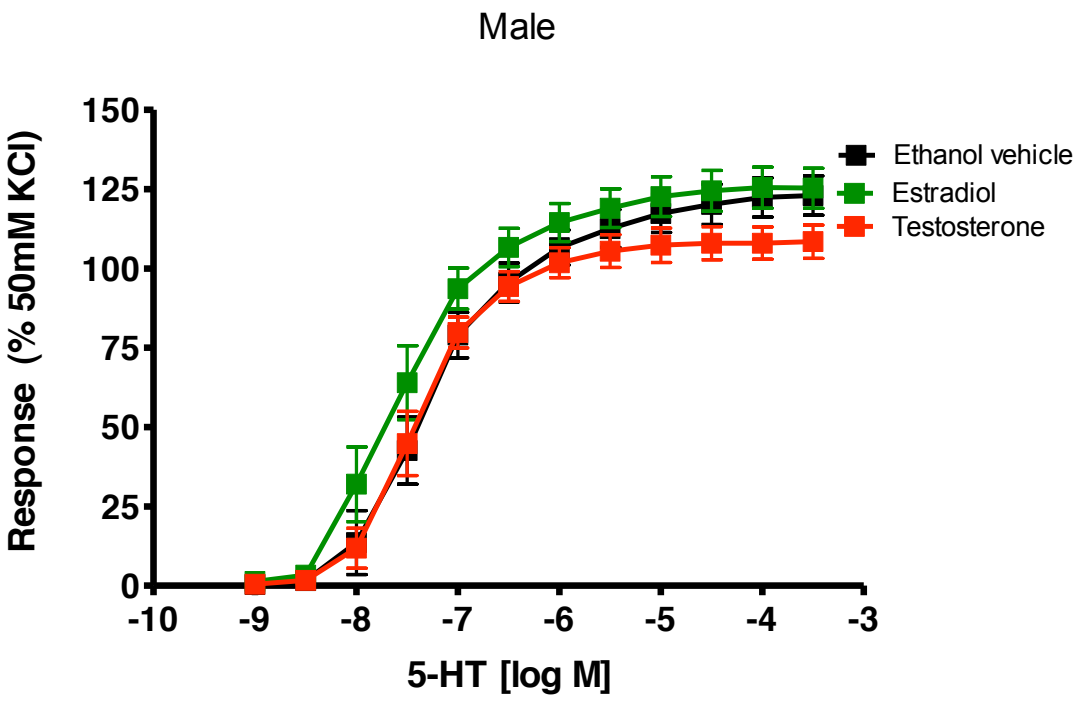


A

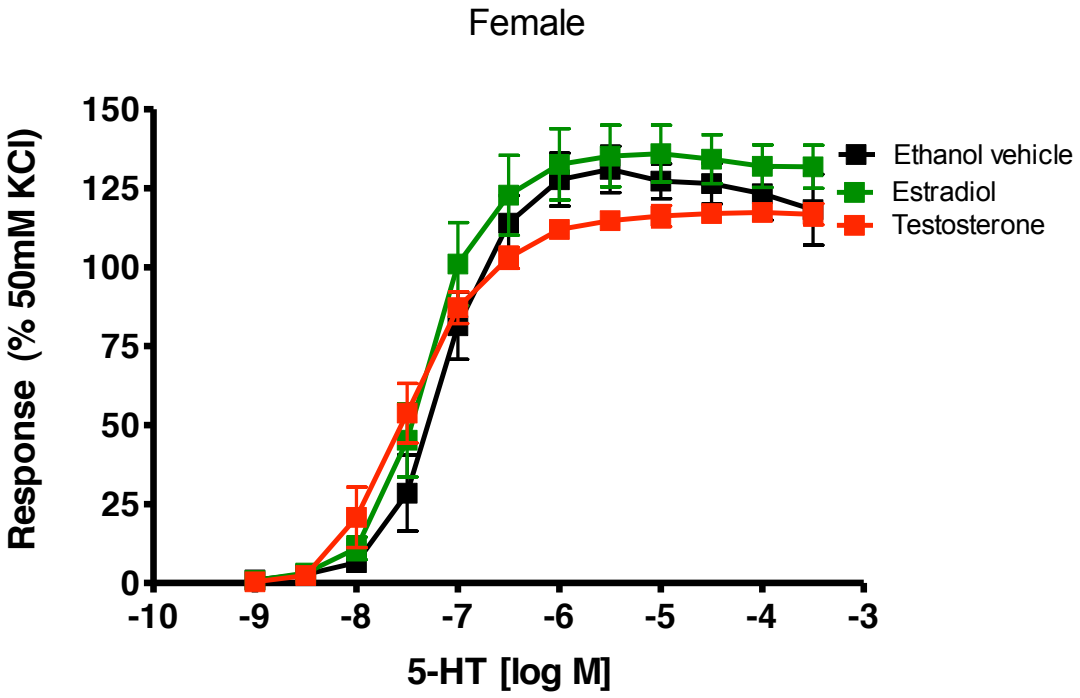


B

Figure 8.3 Cumulative concentration-response curves to 5-HT following selected sex hormones (10^{-5} M) and ethanol vehicle in IPAs from male (A) and female (B) mice (n= 6-7 mice per group). Results expressed as mean ± SEM. Male pEC50: * $p<0.05$ versus testosterone and vehicle. Male Emax: *** $p<0.001$ versus vehicle. Female pEC50: ** $p<0.05$ versus estradiol and 2-ME. Female Emax: * $p<0.05$ versus estradiol using Newman-Keuls multiple comparison test.



A



B

Figure 8.4 Cumulative concentration-response curves to 5-HT following selected sex hormones (10^{-9} M) and ethanol vehicle in IPAs from male (A) and female (B) mice (n= 4-6 mice per group). Results expressed as mean ± SEM. Newman-Keuls multiple comparison test revealed no significant difference in pEC50 or Emax between groups.

