Pharmacology of vascular responses to trace amines

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Summary

Trace amines including β-Phenylethylamine (β-PEA) and tyramine are vasoactive monoamines closely related to the classical neurotransmitters, noradrenaline, serotonin (5-HT) and dopamine. As vasoactive substances, the trace amines are considered sympathomimetic, eliciting vasoconstrictor responses through noradrenaline release, although they can also induce vasodilator responses. The trace amines are well-known agonists of trace amine-associated receptors (TAARs), of which TAAR1 is expressed in rat aorta. Pharmacological characterisation of TAAR1 has proven difficult as TAAR1 is located in intracellular compartments and is poorly expressed at the cell surface. Previous studies have attributed both trace amine-induced vasoconstrictor and vasodilator responses to TAAR1. The aim of the current thesis was to pharmacologically characterise both the vasoconstrictor and vasodilator actions of the trace amines.

β-PEA- and tyramine -induced vasoconstrictor response in rat aortic rings were both significantly potentiated by endothelium removal or inhibition of endothelial nitric oxide synthase (eNOS). Vasoconstrictor responses to β-PEA were found to be resistant to blockade of post-synaptic uptake-2 transporters, antagonists of 5-HT_2 receptors, α_1-adrenoceptors, D_1 and D_2-class dopamine receptors and the mouse-specific TAAR1 antagonist, EPPTB. This indicates that β-PEA-induced contractile responses are likely mediated by a currently unidentified cell surface receptor or receptors. As EPPTB, is a poor tool for the study of rat TAAR1, it is possible that TAAR1 located at the plasma membrane mediates these responses.

β-PEA-induced vasodilator responses in rat aortic rings were partially attenuated by endothelium removal or inhibition of eNOS. Vasodilator responses to β-PEA were resistant to antagonists of muscarinic M3 receptors, β_2-adrenoceptors and EPPTB. β-PEA-induced vasodilator responses were completely abolished by inhibition of uptake-2 transporters indicating that vasodilation is mediated by an intracellular receptor such as TAAR1.

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Chapter 1 General Introduction

1.1 Trace amines

The classic aminergic neurotransmitters, dopamine, noradrenaline, adrenaline and serotonin (5-HT) play crucial roles in the central and peripheral nervous systems (Lindemann and Hoener 2005). In addition to their roles as neurotransmitters, they also are essential regulators of vascular tone (Guimaraes and Moura 2001; Fidalgo et al. 2013). The trace amines are a secondary group of endogenous amines that overlap considerably with the classical biogenic amines. They are similar in structure, biosynthesis and catabolism and are capable of displacing the classical neurotransmitters from their storage vesicles (Lindemann et al. 2005; McGeer 2013).

The term trace amine was coined by Boulton (1974) as a way of describing monoamines with endogenous tissue concentrations below 100ng/g, more than two orders of magnitude less than that of the classical aminergic neurotransmitters (Boulton 1984). Initial investigations of the trace amines predominantly focused on *p*-tyramine, β-phenylethylamine (β-PEA) and to a lesser degree *p*-octopamine, consequently the name trace amine has become synonymous with these amines (Berry et al. 2017). In addition to being found in the nervous system, trace amines are also abundant in the diet being found in various foods such as probiotic yoghurt (Marcobal et al. 2006), fermented sausages (Suzzi and Gardini 2003), cheeses (Bonetta et al. 2008), chocolate and wine (Branchek and Blackburn 2003).

Dysregulation of the trace amines has been linked with a variety of psychiatric disorders including schizophrenia and depression (Berry et al. 2017). In addition, potential roles for trace amines have also been identified in conditions such as attention deficit hyperactivity disorder (ADHD), primary headaches, Parkinson's disease, addiction, eating disorders and hypertension (Lindemann et al. 2005; Broadley 2010). Considering the links between trace amines and the broad range of neurological disorders it is hardly surprising the trace amine field is dominated by the neurological effects of trace amines. Conversely, very little research characterises the pharmacology of the trace amines in the periphery.

1.2 Trace amine synthesis and metabolism

Synthesis of neurotransmitters and trace amines within the central nervous system utilises many of the same enzymes (Figure 1.2.1, Flatmark 2000). With the exception of octopamine, trace amines are synthesised by the enzymatic decarboxylation of their respective precursor amino acids by L-aromatic amino acid decarboxylase (L-AADC, Lindemann and Hoener 2005). P-tyramine is synthesised by L-AADC action on L-tyrosine, β-PEA from L-phenylalanine and tryptamine from L-tryptophan (Zucchi et al. 2006). Octopamine is produced by the further conversion of tyramine by dopamine-β hydroxylase (Burchett and Hicks 2006).

The trace amines have a remarkably high turn over rate, with a half-life of less than 30 seconds (Durden and Philips 1980). Catabolism of the trace amines to biologically inactive molecules primarly occurs via monoamine oxidase (MAO, Berry 2004). MAO is an intracelluar enzyme, responsible for deaminiation of primary and secondary amines (Broadley 2010). Two isozymes of MAO exist, MAO-A and MAO-B, with selectivity for different amines. MAO-B selectively deaminates non polar amines such as β-PEA (Figure 1.2.1, Yang and Neff 1973; Broadley 2010). Other trace amines demonstrate less selectivity and can be metabolised by both MAO-A and MAO-B (Philips and Boulton 1979; Berry 2004). A secondary route of metabolism is via N-methyl transferase (NMT) or phenylethanolamine-N-methyltransferase (PNMT) to form secondary amines (Saavedra et al. 1973; Saavedra et al. 1974).