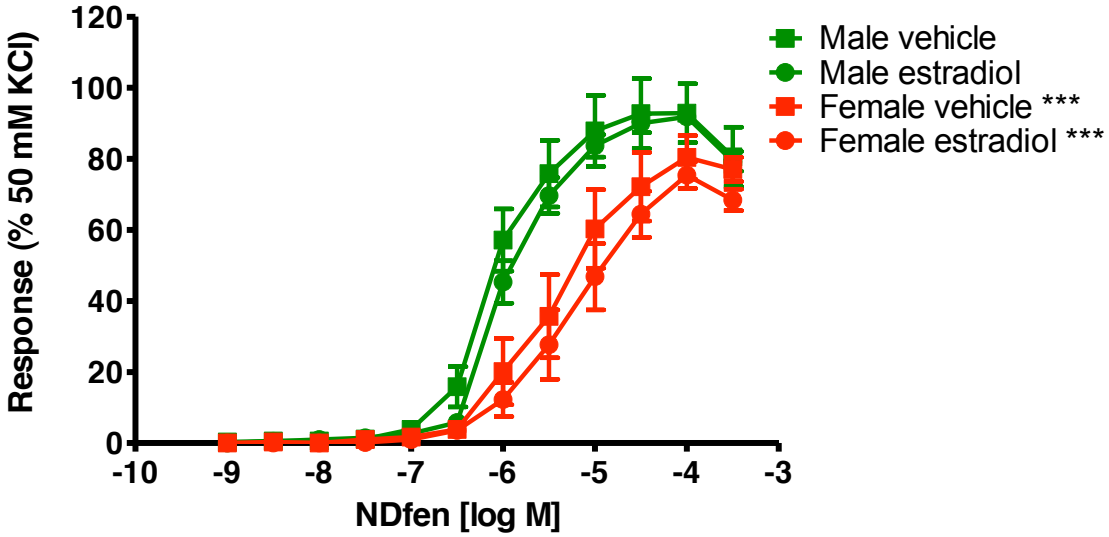


Figure 8.5 Cumulative concentration-response curves to NDfen following estradiol (10^{-9} M) and ethanol vehicle in IPAs from male and female mice (n=6-7 mice per group). Results expressed as mean ± SEM. pEC50: *** $p<0.001$ versus respective male treatment group using Bonferroni multiple comparison test.

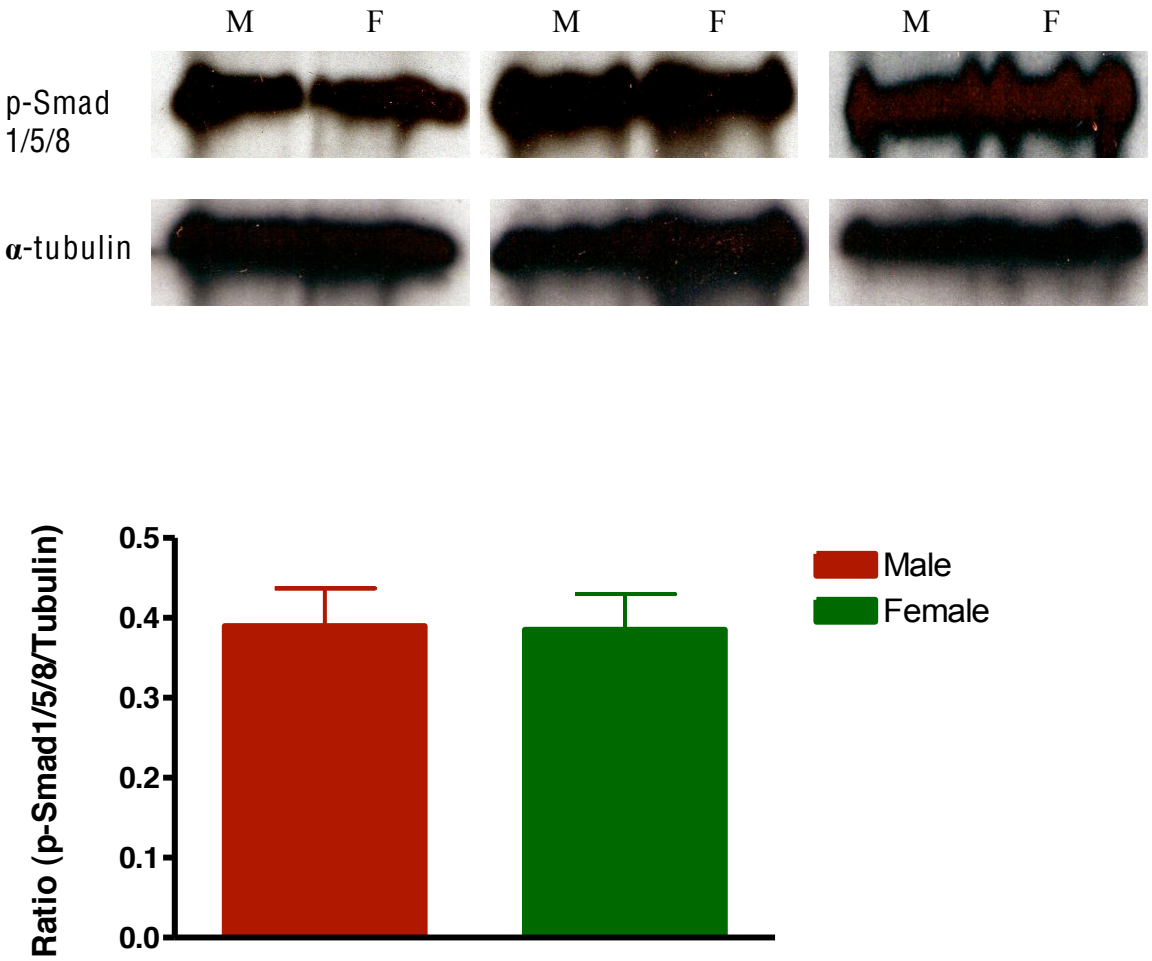


Figure 8.6 Representative autoradiograms and densitometric analysis of p-Smad1/5/8 expression in male and female mouse lung (n=6 mice per group). Results expressed as mean ± SEM. An unpaired two-tailed t-test revealed no significant difference between groups.

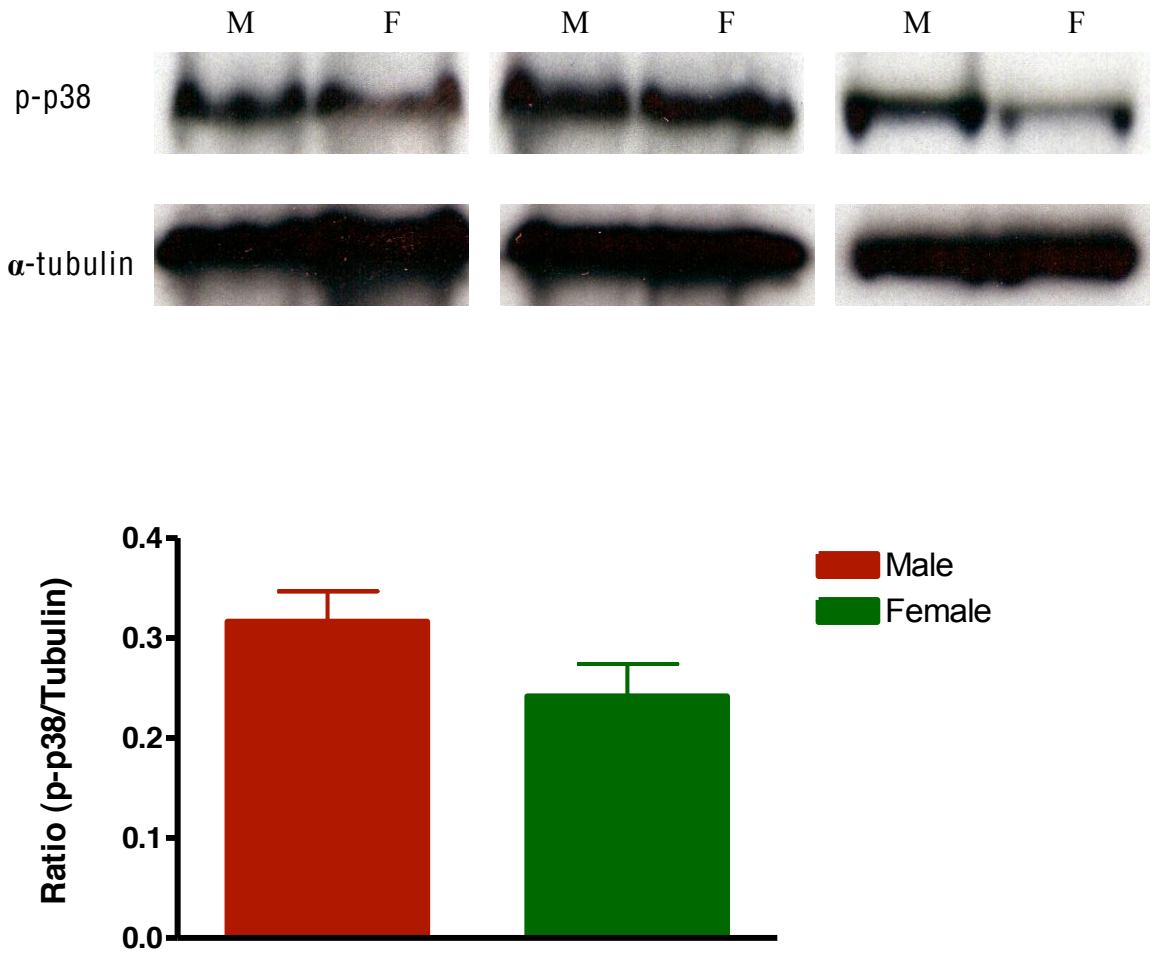


Figure 8.7 Representative autoradiograms and densitometric analysis of p-p38 expression in male and female mouse lung (n=6 mice per group). Results expressed as mean ± SEM. An unpaired two-tailed t-test revealed no significant difference between groups.

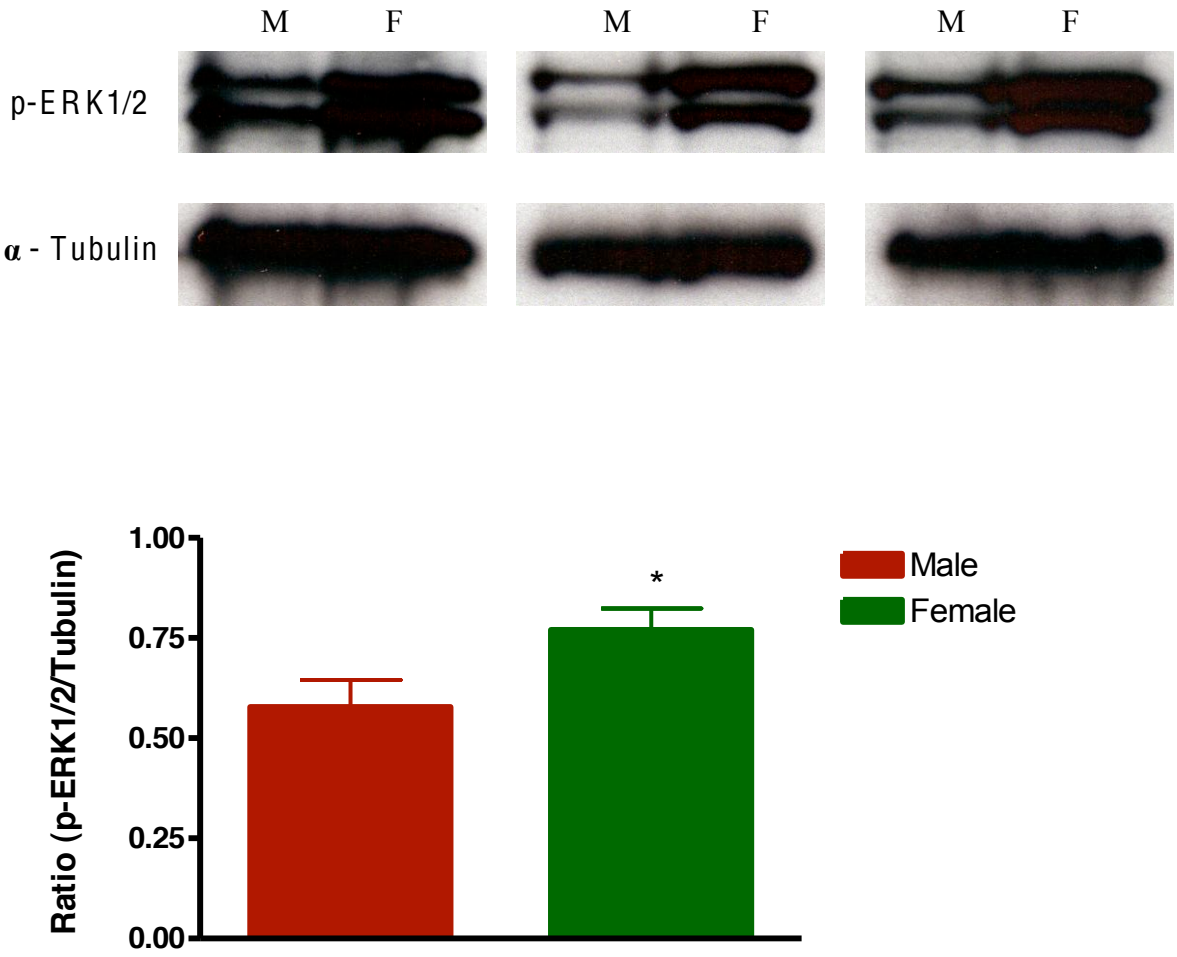


Figure 8.8 Representative autoradiograms and densitometric analysis of p-ERK1/2 expression in male and female mouse lung (n=6 mice per group). Results expressed as mean ± SEM. * $p<0.05$ versus male using an unpaired two-tailed t-test.

8.4 Discussion

The key findings from this chapter are the discovery that steroid sex hormones from both male and female mice have a vasodilator effect on pulmonary arteries pre-constricted with phenylephrine. A pharmacological concentration of 10^{-5} M (10^{-5} M was chosen as a concentration that should reliably uncover any rapid non-genomic vasodilator/vasoconstrictor effect each hormone may have. It would also allow a comparison with a physiological concentration giving greater insight into the vasoactive effects of each hormone) estradiol, 2-ME and testosterone reduced the vasoconstriction to 5-HT in IPAs from male mice but not in female mice. Estradiol and testosterone at a physiological concentration of 10^{-9} M, however, were without effect on 5-HT in either gender. Estradiol at 10^{-9} M was also without effect on NDfen which was found to be a more potent vasoconstrictor in male mice. Finally, p-ERK1/2 was found to be increased in female mouse lung compared with males with p-Smad1/5/8 and p-p38 MAPK levels similar between genders.