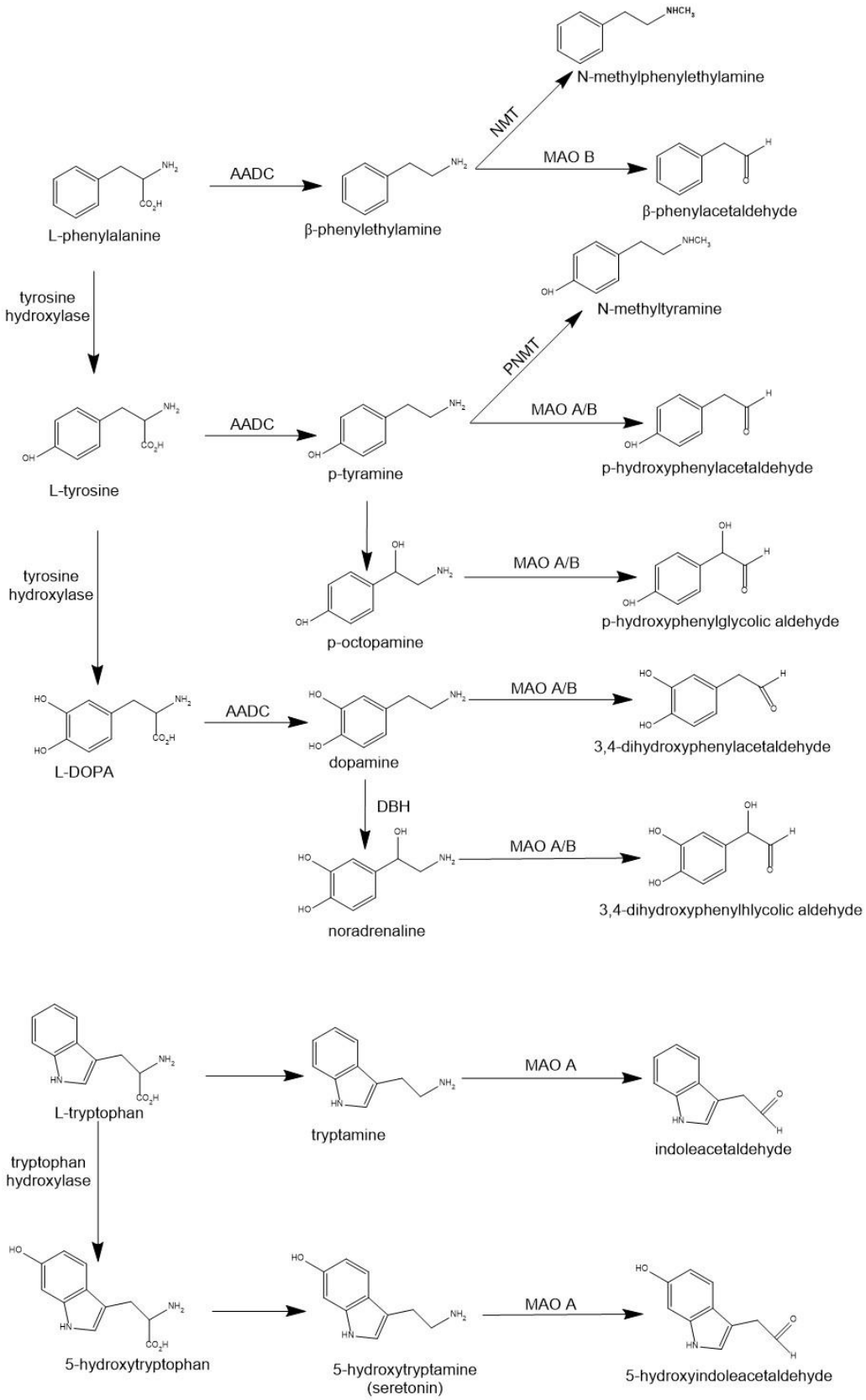

Figure 1.2.1. Synthetic and metabolic pathways for trace amines and catecholamines. AADC, L-aromatic amino acid decarboxylase, DBH dopamine-β-hydroxylase; MAO, monoamine oxidase; NMT N-methyl transferase; PNMT, phenylethanolamine N-methyltransferase.

1.3 G-Protein-Coupled Receptors

G-protein-coupled receptors (GPCRs), also known as 7TM receptors as they contain seven membrane spanning α-helices, represent the largest family of membrane proteins in the human genome (Kobilka 2007). As membrane proteins, GPCRs are activated by a diverse array of extracellular ligands (Latorraca et al. 2017). Upon ligand binding, GPCRs undergo conformational change, resulting in heterotrimeric G-protein coupling and activation (Reiter et al. 2012). These heterotrimeric G-proteins are comprised of an α-subunit ($G_{\alpha i/o}$, $G_{\alpha S}$, $G_{\alpha Q/11}$ and $G_{\alpha 12}$) interacting with a βγ complex (Kobilka 2007). Activation of GPCRs promotes the exchange of a molecule of GDP for a molecule of GTP within the active site of the α-subunit which leads to dissociation of the α-subunit and βγ complex (Reiter et al. 2012). The α-subunit and βγ complex then promote the formation of second messengers such as cyclic adenosine monophosphate (cAMP) or phosphoinositides (Wang et al. 2018).

GPCR signalling was originally thought to be mediated only via intracellular G-proteins. However, following the discovery that β-arrestin could mediate GPCR signalling independently of G-proteins (Figure 1.3.1B, Lefkowitz 2013; Lefkowitz and Shenoy 2005) defined a new paradigm in GPCR signalling known as “biased activation”. Furthermore, dimerisation of GPCRs is required for some signalling pathways (Figure 1.3.1C, Terrillon and Bouvier 2004). It has also been established that GPCRs have the ability to transactivate other types of receptors such as receptor tyrosine kinases (RTKs, Figure 1.3.1D, Cattaneo et al. 2014). Biphasic activation is another unique GPCR activation mechanism in which the GPCR is activated in two phases, early phase and late phase (Figure 1.3.1E, Wang et al. 2018). Finally, many GPCRs are also localised to intracellular cell membrane compartments following extracellular ligand binding, where they are associated with different signalling systems and regulation (Figure 1.3.1F, Jong et al. 2018).

β-Arrestins were first discovered for their roles in receptor desensitisation, although they also mediate receptor internalisation via clathrin coated pits (Smith et al. 2018). It is now established that in addition to acting as a negative regulator of GPCR signalling, β-arrestins also couple to numerous signalling mediators including mitogen activated kinases (MAPKs), the tyrosine kinase SRC, nuclear factor-κB (NF- κB) and phosphoinositide 3-kinase (PI3K), by acting as an adaptor or scaffold (Eichel et al. 2016; Gao et al. 2004; Luttrell et al. 1999).

Most drugs that activate or block GPCRs are considered to equally target distinct downstream signalling pathways mediated by G-proteins and β-arrestins and are considered balanced agonists (Smith et al. 2018). However, it has long been known that selective agonists and antagonists can specifically target particular receptor-linked signal transduction pathways (Roth and Chuang 1987). Numerous ligands have been described that can selectively activate some pathways whilst actively blocking others (Luttrell 2014). In contrast to a balanced agonist, a biased agonist selectively activates G-proteins whilst blocking β-arrestin or vice versa (Smith et al. 2018).