The first series of myography experiments designed to assess the vasoactive effects of sex hormones conclusively show that both male (testosterone) and female (estradiol and 2-ME) sex hormones have vasorelaxant effects on murine IPAs. This is further evidence for the ability of steroid hormones to induce rapid non-genomic vasoactive effects on vascular tissue. Both estradiol and testosterone have previously been shown to dilate rat pulmonary vessels pre-contracted with prostaglandin-F-2α with testosterone inducing the largest relaxation, an effect found to be greater in male pulmonary arteries (English et al., 2001). This differs to the mouse IPAs pre-constricted with phenylephrine utilised in this investigation where there was no difference in testosterone induced relaxation between male and female IPAs and no significant differences emerged between testosterone and estradiol in either gender. There was an enhanced response to estradiol in male mice compared with female mice at several concentrations tested. This would indicate a greater

activation of vasodilator pathways in male mice. The fact that the three hormones did not differ significantly in effect (with the exception of a greater relaxation induced by estradiol at 10 μM compared with 2-ME in male IPAs) may indicate that similar pathways were utilised in inducing the vasorelaxation. These may involve NOS and NO activation which has been demonstrated to be involved in estradiol induced vasorelaxation in several vascular beds (McNeill et al., 1996; Chen et al., 1999; Stefano et al., 2000; Scott et al., 2007). Other studies, however, have found the vasoactive properties of sex hormones to be independent of NO (Chester et al., 1995; Sudhir et al., 1995; Naderali et al., 1999; Teoh et al., 2000a; Teoh 2000b; Shaw et al., 2000). There is sufficient evidence that estradiol exerts its vasoactive effects by different mechanisms depending on gender (Chester et al., 1995; McNeill et al., 1996), species, arterial location (Opgaard et al., 2002), the inducer of vasoconstriction used in the experiment (Teoh et al., 2000a) and even depending on whether the animal is pregnant or not (Scott et al., 2007). A more consistent finding across the literature on the vasoactive properties of sex hormones is the high concentration (relative to physiological concentrations) of hormone necessary to induce significant vasodilation. Generally, supraphysiological (>0.1 μM, more commonly >3 μM) concentrations have been required to induce significant relaxation (Naderali et al., 1999; Shaw et al., 2000; Opgaard et al., 2002; Scott et al., 2007). The results presented in this thesis agree with this conclusion. 0.1 μM and 3 μM in male and female mice respectively was the lowest concentration of hormone that induced significant vasorelaxation. Although each of the three hormones induced vasodilation in male mice at lower concentrations than in female mice, the extent of the vasodilation was small (< 10% of phenylephrine pre-constriction at concentrations less than 1 μM). Even the concentrations required to induce a small response, therefore, are higher than the normal physiological concentration of estradiol in females (<2 nM) and the plasma testosterone concentration (10-38 nM) in males. It is unlikely, therefore, that any rise or fall in either male or female sex hormones

would directly affect the tone of pulmonary arteries. As outlined in the introduction, however, physiological concentrations of estradiol can influence HPV (Lahm et al., 2007) and both estradiol and testosterone (1-30 nM) alters 5-HT and ET-1 induced vasoconstriction in porcine pulmonary arteries (Teoh et al., 2000a). In this investigation, both pharmacological (10^{-5} M) and physiological (10^{-9} M) concentrations of male and female sex hormones were tested in their respective abilities to alter the vasoconstriction induced by 5-HT in IPAs. Estradiol, 2-ME and testosterone decreased the Emax of 5-HT at 10^{-5} M in male IPAs, consistent with the vasodilator effect of these hormones at this concentration with estradiol having the most pronounced effect. Hormones had more divergent effects at 10^{-5} M when tested in female IPAs. While no hormone affected the 5-HT response compared with vehicle, there was a trend towards enhanced vasoconstriction for testosterone and a trend towards decreased vasoconstriction for estradiol. This resulted in significant differences between testosterone and estradiol and between testosterone and 2-ME. Similar results have previously been reported for porcine coronary arteries with estradiol reducing 5-HT induced vasoconstriction and testosterone enhancing it (Teoh et al., 2000a).

Interestingly, testosterone was the only hormone to alter 5-HT response between male and female IPAs with testosterone increasing the Emax of 5-HT in females compared with male IPAs. It would appear that the ability of testosterone to reduce 5-HT induced vasoconstriction is strongly male specific with no such effect and even a trend toward enhanced vasoconstriction occurring in females. This may indicate different mechanisms of action for testosterone in male and female IPAs and it is certainly clear that testosterone induces different signalling systems in different species and vascular beds. NO, Ca^{2+} and K^{+} channels, prostacyclin and androgen receptor (AR) mediated processes have all been suggested as modulators of testosterone action (Littleton-Kearney and Hurn, 2004). Testosterone and estradiol at 10^{-9} M had no significant effect on the 5-HT CCRCs in either

male or female IPAs. There was an observable trend, however, towards enhanced 5-HT induced vasoconstriction following 10^{-9} M estradiol, an interesting observation as it is a trend in the opposite direction from the reduced vasoconstriction 10^{-5} M estradiol had on 5-HT. It is unclear if this is evidence of a true pharmacological effect. Enhanced vasoconstriction to phenylephrine has been observed in rabbit pulmonary arteries following estradiol but only at concentrations exceeding 10^{-6} M (Opgaard et al., 2002). Additionally, a physiological concentration (7 nM) of estradiol can increase the pressor response to noradrenaline in rat mesenteric arteries (Vargas et al., 1995). As described in the introduction to this chapter, the effects of estradiol can differ between vascular beds and the study by Vargas et al., 1995 did not study pulmonary arteries. Currently, there is no evidence for a direct vasoconstrictor effect of estradiol in mouse IPAs. A similar 20 minute exposure to 1 nM testosterone has previously been shown to reduce vasorelaxation induced by bradykinin and calcium ionophore A23187 (Teoh et al., 2000b) and to enhance vasoconstriction induced by 5-HT, ET-1 and U46619 (Teoh et al., 2000a). This latter study also tested estradiol at 10^{-9} M and found this concentration was sufficient to reduce vasoconstriction induced by 5-HT, ET-1 and U46619. Both studies were performed on mixed gender porcine coronary arteries which may be more sensitive to the vasoactive effects of sex hormones than murine IPAs. A final study examining whether estradiol (10^{-9} M) would act as a risk or modulating factor for NDfen induced vasoconstriction failed to show any influence of estradiol on NDfen. In fact, female gender acted as a protecting factor against the effect of NDfen with the potency of NDfen being greater in male IPAs possibly indicating enhanced activation of 5-HT_{2A} mediated pathways in IPAs in young adult male mice.