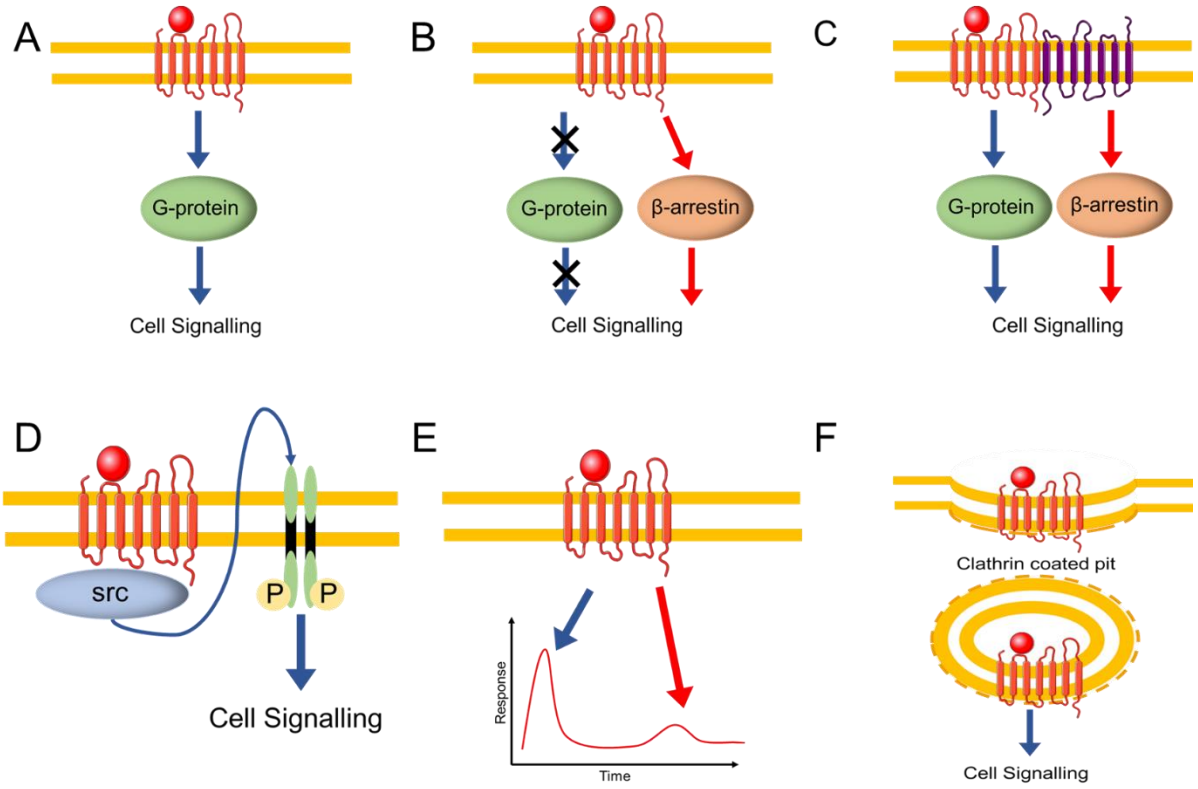

Figure 1.3.1. Types of GPCR activation. (A) Classical model, (B) biased activation, (C) dimerisation activation, (D) transactivation, (E) biphasic activation, (F) intracellular activation. Bias activation

1.3.1 Dimerisation activation

Classically GPCRs were assumed to exist and function as monomeric receptors (Terrillon and Bouvier 2004). It is now widely accepted that some GPCRs can form both homodimers and heterodimers (Angers et al. 2002). Compared with monomers, dimers often display a different agonist affinity, efficacy and trafficking properties in addition to mediating alternative signalling pathways and biological functions (Gomes et al. 2001).

GPCRs that display dimerisation activation can be classified into three categories. Firstly, GPCRs that initiate signalling only after the formation of a dimer complex, such as the γ-aminobutyric acid type B receptor ($GABA_BR$, Wang et al. 2018; White et al. 1998). Secondly, dimeric GPCRs that compared with their monomeric counterpart, couple with different G-proteins and downstream signalling pathways (Terrillon and Bouvier 2004). For example, dopamine D_1 monomers are coupled to $G_{\alpha s}$ and D_2 monomers are coupled to $G_{\alpha l}$, whereas the D_1/D_2 heterodimer signals through $G_{\alpha q}$ (Lee et al. 2004). Finally, dimerisation of GPCRs can switch the coupling of a GPCR from a G-protein to β-arrestin (Wang et al. 2018). For example, the heterodimers between μ opiod and δ opiod receptors triggers exclusive β-arrestin signalling (Rozenfeld and Devi 2007).

1.3.2 Transactivation

GPCR transactivation was first reported for the epidermal growth factor receptor (EGFR, Daub et al. 1996). Daub et al. (1996) demonstrated that GPCR agonists caused tyrosine phosphorylation and activation of EGFR that was inhibited by selective antagonists of GPCRs. Transactivation is a feature of many GPCRs including β_1- and β_2-adrenoceptors and 5-HT receptors (Kruk et al. 2013; Maudsley et al. 2000; Noma et al. 2007). Transactivation is not limited to EGFR but has been reported for several other receptors including RTKs, members of the transforming growth factor β (TGF-β) family and pattern recognition receptors (Daub et al. 1996; Burch et al. 2010; Abdulkhalek et al. 2012; Wang et al. 2018).

Evidence suggests two mechanisms of GPCR transactivation of receptors (Wang 2016b). The first mechanism known as the “triple membrane pass signal” (TMPS) pathway as the signal generated by the GPCR agonist crosses the plasma membrane three times (Wang 2016b). In this model, the GPCRs activate membrane-bound matrix metalloproteinases (MMPs, Cattaneo et al. 2014). MMPS then cleave ligands that are released into the extracellular space where they bind and activate membrane receptors, for example the EGFR ligand heparin-binding EGF-like factor (HB-EGF, Liebmann 2011).

In other situations, transactivation of receptors by GPCRs can also be considered ligand-independent (Cattaneo et al. 2014). In this model GPCR ligand binding leads to activation of intracellular protein tyrosine kinases (PTKs) such as Src which phosphorylates tyrosine residues of membrane bound receptors such as EGFR allowing interaction with downstream signalling pathways (Wang 2016b). Several mechanisms have been suggested to explain the pathways leading to enhanced activity of PTKs. In one such mechanism, GPCR activation stimulates reactive oxygen species (ROS) production by NADPH oxidase (Liebmann 2011; Cattaneo et al. 2014). ROS are suggested to destabilise the equilibrium of the intracellular phosphorylation and enhance the activity of PTK by either inactivating protein tyrosine phosphatases or stimulating proteolysis of regulatory proteins that block PTK activity (Liebmann 2011). ROS-independent mechanisms have also been suggested. Src family kinases are associated with GPCRs through direct interaction with cytoplasmic receptor domains (Cattaneo et al. 2014). This interaction is thought to activate Src kinases leading to phosphorylation of RTKs such as EGFR (Wang 2016b).

1.3.3 Biphasic activation

Biphasic activation of GPCRs was first observed in the angiotensin II type 1 receptor (AT1R, Schorb et al. 1995). However, since then it has been observed in several other GPCRs including, β_2- and β_3-adrenoceptors (Gesty-Palmer et al. 2006; Hadi et al. 2013). Biphasic activation is characterised by an early phase (5 - 10 minutes) and late phase (90-120 minutes, Wang et al. 2018). Often the intensity and duration of these phases differ significantly suggesting the two phases have distinct functions (Wang et al. 2018).

Three mechanisms of biphasic activation have been identified so far. In the first mechanism, the early phase is mediated by the G-protein pathway and the late phase by the β-arrestin pathway (Gesty-Palmer et al. 2006). The second mechanism is the reverse of the first, where β-arrestin mediates the early phase and the late phase is mediated by G-proteins (Gong et al. 2008). In the final mechanism, the early and late phase are mediated by distinct G-proteins (Hadi et al. 2013).