Estradiol and its metabolites also affect vascular SMC proliferation with the effect being anti-mitogenic in most studies to date. Multiple mechanisms of actions are suggested for the anti-mitogenic, anti-migratory effects of estrogens including inhibition of MAPK

pathways, decreased release of mitogens such as ET-1 and PDGF in addition to an inhibition of ROS production (Dubey and Jackson, 2001). Interaction with pathways such as these in PASMCs possibly explains the beneficial role estradiol and 2-ME have in animals models of PAH as outlined in section 1.2.3.2.4. The effect on endothelial cell growth by estradiols appears concentration dependent. Lower concentrations (10-100 nM) of estradiol metabolites can induce proliferation of vascular endothelial cells whereas higher concentrations (>100 nM) inhibit endothelial cell proliferation (Dubey and Jackson, 2001). In rat lung ECs, estradiol induced proliferation of both male and female cultured cells at physiologically relevant concentrations (3-30 nM) while testosterone only induced proliferation of male ECs. The extent of proliferation induced by estradiol depended on the relevant ratio of estradiol to both progesterone and testosterone with testosterone increasing the proliferative effect of estradiol on female ECs in a 1:1 ratio. Estradiol induced proliferation in these cells was dependent on ERs (Liu et al., 2002) highlighting the complexity of hormonal effects on cellular functions. 2-ME but not estradiol induces apoptosis of bovine PAECs. This was partly dependent on stress-activated protein kinase (SAPK). The EC_{50} value for inducing apoptosis was 0.45 μM which is similar to the concentration required for inhibition of proliferation (Yue et al., 1997). Fas, Bcl-2, β-galactosidase and ROS scavenging may also contribute to its role in preventing apoptosis (Yue et al., 1997; Tsukamoto et al., 1998; Dubey and Jackson, 2001).

There is currently no direct link between sex hormones and the development of PAH. If hormonal agents do play a role, the effects are likely to be more subtle than those observed in animal models using high plasma concentrations. It may be that alterations in the balance between estradiol and its metabolites, progesterone and testosterone or their receptors are more important than any single rise or fall in hormone concentration. Evidence for this is supported by the finding that progesterone receptors are present within the intimal lesions in human PAH but ERs are absent. Neither of these receptors are

present in healthy lung tissue (Barberis et al., 1995). As mentioned above, the relevant ratio of progesterone and testosterone to estradiol modulates the proliferative effect of estradiol on vascular ECs. The balance of circulating hormones can affect the response to other circulating vasoconstrictors and growth factors. Female mesenteric arteries from rats display increased responsiveness to vasoconstrictors compared with males and pre-treatment of males with estradiol increases the responsiveness of male arteries (Altura, 1975). Inherent differences in many of the candidate systems put forward as modulators in the pathophysiology of PAH may also exist between male and females that increase the risk in females when challenged with pulmonary hypertensive risk factors. It is already established that estrogens can modulate MAPK pathways including ERK1/2 and p38. To determine if these two pathways, relevant to both 5-HT and Dfen induced proliferation of pulmonary cells and dysfunctional BMPR2 signalling, differs in male and female mouse lung, western blot analysis of these proteins were performed. The canonical BMPR2 signalling protein: p-Smad1/5/8 was also analysed. Only p-ERK1/2 was significantly different, being raised in female mouse lung compared with males. p-ERK1/2, as previously described throughout this thesis, is implicated in 5-HT induced proliferation of PASMCs (Lee et al., 1999; Liu et al., 2004; Lawrie et al., 2005), BMP-2 and BMP-7 induced proliferation of PASMCs in IPAH patients (Takeda et al., 2004) and also perhaps in Dfens mechanism of action (Lee et al., 2001). It could be hypothesised that if the increased p-ERK1/2 observed in female mouse lung was raised in PASMCs these cells may be less resistant to any factor that increases p-ERK1/2 promoting proliferation and also acting to suppress the anti-proliferative p-Smad1/5/8 pathway through direct inhibition (Massague, 2003). It may also be that a combination of small differences between male and female physiology are additive in creating a pulmonary vasculature at greater risk of PAH and some of these differences will be discussed in the final chapter.

Chapter 9

General discussion

9.1 General discussion

Recent years have provided extensive knowledge in understanding the pathophysiology of PAH including genetic, cellular and molecular advances. The result of this research has been the discovery of BMPR2 mutations as the principal genetic basis for the disease, the identification of candidate molecular mechanisms that modulate the severity of the disease and the emergence of new vasodilator therapies for improving survival and quality of life. Furthermore, PAH has revealed itself to be a multifactorial disease with neither one factor or mutation responsible for the severe disease phenotype observed in human patients. The creation of the $BMPR2^{+/-}$ mouse as a genetic model to mimic the haploinsufficent state of FPAH in humans offers the opportunity to assess how a proven genetic risk factor may interact with other genetic or acquired factors speculated to be causative or modulating in the disease progression. The large body of evidence outlined previously in this thesis implicating the serotonergic system, in particular heightened expression of the 5-HTT as a contributor to PAH and the existence of a 5-HTT+ mouse led to a new 5-HTT+/$BMPR2^{+/-}$ mouse being developed to further explore genetic interaction and subsequent interaction with possible secondary risk factors.

In chapter 3, the pulmonary phenotype of cross TG mice and single TG animals was extensively assessed in both male and female mice and the pulmonary vascular response to 14 days hypoxia compared with WT animals. The most significant finding from this set of experiments was the divergent effect that overexpression of the 5-HTT and BMPR2 deficiency had on pulmonary phenotype and that these effects were only evident in female animals. 5-HTT+ overexpression caused a rise in mRVP and vascular remodelling in room air in females and these animals had a more severe pulmonary hypertensive phenotype under hypoxia. The increase in vascular remodelling in female 5-HTT+ mice is a novel finding and further strengthens a role for the 5-HTT as a modulating factor in PAH. Future studies will need to address the question of what molecular mechanisms are

responsible for linking the 5-HTT to an increase in RVP and remodelling. Indeed, studies such as these are already under way with the recent discovery that Kv1.5 downregulation by the 5-HTT in PASMCs is dependent on the nuclear factor of activated T-cells (NFAT) c2 transcription factor (Guignabert et al., 2009). NFATc2 was found to mediate 5-HTT downregulation of Kv1.5 in both human PASMCs and in PASMCs from the SM22-5-HTT+ mice discussed in chapter 3. Moreover, gene expression studies have been performed on 5-HTT$^{-/-}$ mice that indicate a decrease in inflammatory genes and changes in cell cycle genes that tend towards lower proliferation (Crona et al., 2009). The logical progression from this study would be a similar analysis of gene expression in the 5-HTT+ mouse used in this investigation that may identify genes or groups of genes that contribute to the pulmonary hypertensive phenotype. Female BMPR2$^{+/-}$ mice, while phenotypically normal in room air, did develop more severe vascular remodelling following hypoxia than mice without BMPR2 deficiency. As discussed in chapter 3, this investigation is the first to show heightened vascular remodelling in the BMPR2$^{+/-}$ mouse following hypoxia, a particularly important and relevant finding to PAH in humans as it demonstrates that the interaction of two known risk factors for PAH (namely BMPR2 mutation and female gender) can predispose the pulmonary vasculature to more extensive changes following hypoxia, a factor in several diseases associated with PAH. No synergy was observed between overexpression of the 5-HTT and BMPR2 deficiency in the *in vivo* experiments that resulted in an exaggerated pulmonary hypertensive phenotype. Developing a suitable model for PAH has proven difficult with neither the hypoxic or MCT rodent models reproducing the full complexity of vascular lesions observed in human patients and genetic models also have limitations. Despite mimicking the genetic background of a haploinsufficent FPAH patient with L/L allele genotype for 5-HTT, the level of BMPR2 and 5-HTT in Cross TG mice are higher and lower respectively than levels seen in PAH patients. It may be that synergistic effects in PASMCs from Cross TG mice that

predispose these cells to proliferation and vasoconstriction are too subtle to create a more pronounced pulmonary hypertensive phenotype following the hypoxic challenge used in this investigation. Long et al., 2006 demonstrated that $BMPR2^{+/-}$ mice develop a pulmonary hypertensive phenotype following 2 weeks of 5-HT infusion and that PASMCs from these mice proliferate to a greater degree than WT PASMCs following 5-HT. Taken together, these results indicate that loss of BMPR2 can enhance the deleterious effect of 5-HT in murine PASMCs. The proliferative effect of 5-HT and inhibition of p-Smad1/5/8 was dependent on $5\text{-}HT_{2A}$ receptor activation rather than 5-HT uptake via the 5-HTT which may explain the lack of synergy in the Cross TG mice used in this investigation, however, the 5-HTT may have contributed to the PAH observed *in vivo* in the study by Long et al., 2006. It would be useful in future studies to infuse female $BMPR2^{+/-}$ mice with 5-HT in the same way Long et al., 2006 did for the males. These results could be compared for gender differences and could subsequently be compared with the Dfen results presented in this thesis. This would provide further information on the interaction between BMPR2 deficiency, gender and the serotonergic system as well as offer clues as to whether 5-HT alone mediates the *in vivo* effects of Dfen.

Enhancement of $5\text{-}HT_{2A}$ signalling is supported by experimental results in IPAs from 5-HTT+, $BMPR2^{+/-}$ and Cross TG mice that demonstrated greater vasoconstriction to the $5\text{-}HT_{2A}$ agonist NDfen compared with WT IPAs. The greatest vasoconstriction was observed in $BMPR2^{+/-}$ mice supporting the work by Long et al., 2006 of enhanced $5\text{-}HT_{2A}$ mediated serotonin signalling in these mice. An enhancement of $5\text{-}HT_{2A}$ receptor signalling may be an important factor in transducing the genetic changes of 5-HTT+ and $BMPR2^{+/-}$ into pathophysiological changes in the pulmonary vasculature, both in the TG animals in this thesis and also in human patients. This is due to its diverse contributory role in 5-HT (Maclean et al., 1996; Long et al., 2006) and NDfen (Ni et al., 2005) induced vasoconstriction, hypoxic induced proliferation of PAFs (Welsh et al., 2004) and 5-HT

induced proliferation of PASMCs, an effect associated with enhanced activation of p-ERK1/2 (Long et al., 2006) (figure 9.1).

Further support for cross-talk between 5-HT and BMPR2 pathways in murine PASMCs came from the Dfen pharmacology experiments in chapter 6. IPAs from female Cross TG mice were significantly more sensitive to Dfen induced vasoconstriction than WT or single TG animals and displayed vasoconstriction at clinically relevant concentrations. Unlike the pulmonary hypertensive phenotype observed in WT and $BMPR2^{+/-}$ mice following treatment with Dfen, the vasoconstriction exerted by Dfen was independent of peripheral 5-HT synthesis (at least in WT and $Tph1^{-/-}$ mice). The enhanced vasoconstriction observed in Cross TG mice may reflect an enhancement of intracellular serotonergic signalling capable of being activated by Dfen, enhancement of 5-HT receptors or a direct inhibitory action of Dfen on Kv channel function.

This investigation has found evidence of abnormal KCNQ channel mediated vasoconstriction in 5-HTT+, $BMPR2^{+/-}$ and Cross TG mice. Changes in KCNQ channel expression and/or function related to 5-HTT overexpression or loss of BMPR2 may contribute to the pathophysiology of PAH given the importance of K^+ channels in PASMC vasoconstriction, remodelling and apoptosis and the negative effect that factors such as hypoxia, 5-HT and Dfen have on Kv channels in PASMCs. The recent finding that flupirtine, a KCNQ channel activator, can reduce hypoxic PAH and the pulmonary hypertensive phenotype of 5-HTT+ mice (Morecroft et al., 2009) indicates a possible involvement of KCNQ dysfunction in the disease and offers a new target for potential therapeutic research.

Experiments in $Tph1^{-/-}$ mice conclusively showed that Dfen requires peripheral 5-HT synthesis to induce PAH, a process thought to involve massive 5-HT release related to Dfens role as a 5-HTT substrate. This is a key discovery that not only answers an important question regarding the mechanism of action of Dfen but also supports the

'serotonin hypothesis' of PAH. This is also the first investigation to look at the effect of BMPR2 haploinsufficiency on Dfen APAH *in vivo*. $BMPR2^{+/-}$ mice were not any more susceptible to the cardiopulmonary effects of Dfen with the exception of small but significant levels of remodelling in male $BMPR2^{+/-}$ following Dfen compared with WT. Evidence linking BMPR2 deficiency and Dfen induced PAH in human patients is inconclusive. As mentioned in chapter 4 the functional significance of many of the BMPR2 mutations found in PAH patients who had taken anorexigens is not clear. The very fact that BMPR2 mutations have been found in APAH patients taking Dfen and that they correlate with a shorter exposure time would indicate some level of interaction between BMPR2 mutation and Dfen induced PAH although this may be dependent on the nature of the BMPR2 mutation.

The interaction between 5-HT and BMPR2 was further explored in chapter 5 by analysing expression of BMPR2, p-SMAD1/5/8 and p-ERK1/2 in IPAs from $Tph1^{-/-}$ mice. Although no significant differences were found between $Tph1^{-/-}$ and WT mice, there was a trend (more than two fold increase) towards heightened BMPR2 expression in $Tph1^{-/-}$ mice. This result is suggestive, although not conclusive, of a reciprocal influence between the two pathways (figure 9.1). Increased BMPR2 expression in $Tph1^{-/-}$ mice may contribute to the attenuated hypoxic induced PAH observed in these mice (Morecroft et al., 2007).