1.3.4 Intracellular activation

GPCRs are well characterised for their position at the cell surface where they transform an extracellular stimulus into an internal signal (Kobilka 2007). In addition, GPCRs are located at the endoplasmic reticulum (ER) where they are synthesised, folded and sorted for transport to the cell surface or on endosomes that have been internalised during receptor desensitisation (Jong et al. 2018). Emerging evidence suggests that certain intracellular membranes are the predominant location of a number of GPCRs where they may couple to different signalling systems (Irannejad et al. 2013; Jong et al. 2014; Branco and Allen 2015; Irannejad et al. 2017). The majority of identified intracellular GPCRs are localised to the nuclear membrane (O'Malley et al. 2003; Jong et al. 2018). However, to date intracellular GPCRs have also been identified on endosomes (Samaraweera et al. 2001), vesicles and mitochondria (Bénard et al. 2012).

Many GPCRs are activated at the cell surface prior to being internalised and transported to their respective intracellular site of action (Jong et al. 2018). However, GPCRs can also be activated at subcellular locations (Wang et al. 2018), thus requiring intracellular transport of ligands. GPCR ligands may enter the cell through simple diffusion, endocytosed or actively transported into the cell through channels and pores or synthesised internally (Boivin et al.

2008; Barlow et al. 2010). Furthermore, since the ligand binding sites are located inside the vesicle, luminal region of the ER or nucleus this requires ligands to cross both the plasma membrane and intracellular membrane to activate the GPCR (O'Malley et al. 2003). Whilst highly permeable ligands may easily cross intracellular membranes, less permeable and charged molecules may require an active transport process (Jong et al. 2018).

1.4 Trace amine-associated receptors

1.4.1 Identification and history

The search for a trace amine-specific receptor has been a subject of interest for many years, but ultimately a distinguishable receptor was not identified. A saturable binding site for β-PEA in the rat forebrain was first described by Hauger et al. (1982). However, this was later shown to be β-PEA binding to MAO-B (Li et al. 1992). High affinity and saturable binding sites in the rat brain for tyramine (Ungar et al. 1977) and tryptamine (Perry 1986) have also been reported although not further characterised beyond their initial identification.

Definitive evidence of a GPCR for the trace amines was made simultaneously by two independent groups (Borowsky et al. 2001; Bunzow et al. 2001). Whilst searching for novel catecholamine receptors, Bunzow et al. (2001) screened a cDNA template synthesised from a rat pancreatic tumour cell line using a pair of degenerate oligonucelotides based on the third and sixth transmembrane domains of known catecholamine GPCRs and discovered the first rat and human trace amine receptor. The receptor they identified was named trace amine receptor 1 (TAR1). Borowsky et al. (2001) was searching for novel 5-HT_1-like receptors using the same technique in genomic DNA. They identified a family of 15 new GPCRs, they named trace amine receptors based on the ability of β-PEA and tyramine to functionally activate two family members (Borowsky et al. 2001). This new receptor family was abbreviated as TA_x where x designates individual isoforms (Borowsky et al. 2001). Identification of receptors for the trace amines triggered a surge of interest, largely as pharmacological profiling demonstrated numerous psychotropic substances displayed high affinity for TAR1 (Bunzow et al. 2001). Both studies by Bunzow et al. (2001) and Borowsky et al. (2001) localised the trace amine GPCR family to human chromosome 6q23.2. A locus with strong association for susceptibility to schizophrenia (Levinson et al. 2000; Duan et al. 2004) and mood disorders (Venken et al. 2005). Consequently, trace amine receptor research became almost entirely devoted to the central nervous system effects of TAR1 (Gainetdinov et al. 2018).

Immediately following the discovery of the TAR GPCR family, there was little consistency within the nomenclature. Some researchers used the TA_x nomenclature of Borowsky et al. (2001), others the TAR nomenclature of Bunzow et al. (2001) and also a third nomenclature of TRAR (Duan et al. 2004). To reduce confusion a revised version of the nomenclature based on chromosomal location, phylogenetic relationships and examination of gene sequences was proposed (Lindemann et al. 2005). The TARs were subsequently renamed the trace amine-associated receptors (TAARs, Table 1.4.1).

At present, the only family member to have been deorphanized by IUPHAR is TAAR1 (Gainetdinov et al. 2018). The IUPHAR recommends receptors be named after their endogenous ligand. However, a single ligand for the TAAR family as a whole has yet to be identified (Lindemann et al. 2005; Ferrero et al. 2012). TAAR1-TAAR4 respond to primary trace amines (Bunzow et al. 2001; Borowsky et al. 2001), whereas TAAR5-TAAR9 respond to tertiary amines (Ferrero et al. 2012; Wallrabenstein et al. 2013). Molecular docking experiments have suggested some family members (TAAR6 and TAAR8) are activated by diamines such as putrescine and cadaverine (Li et al. 2015; Izquierdo et al. 2018).

Table 1.4.1. Trace amine associated receptor nomenclature, adapted from Lindemann et al. (2005). ψ denotes a pseudogene.

TAAR	Human		Chimpanzee		Rat		Mouse	
	Old name	bp	Old name	Bp	Old name	bp	Old name	Bp
TAAR1	TA1	1020	Novel	1020	TA1	999	TA1	999
TAAR2	GPR58	921 1056	Novel ψ	920	Novel	1020	Novel	1020
TAAR3	GPR57ψ	130	Novel ψ	1055	Novel	1029	Novel	1032
TAAR4	TA2ψ, 5-HT-4ψ	1049	Novel ψ	1030	TA2	1044	Novel	1044
TAAR5	PNR	1014	Novel	1049	Novel	1014	Novel	1014
TAAR6	TA4, TRAR4	1038	Novel	1014	TA4	1038	Novel	1038
TAAR7	Novel ψ		Novel ψ	1038				
TAAR7a					TA8	1077	Novel	1077
TAAR7b					TA12	1077	Novel	1077
TAAR7c					Novel	1077	Novel ψ	1055
TAAR7d					TA15	1077	Novel	1077
TAAR7e					TA14	1077	Novel	1077
TAAR7f					TA13ψ	1089	Novel	1077
TAAR7g					TA9	1077		
TAAR7h					TA6	1077		
TAAR7i					Novel ψ	1067		
TAAR8	TA5, GPR102	1029	Novel ψ	1027				
TAAR8a					TA11	1035 1125	Novel	1035
TAAR8b					TA7	1035 1125	Novel	1035
TAAR8c					TA10	1045 1125	Novel	1035
TAAR9	TA3	1047	Novel ψ	1048	TA3	1017	Novel	1047

1.4.2 Diversity and evolution of TAARs

Evidence of an ancestral TAAR-like protein first appeared in lamprey (Gloriam et al. 2005; Hashiguchi and Nishida 2007; Libants et al. 2009). However, it was not until the divergence of jawed vertebrates from jawless fish that a signature TAAR motif appeared (Gainetdinov et al. 2018). Due to the absence of a conserved TAAR motif in the lamprey, it has been argued that ancestral TAARs emerged only following divergence from the lamprey (Tessarolo et al. 2014). Interestingly, lamprey innately avoid sources of PEA (Di Rocco et al. 2016), a response mediated by TAARs in jawed vertebrates (Gainetdinov et al. 2018). This behaviour suggests the possibility of ancestral TAARs being present in the lamprey.

Among mammals there is considerable variation in the number of functional TAARs. At present the highest number of functional TAARs identified is in the flying fox, with a total of 26 functional TAAR genes (Eyun et al. 2016). The only vertebrate species known to contain no functional TAAR genes is the bottlenose dolphin (Eyun et al. 2016). The TAARs appear to be divided into nine subfamilies (TAAR1-TAAR9, Berry et al. 2017). These subfamilies have undergone repeated species-specific expansion, duplication and pseudogenisation (Lindemann et al. 2005; Eyun et al. 2016). This has resulted in wide variability in the number of functional TAARs found between species and species specific isoforms have even appeared (Lindemann et al. 2005).

The mammalian TAARs appear to have diverged along two distinct evolutionary paths. Firstly, the primary amine-detecting TAARs (TAAR1-4) and secondly the tertiary amine-detecting TAARs (TAAR5-9) (Ferrero et al. 2012). The primary amine-detecting TAARs appear to be older, more highly conserved, and with the exception of TAAR4, generally consist of a single isoform in the majority of genomes (Eyun et al. 2016). Tertiary amine-detecting TAARs arose more recently and considerable numbers of species-specific isoforms have emerged (Eyun et al. 2016).

1.4.3 TAAR1

TAAR1 is the most highly characterised member of the TAAR family and is regarded as the primary receptor for both tyramine and β-PEA (Lewin 2006). However, despite being rarely studied, TAAR4, a human pseudogene, is also a target for β-PEA and tryptamine (Borowsky et al. 2001). TAAR1 is also a target for the endogenous thyroid hormone metabolites thyronamine (T_0AM) and 3-idothyronamine (T_1AM) (Frascarelli et al. 2008; Scanlan et al. 2004). However, T_1AM reportedly interacts with TAAR5, and has demonstrated selectivity for multiple targets including α_2 and β_2-adrenoceptors, TRM8 calcium channels, the dopamine transporter (DAT), noradrenaline transporter (NET) and vesicular monoamine transporter (VMAT, Rutigliano et al. 2017). This indicates TAAR1 may not be the primary target of T_1AM.

Unlike other TAARs which have generally undergone recurrent pseudogenisation and duplication TAAR1 has been evolutionarily stable (Vallender et al. 2010). Despite being evolutionarily conserved TAAR1 displays considerable sequence divergence across species (Lindemann et al. 2005). There is 87% sequence homology between mouse and rat, 79% between rat and human and 76% between human and mouse (Borowsky et al. 2001). There is also species specific pharmacological profiles with considerable (10 fold or more) differences in drug potency (Vallender et al. 2010). Comparison of human and rat TAAR1 has shown β-PEA has a similar potency between receptor forms. In contrast tyramine was demonstrated to have a lower potency at human TAAR1 than β-PEA, whereas it has a higher potency than β-PEA for rat TAAR1 (Wainscott et al. 2007). It is unclear what effect, if any, these differences have in-vivo, although these species dependent differences must be taken into consideration.

TAAR1 is expressed primarily in the central nervous system, particularly in brain structures associated with psychiatric disorders (Berry et al. 2017). However, high levels of TAAR1 expression have been confirmed in a number of organs involved in nutrient sensing. These

include pancreatic β-cells (Revel et al. 2013), enterochrommafin mucosal cells of the intestines (Kidd et al. 2008; Raab et al. 2016) and D cells of the stomach (Raab et al. 2016). Furthermore, TAAR1 expression has been confirmed in human leukocytes including peripheral mononuclear cells (PMNCs, Nelson et al. 2007), B lymphocytes (Wasik et al. 2012), monocytes, polymorphonuclear leukocytes, NK cells and T lymphocytes (Babusyte et al. 2013). Consequently, TAAR1 has been postulated as a novel therapeutic target for immunomodulatory disorders. In the rat cardiovascular system TAAR1 expression has been confirmed in the aorta and heart suggesting TAAR1 may play a role in regulating cardiac output (Chiellini et al. 2007; Fehler et al. 2010).

Pharmacological characterisation of TAAR1 has proven difficult due to its poor extracellular plasma membrane localisation in model cell systems. Bunzow et al. (2001) reported that M1 flag-tagged rat TAAR1 displayed a largely intracellular distribution when stably expressed in HEK293 cells. Later work by Miller et al. (2005) observed primarily intracellular distribution of an EGFP-rhesus monkey TAAR1 chimera transiently expressed in HEK293 cells. Further evidence for localisation of TAAR1 intracellularly came when Xie et al. (2008) demonstrated TAAR1 to be associated with the intracellular membrane fraction.

1.4.4 Cardiovascular TAAR1

Although TAAR1 expression has been confirmed in rat aorta and heart (Chiellini et al. 2007; Fehler et al. 2010), the role of TAAR1 has yet to be elucidated. At high micromolar concentrations, trace amines including β-PEA, tryptamine, tyramine, octopamine induce vasoconstrictor responses in rat aorta (Fehler et al. 2010; Broadley et al. 2013). The aforementioned studies attributed the vasoconstrictor actions of the trace amines to activation of TAAR1 by comparing the potency orders of the trace amines generated in rat aorta against the potency orders for cAMP generation in rat TAAR1 transfected HEK293 cells (Borowsky et al. 2001).

In the perfused mesenteric vascular bed, tryptamine is the only trace amine reported to induce a pressor response (Anwar et al. 2012). This could be abolished by the selective 5-HT_{2A} receptor antagonist, ritanserin (Anwar et al. 2013), demonstrating the pressor response to tryptamine was not mediated by TAAR1. Conversely, β-PEA, tyramine, tryptamine and 5-HT elicited a vasodilator response, mediated through endothelial release of nitric oxide, when the vascular contractile tone was increased using phenylephrine (Anwar et al. 2012). As this response was resistant to 5-HT_7 antagonism, the authors attributed the vasodilator response to TAAR1 based on the affinity of the trace amines for rat TAAR1 in transfected HEK293 cells. However, this does not take into account that the 5-HT induced vasodilator response in these vessels was also resistant to 5-HT_7 antagonism and was not further investigated. Furthermore, 5-HT does not share the affinity of the trace amines for TAAR1 (Borowsky et al. 2001), therefore it is unlikely 5-HT-induced vasodilation was through TAAR1. A conflicting study by Narang et al. (2014), demonstrated β-PEA induced vasodilation is independent of endothelium-derived nitric oxide release. The authors suggested that β-PEA alters vascular tone through dual indirect sympathomimetic and α_1-adrenoceptor blocking actions. In perfused mesenteric vessels, low concentrations (30 μM) of β-PEA enhanced nerve mediated vasoconstriction by increasing noradrenaline availability and blocking of presynaptic α_2-adrenoceptors. However, at high micromolar concentrations (100 μM), β-PEA prevented contractile responses despite increased noradrenaline availability (Narang et al. 2014). However, Fehler et al. (2010) and Broadley et al. (2013) have demonstrated that β-PEA-induced contraction is resistant to α_1- and α_2-adrenoceptor blockade and therefore the possibility trace amines evoke their vascular actions via an alternative receptor, such as TAAR1, cannot be overlooked.