The *in vivo* experiments revealed that a 50% reduction in BMPR2 in all cells can enhance vascular remodelling following hypoxia in female mice. More severe loss of BMPR2 in PAECs or PASMCs would be thought to exaggerate the predisposition to vascular remodelling and experiments over the last several years are beginning to confirm this hypothesis. As discussed in chapter 3, severe loss of BMPR2 in PASMCs through dominant negative inhibition induces a pulmonary hypertensive phenotype in mice, both male and female (West et al., 2004; Tada et al., 2007). The SM22-tet-$BMPR2^{delx4+}$ mouse develops more severe PAH following hypoxia, further supporting an interaction between

genetic and environmental elements (West et al., 2004), however, the SM22-tet-BMPR2^{delx4+} mouse displays relatively minor vascular remodelling. More recently, West et al., 2008a developed a mouse expressing a smooth muscle specific BMPR2^{R899X} mutation. This is an arginine to stop mutation present in the cytoplasmic tail of BMPR2 and is also found in FPAH. This is in contrast to the BMPR2^{delx4+} mutation which results in a premature stop 18 amino acids into the kinase domain resulting in loss of the entire intracellular domain including kinase activity. About one third of the mutant SM22-rtTA x TetO7-BMPR2^{R899X} mice had increased RVP and these mice had extensive vascular remodelling. The structural changes observed may partly be the result of the elevated p-ERK1/2 and p-p38 MAPK observed in the lungs of the TG mice (West et al., 2008a). This evidence highlights an important point regarding the mutation specific effects of BMPR2 disruption. Different BMPR2 mutations will have different effects on the stoichiometry of BMP cell surface receptor expression and the nature of intracellular signalling such as the degree of Smad activation and proteins controlled by the cytoplasmic tail such as LIMK-1. As more data is acquired on the mutation specific effect of BMPR2 mutations, this will allow new genetic models to be created and the pulmonary hypertensive phenotype to be compared between models. For example, the mouse used in this investigation mimics the haploinsufficent state that occurs in the majority (~70%) of human FPAH patients (Machado et al., 2006; Morrell, 2006). That still leaves 30% of FPAH patients who carry missense mutations that may lead to a dominant negative effect. Missense mutations have recently been shown to correlate with a more severe disease phenotype in human patients compared with mutations resulting in haploinsufficiency (Austin et al., 2009a). Creating a mouse carrying a dominant negative mutation in all somatic cells will allow a comparative study to be performed with the haploinsufficent BMPR2$^{+/-}$ mouse offering new insights into the interaction of specific mutation types with other risk factors. Microarray studies may also uncover potential genes of interest that are divergently modified between the

different genetic models.

Gender emerged unexpectedly as a crucial and in some experiments the most critical factor in determining the extent of vasoconstriction, vascular remodelling or RVP present in mice. The effect of gender was also dependent on the genetic background of the animal. In the hypoxic *in vivo* study in chapter 3 female TG mice often had a greater pulmonary hypertensive phenotype than the males, a situation most marked in the 5-HTT+ mouse. Female 5-HTT+ mice also had increased RVP and vascular remodelling in room air, a phenotype not present in the males. The interaction of genetics and gender was also observed during the Dfen pharmacology experiments in chapter 6 (Dfen induced vasoconstriction was similar in male and female WT mice, greater in male 5-HTT+ mice, marginally greater in male $BMPR2^{+/-}$ mice but greater in female Cross TG mice which were the most sensitive of all mice to Dfen induced vasoconstriction) and NDfen pharmacology experiments in chapter 8 (where male mice were more sensitive to NDfen). The most profound result of all regarding gender differences was the absence of a pulmonary hypertensive phenotype in male WT and $BMPR2^{+/-}$ animals following Dfen which induced robust vascular remodelling and elevated RVP in females. $BMPR2^{+/-}$ male mice did show remodelling following Dfen but only at 50% of the level observed in females and this was not accompanied by elevated RVP. The experiments just outlined indicate an interaction between female gender and 5-HTT and BMPR2 pathways. In chapter 8, protein expression of p-ERK1/2 was found to be elevated in the lungs from female WT mice compared with males. As shown in figure 9.1, p-ERK1/2 can be increased in the context of dysfunctional BMP mediated signalling and increased serotonergic signalling. Increased p-ERK1/2 activity in female PASMC that are $BMPR2^{+/-}$ or that have increased expression of the 5-HTT and $5\text{-}HT_{2A}$/$5\text{-}HT_{1B/1D}$ receptors may further shift the balance of the cell towards proliferation and increased vascular remodelling (figure 9.1). Elevated p-ERK1/2 may be one of many factors that act

collectively to increase the risk of PAH in female mice and human female PAH patients. One candidate gene that may also contribute to the increased risk of PAH in females is Cytochrome P450 1B1 (CYP1B1), an enzyme involved in the breakdown of estrogens and expressed in the lung. CYP1B1 has been found to be decreased up to 10 fold in immortalised lymphoblastic cell lines derived from female FPAH patients with BMPR2 mutation compared with male affected BMPR2 mutation carriers or unaffected BMPR2 mutation carriers (West et al., 2008b). More recently, a polymorphism in CYP1B1 resulting in decreased activity and previously linked to several cancers was associated with a 4 fold higher penetrance of PAH in $BMPR2^{+/-}$ females (Austin et al., 2009b). $BMPR2^{+/-}$ females with PAH were found to have an altered balance in estrogen metabolites compared with unaffected $BMPR2^{+/-}$ females. The balance was shifted so that the proportion of 16α-hydroxyestrone (16α- OHE_1) was greater. 16α- OHE_1 is a significantly greater mitogenic compound than other estrogen metabolites and its increase is thought to be a direct consequence of diminished CYP1B1 (Austin et al., 2009b). Similar to data outlined in the discussion in chapter 8 regarding the balance between testosterone, estradiol and progesterone, it may be that the balance between pro-proliferative and anti-proliferative estrogen metabolites is critical to the overall effect on vascular cells. The discovery that decreased CYP1B1 interacts with BMPR2 deficiency in human PAH patients offers the potential for future animal models in which to further explore the interaction of BMPR2 mutation with female gender. This may involve creating a mouse that is $BMPR2^{+/-}$ and deficient in CYP1B1 and then exposing this model to additional modifying factors such as 5-HT, hypoxia and Dfen. It is interesting to note that in addition to the discovery of CYP1B1 polymorphisms in human female FPAH patients, it has recently been shown that PAH patients with BMPR2 mutations have an earlier age at diagnosis compared with non-carriers, but that this only occurs for women. Within the male subgroup, no statistical

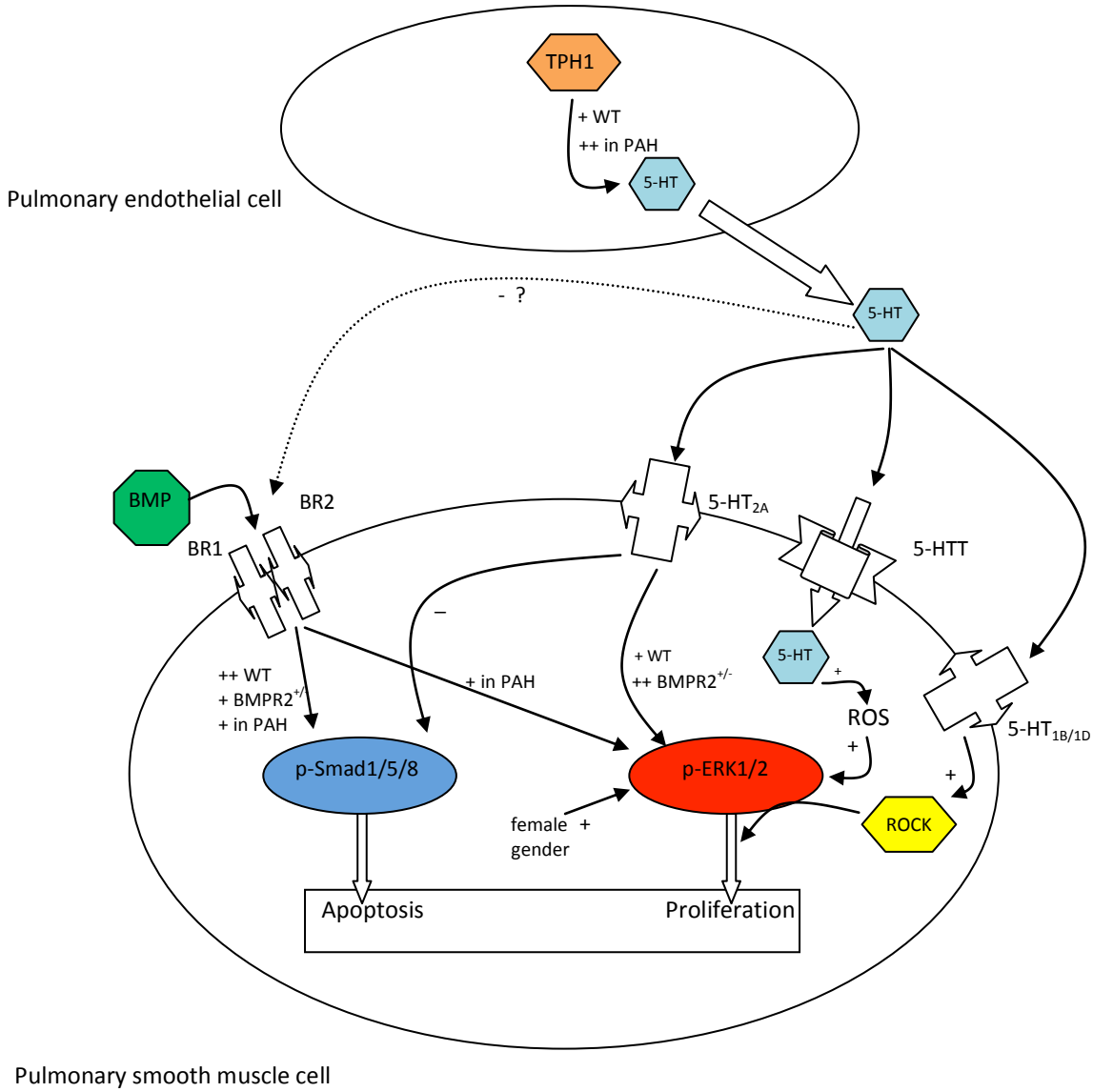


Figure 9.1 Interaction of serotonergic and BMPR2 signalling pathways on pulmonary smooth muscle cell proliferation.

The balance between p-ERK1/2 and p-Smad1/5/8 is thought to be crucial in determining the proliferative state of a PASMC. Evidence from BMPR2$^{+/-}$ cells or cells from PAH patients suggests there is enhancement of p-ERK1/2 signalling, secondary to an increase in serotonergic activity or aberrant BMPR1A/1B (BR1) and BMPR2 (BR2) signalling. This promotes proliferation. Conversely, a decrease in p-Smad1/5/8 further shifts the balance towards proliferation. The elevated p-ERK1/2 expression in female mouse lung may contribute to the greater risk associated with female gender, especially when combined with dysfunctional BMPR2 or enhanced 5-HT signalling. The dashed arrow represents a potential new level of cross-talk between 5-HT and BMPR2 suggested by a trend towards enhanced BMPR2 expression in pulmonary arteries from Tph1$^{-/-}$ mice. This will require greater clarification in future studies.

differences emerged in age at diagnosis between BMPR2 mutant carriers and non carriers (Austin et al., 2009a).

Direct interactions may also exist between BMPR2 or the 5-HTT and estrogens. Estradiol has been shown to suppress BMPR2/p-Smad1/5 signalling in several cell lines through Smad inhibition by ER-α (Yamamoto et al., 2002). The cytoplasmic tail of BMPR2 contains estrogen sulfotransferase binding sites (Hassel et al., 2004) and this enzyme has a high affinity for estrogens and is involved in controlling local estrogen concentration (Song, 2001). The state of the 5-HTT can also be influenced by estrogens with increased circulating levels of estradiol upregulating 5-HTT expression in the brains of rats (McQueen et al., 1997; Rivera et al., 2009). It is still unclear why the female pulmonary vasculature is more susceptible to PAH and what interactions may occur between loss of BMPR2 or heightened serotonergic signalling and estrogenic effects in the lung. The use of genetic models in animals can offer clues as to which molecular pathways may warrant further study. Microarray studies have indicated increased expression of cytokines and markers of immune response in SM22-tet-BMPR2^{delx4+} mice, an effect most prominent in female mice; with 8 week old SM22-tet-BMPR2^{delx4+} female mice having twice the expression of IL-6 than males (Tada et al., 2007). Further studies such as this will help characterise pathways that differ between males and female and which are likely to contribute to disease progression.

To conclude, this investigation has uncovered important interactions between genetic pre-disposition, gender and modifying factors in PAH that implicate the serotonergic system (both overexpression of the 5-HTT and peripheral 5-HT synthesis) as an important modulating factor in PAH. BMPR2 haploinsufficiency alone is not sufficient to induce PAH or to worsen Dfen associated PAH but is a risk factor for hypoxic induced vascular remodelling. This investigation also uncovered increased vasoconstriction to NDfen in all TG mice and an increased contractile response to 5-HT in BMPR2$^{+/-}$ mice. This is an

important finding since increased pulmonary vasoconstriction is thought to be an important aspect in the initial stages of PAH. The enhanced vasoconstriction may partly relate to changes in K^+ channel function and this investigation found impaired KCNQ channel function in TG mice, an alteration that may have detrimental consequences in the pulmonary vasculature given the recent discovery of the role these channels play in setting the resting V_m of PASMCs and the ability of KCNQ activators to reduce the symptoms of PAH in animal models. The resistance of male mice to many of the changes characteristic of PAH is something that was unexpected and also an important consideration for future studies in animal models of PAH. Gender must be considered an essential component in experimental design and interpreting results, especially in studies using hypoxia or Dfen. The female vasculature appears more susceptible to the changes characteristic of PAH and an increase in p-ERK1/2 may be an important candidate in raising this risk. The use of novel genetic animal models containing mutations or polymorphisms derived from human PAH patients have provided and will continue to provide new data on the cellular and molecular mechanisms underlying a highly complex disease. Future genetic models utilising haploinsufficent and dominant negative BMPR2 dysfunction combined with conditional knockout studies to unravel the complex cellular specific roles of such mutations will provide the basis for exploring the interaction of modulating factors such as 5-HT, K^+ channels and female gender both at the genetic and pharmacological level. Identification of the modifying factors that interact with BMPR2 deficiency and the unravelling of the molecular pathways that underlie those interactions will allow a better understanding of the pathophysiology of PAH and to aid the search for targeted therapies.

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