The previous studies in rat aorta and perfused mesenteric vasculature have relied heavily on resistance of trace amine induced responses to blockade of α_1- and α_2-adrenoceptors, β_2-adrenoceptors, 5-HT_{2A} and inhibition of neuronal transport and oxidation of trace amines (Anwar et al. 2012; Anwar et al. 2013; Broadley 2010; Broadley et al. 2013; Fehler et al.

2010). The major limitation of this method, is the lack of TAAR1 selective tools, preventing the confirmation of a definitive role for TAAR1 in the vasculature. In lieu of the lack of TAAR1 selective tools, these studies compared the potency orders of the vascular responses to the trace amines to those of cAMP generation in TAAR1 expressing cell lines (Table 1.4.2). In cell lines the potency order for the trace amines at TAAR1 is, Tyramine > β-PEA > Tryptamine > Octopamine (Bunzow et al. 2001). The potency order of the trace amines for vasodilator responses to trace amines was identical to that of cell lines, supporting the hypothesis that TAAR1 mediates the vasodilator effects of the trace amines (Anwar et al. 2012). Although similar, the potency order of vasoconstrictor actions of the trace amines does not fully replicate that of cell lines or vasodilator responses (Fehler et al. 2010). However, without a TAAR1 selective tool the role of TAAR1 in the vasculature is unlikely to be fully defined.

More recently it has been shown that the onset of octopamine-induced contractile responses are significantly reduced in the presence of α_1-adrenoceptor blockade (Broadley and Richards 2015). The authors suggest that the slower contractile response could represent activation of TAAR1 and as such there is a fundamental difference in the vasoconstrictor profile of TAAR1 and α_1-adrenoceptors. The authors continue to suggest that enzyme kinetics (Lineweaver and Burk 1934) can be applied to calculate the affinity of octopamine for TAARs and α_1-adrenoceptors, thereby acting as a tool to unravel TAAR1 responses (Broadley et al. 2013; Broadley and Richards 2015).

The slow onset of constriction is a novel finding that has not been previously recognised. As bolus doses have been utilised in isolated tissue studies (Fehler et al. 2010; Broadley et al. 2013), in-vivo (Bianchetti et al. 1982; Colombo et al. 1989) and in perfusion studies (Anwar et al. 2012; Anwar et al. 2013) it is possible that the slow contractile response to trace amines were unable to fully develop. Many of the previous investigations may therefore have underestimated the vasoconstrictor and pressor responses to trace amines and may have failed to notice the delayed response entirely.

Pilot in vivo studies, from our laboratory, have demonstrated that β-PEA and amphetamine-induced pressor responses in rats are not inhibited by α_1-adrenoceptor blockade. In the presence of prazosin, infusion of β-PEA causes an initial depressor response immediately followed by a pressor response and an extended pressor response. Following infusion, a sustained, extended secondary pressor response to β-PEA has been observed (Figure 1.4.1). This could represent the slower onset contractile response seen in isolated tissue studies.

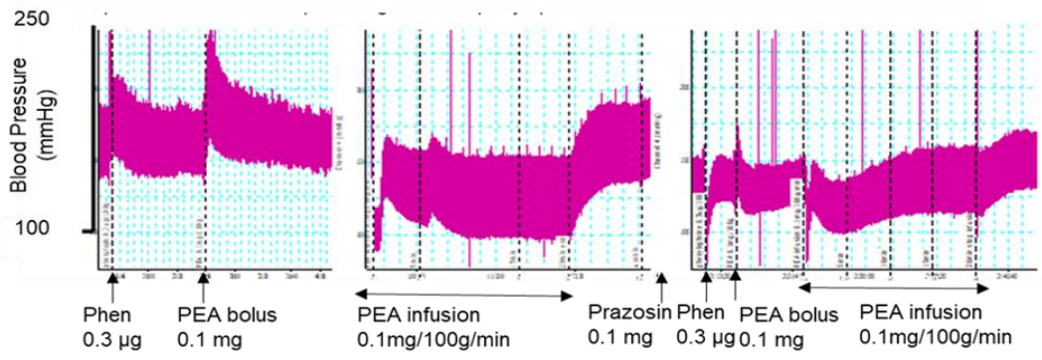

Figure 1.4.1. β-Phenylethylamine (PEA) bolus and infusion on rat blood pressure before and after administration of prazosin. Doses are per 100g. Phen = phenylephrine. ←→ Duration of infusion

The trace amines β-PEA, octopamine and tryptamine induce a negative inotropic effect in isolated rat hearts when used in micromolar concentrations (Frascarelli et al. 2008). Prior to this study β-PEA, octopamine and tyramine were considered positive inotropic agents due to their sympathomimetic actions (Kopin 1968) The TAAR1 agonists T_1AM and thyronamine also induce a negative inotropic effect in isolated rat hearts (Chiellini et al. 2007; Frascarelli et al. 2008). The authors suggest that activation of TAAR1 is most likely to be responsible as the potency rank order of T_1AM and thyronamine to activate TAAR1 functionally in-vitro mirrored the potency rank order to decrease cardiac output (Scanlan et al. 2004; Chiellini et al. 2007; Frascarelli et al. 2008). Interestingly, these endogenous thyroid hormone derivatives are more potent agonists of TAAR1 than the trace amines themselves (Bunzow et al. 2001; Chiellini et al. 2007). This raises the question of whether Thyronamine and T_1AM are the natural ligands of TAAR1 and not the trace amines.

The amalgamation of these findings raises significant questions regarding the trace amines and TAAR1 signalling within the cardiovascular system. Evidence suggests trace amines exert their vasoconstrictor effects through a combination of both direct/indirect sympathomimetic action and through possible direct interaction with TAARs. The vasoconstrictor responses of trace amines are slow in onset (Broadley et al. 2013; Broadley and Richards 2015) which could be representative of kinetics of drug-receptor interaction or post-receptor signalling cascades. It is clear that trace amines have a differential effect in the mesenteric vessels where they induce a predominately vasodilator response (Anwar et al. 2012). TAAR1 may mediate dual vasoconstrictor and vasodilator responses that is dependent on the vascular bed. These dual responses could represent activation of differential signalling cascades or could be dependent on activation of TAAR1 at the level of the endothelium or smooth muscle. The effects of thyronamine and T_1AM are currently unknown within the vasculature, however it is likely they recapitulate the effects seen with trace amines.

*Table 1.4.2. Published potency orders for vascular responses to trace amines compared with published values for cAMP generation in rat TAAR1 transfected cell lines. * in the presence of 5-HT_{2A} antagonists, ritanserin or ketanserin. ** In the presence of α_1- and α_2-adrenoceptor antagonist benextramine.*

-LogEC_{50} values (M)				
Vasodilation in mesenteric vascular bed (Anwar et al. 2012)	Tyramine 4.2 ± 1.5	β-PEA 3.63 ± 0.78	Tryptamine* 2.86 ± 0.09	Octopamine 2.59 ± 0.68
Vasoconstriction in rat aorta (Fehler et al. 2010)	Tryptamine* 5.51 ± 0.12	β-PEA 4,46 ± 0.15	Octopamine 4.36 ± 0.17	Tyramine 3.71 ± 0.29
cAMP generation in rat TAAR1 transfected cell lines (Bunzow et al. 2001)	Tyramine 7.16	β-PEA 6.62	Tryptamine 6.51	Octopamine 5.89
Vasodilation of rat aortic rings (Varma et al. 1995)	Tyramine 4.04 ± 0.20	β-PEA 3.28 ± 0.12	Octopamine** 2.67 ± 0.14	Tryptamine ND

1.4.5 Synthetic TAAR1 agonists and antagonists

Substantial efforts have been committed to the development of synthetic TAAR1 agonists. Investigators at Hoffmann-La Roche generated the first full TAAR1 agonist, RO5166017, through structural modification of the α_2-adrenoceptor agonist S18616 (Revel et al. 2011). Since then, Hoffman-La Roche have subsequently identified other full agonists, such as RO5256390, and partial agonists such as RO5203648 (Revel et al. 2012; Revel et al. 2013). Although the "RO compounds" are highly selective for mouse and rat TAAR1 in radioligand binding assays, at micromolar concentrations the "RO compounds" produce >80% inhibition of specific ligand binding at alternate receptors including, α_2-adrenoceptors, 5-HT (5-HT_{2A} and 5-HT_3), opioid, imdazoline and muscarinic receptors (Revel et al. 2012; Revel et al. 2013).

Despite efforts to identify novel TAAR1 antagonists, to date only a single antagonist compound has been identified. Screening of Roche compounds based on their ability to inhibit cAMP production in cells expressing chimeric human/rat TAAR1 led to the identification of a single compound (Stalder et al. 2011). This was identified as a benzamine derivative, N-(3-Ethoxy-phenyl)-4-pyrrolidin-1-yl-3-trifluoromethyl-benzamide (EPPTB). EPPTB demonstrates strong selectivity and high affinity for mouse TAAR1 with an IC_{50} of 27.5 nM (Bradaia et al. 2009). However, EPPTB has a lower affinity for rat and human TAAR1 with IC_{50}'s of 4539 nM and 7487 nM respectively (Bradaia et al. 2009). Furthermore, EPPTB reduced basal cAMP levels in cell lines, indicating TAAR1 was activated and EPPTB acts as an inverse agonist of TAAR1. Unfortunately, the use of EPPTB for in-vivo studies is limited due to its high clearance and poor solubility (Stalder et al. 2011).

1.5 5-HT receptors

As trace amines are structurally similar to 5-HT (Figure 1.2.1, Lindemann and Hoener 2005), it is possible trace amines may interact with 5-HT receptors. 5-HT receptors can be divided into one of seven families, 5-HT_{1-7}, that comprise a total of 14 structurally and pharmacologically unique receptor subtypes (Andrade et al. 2019).

With the exception of the 5-HT_3 receptor, which is a ligand gated ion channel, all other 5-HT receptor subtypes so far identified are GPCRs (Watts et al. 2012). 5-HT GPCRs can couple to all three canonical signalling pathways through $G\alpha_i$, $G\alpha_{q/11}$ and $G\alpha_s$, allowing them to modulate several different biochemical signalling pathways and illicit complex physiological responses (McCorvy and Roth 2015).

All 5-HT receptor families, excluding 5-HT_6 play important roles in regulating the cardiovascular system (Table 1.5.1, Fidalgo et al. 2013). In humans and animals 5-HT predominantly induces arterial vasoconstriction, largely mediated by 5-HT_{2A} but also partially through 5-$HT_{1B/1D}$ (Watts and Davis 2011; Watts et al. 2012). Nearly all blood vessels exhibit a vasoconstrictor action to 5-HT when mounted in organ baths (Watts et al. 2012). In contrast, very few blood vessels display a relaxation response to 5-HT. In some cases, 5-HT receptors mediating vasoconstriction must first be blocked to unmask 5-HT relaxant responses (McLennan and Taylor 1984). In the rat, 5-HT induces vasodilator responses in the jugular vein and pulmonary and coronary arteries through activation of 5-HT_{2B} and 5-HT_7 receptors (Mylecharane 1990; Mankad et al. 1991; Ellis et al. 1995; Centurión et al. 2004). These vasodilator responses are conserved in larger mammals, including the pulmonary arteries of pigs and coronary arteries of greyhounds (Woodman and Dusting 1994; Glusa and Pertz 2000; Jähnichen et al. 2005). 5-HT induced vasodilation and vasoconstriction has also been reported in rat mesenteric arteries (Anwar et al. 2012). The authors of this study confirmed the vasodilator responses to 5-HT were not mediated by 5-HT_7 receptors. However, the vasoconstrictor action of 5-HT was shown to be mediated by 5-HT_{2A} receptors (Anwar et al. 2012).

The trace amine, tryptamine can induce vasoconstriction of rabbit aorta through direct activation of 5-HT receptors (Stollak and Furchgott 1983). This effect can be attributed to

activation of 5-HT_2 receptors and is unsurprising considering tryptamine is a structural congener of 5-HT (Bradley et al. 1985; Anwar et al. 2012). In the perfused rat mesenteric vascular bed, tryptamine induces a vasoconstrictor response that can be abolished by antagonists of 5-HT_{2A} (Anwar et al. 2012).

Table 1.5.1. Physiology of 5-HT receptors in the cardiovascular system; their agonists and antagonists (adapted from Watts et al. (2012)).

Receptor	Location / Cardiovascular response	Coupling	Agonists	Antagonists	Reference
5-HT_{1A}	Central nervous system (CNS,lower/raise blood pressure)	$G\alpha_i$ ↓cAMP	8-OH-DPAT U92016A	WAY100635 NAN190	Boess and Martin 1994
5-HT_{1B}	Smooth muscle (contraction), sympathetic presynaptic terminal (inhibition of noradrenaline release), CNS lower/raise blood pressure)	$G\alpha_i$ ↓cAMP	CP-930129, sumatriptan, eletriptan (some affinity for 5-HT_{1D})	GR-127935 (some affinity for 5-HT_{1D}), GR55562, isalmoltane, SB236057	McCorvy and Roth 2015
5-HT_{1D}	Smooth muscle (contraction)	$G\alpha_i$ ↓cAMP	PNU-109291, alniditan, eletriptan (some affinity for 5-HT_{1B}), L-703664	SB 272183, LY310762, BRI15572	Hamblin and Metcalf 1991
5-HT_{1E}	None	$G\alpha_i$ ↓cAMP	5-CT (non-selective), BRL54443 (some affinity for 5-HT_{1F})	None	Adham et al. 1994
5-HT_{1F}	Smooth muscle (contraction), trigeminal nerve	$G\alpha_i$ ↓cAMP	LY344370, BRL54443 (some affinity for 5-HT_{1E})	None	Adham et al. 1993
5-HT_{2A}	Platelet (aggregation), smooth muscle (contraction), adrenal gland (epinephrine release), heart (tachycardia, contraction)	$G\alpha_{q/11}$ ↑PLC	DOB (2A/2B, 2C), DOI, alpha-methyl-5-HT (non-selective), TCB-2	R-96544, MDL100907, ketanserin	Roth et al. 1984
5-HT_{2B}	Endothelium (relaxation), smooth	$G\alpha_{q/11}$ ↑PLC	BW723C86	LY272015, RS127445	Loric et al. 1995

	muscle (contraction), cardiac valves (proliferation), cardiomyocte (development)				
5-HT_{2C}	None	$G\alpha_i$ ↓cAMP $G\alpha_{q/11}$ ↑PLC	WAY 163909, DOI (2A, 2B, 2C), MK212, 1-methylpsilocin	RS102221, SB242084	Lucaites et al. 1996
5-HT_3	Vagus nerve (bradycardia)	↑Na^+/K^+ current and neurone depolarisation	2-methyl-5-HT, phenylbiguanide	odansetron, granisetron	Thompson and Lummis 2006
5-HT_4	Cardiomyocyte (contraction)	$G\alpha_s$ ↑cAMP $G\alpha_{q/11}$ ↑Ca^{2+} current	BIMU1, BIMU8, zacopride	GR113808, RS100235	Bockaert et al. 1990,Ponimaskin et al. 2002
5-$HT_{5\ (A, B)}$	None	unknown	None	SB699551	Barnes and Sharp 1999
5-HT_6	None	$G\alpha_s$ ↑cAMP	None	Ro 04–6790, SB 399885, SB271046	McCorvy and Roth 2015
5-$HT_{7\ (a-d)}$	Smooth muscle (relaxation); cardiomyocyte (contraction)	$G\alpha_s$ ↑cAMP	LP12, LP44, AS-19, 5-CT (nonselective)	SB-269970, SB-258719	Obosi et al. 1997

1.6 Adrenoceptors

The close structural similarities of trace amines and noradrenaline suggests trace amines could interact directly with adrenoceptors (Lindemann and Hoener 2005). As sympathomimetic agents, trace amines may also exert their vascular effects through indirect stimulation of adrenoceptors (Broadley 2010). Adrenoceptors were initially divided into two major groups, α- and β-adrenoceptors (Bylund et al. 1994). It is now generally accepted that three groups of adrenoceptors exist, α_1, α_2 and β-adrenoceptors (Bloom et al. 2009). These can then be further subdivided into three more subfamilies (Docherty 2019).

Vascular smooth muscle expresses both α- and β-adrenoceptors, therefore the net response to noradrenaline and adrenaline is dependent on the relative importance of each population (Guimaraes and Moura 2001). In the majority of blood vessels, α-adrenoceptor mediated vasoconstriction predominates, such that β-adrenoceptor mediated vasodilation can only be achieved in the presence of both α_1-adrenoceptor blockade and active tone (Tanaka et al. 2005).

1.6.1 α_1-adrenoceptors

A total of three unique α_1-adrenoceptor subtypes have been identified (Cotecchia et al. 1988; Schwinn et al. 1990; Lomasney et al. 1991; Perez et al. 1991). The accepted nomenclature for these receptors is α_{1A}, α_{1B} and α_{1D} (Hieble et al. 1995). All three receptors couple through $G_{q/11}$ to increase intracellular Ca^{2+} and activate protein kinase C (Hague et al. 2003).

In the vasculature, α_1-adrenoceptors serve as a stimulatory receptor mediating smooth muscle contraction and have a major role in control of blood pressure (Docherty 2010). Although all three α_1-adrenceptor subtypes are present in most blood vessels, the individual contribution of each α_1-adrenoceptor to vascular tone can vary (Bloom et al. 2009). Of the three subtypes α_{1A}- and α_{1D}-adrenoceptors are most commonly implicated in regulating vascular smooth muscle tone (Guimaraes and Moura 2001). By comparison α_{1B}-adrenoceptors appear to have only a minor contribution (Broadley 1996). However, α_{1B}-adrenoceptors appear to have an important role in determining vascular responsiveness (Daly and McGrath 2011). For example, in α_{1B}-adrenoceptor knock out mice, contractions in tail artery develop more slowly but in the aorta are unaffected (Daly et al. 2002)

1.6.2 α_2-adrenoceptors

α_2-adrenoceptors are located throughout the central and peripheral nervous system (Bloom et al. 2009). They are found both pre- and post-synaptically, and although generally considered inhibitory can also mediate excitatory responses (Civantos Calzada and Aleixandre de Artinano 2001). Pre-synaptic α_2-adrenoceptors, located on sympathetic neurones, have a sympatholytic effect, inhibiting noradrenaline release (Bloom et al. 2009). Post-synaptic α_2-adrenoceptors have been positively identified in liver cells, platelets, vascular smooth muscle and vascular endothelium (Civantos Calzada and Aleixandre de Artinano 2001; Vanhoutte 2001). Their activation results in platelet aggregation, vasoconstriction and release of nitric oxide from endothelial cells (Martinotti 1991; Vanhoutte 2001).

Three α_2-adrenocepors have been cloned and characterised in mammals (Bylund et al. 1994). α_{2A}-adrenoceptors are widely distributed throughout the mammalian brain (Bylund et al. 1994). In contrast α_{2B}-adrenoceptors are almost exclusively found in the thalamus, whereas the α_{2C}-receptor is found in the olfactory bulb, cerebral and cerebellar cortex and dorsal route ganglia (Bloom et al. 2009). In humans, pigs and rabbits the α_{2A}-adrenoceptor has a different pharmacological profile compared with that of rat, guinea pig, mouse and cow (Ruffolo et al. 1991). Consequently, researchers will often refer to the latter as the α_{2D}-adrenoceptor (Bloom et al. 2009).

Vasoconstrictor responses to α_2-adrenoceptors are generally restricted to small resistance arteries and arterioles (Guimaraes and Moura 2001). However, evidence suggests small populations of α_2-receptors may be involved in the contractile responses to α-adrenoceptor agonists in rat aortic rings (Piascik et al. 1996). A role for α_2-adrenoceptors in mediating vasodilator responses has also been observed (Martinotti 1991). Vasodilator responses to α_2-adrenoceptor agonists can be abolished by endothelium removal or inhibitors of the nitric oxide pathway (Vanhoutte 2001). This suggests, activation of α_2-adrenoceptors leads to stimulation of endothelial nitric oxide synthase (eNOS). However, it has been suggested that basally released nitric oxide may potentiate the direct inhibitory action of α_2-adrenoceptor agonists on vascular smooth muscle (Bryan et al. 1995).

1.6.3 β_1-adrenoceptors

β_1-adrenoceptors are generally post-synaptic, although can also be found pre-synaptically (Heimburger et al. 1989). They are predominantly located in the heart where they account for 70-80% of cardiac β-adrenoceptors (Brodde 2008). Activation of cardiac β_1-ardrenoceptors leads to a positive chronotropic and iontropic effect (Bylund et al. 1994). This is achieved through positive coupling via $G\alpha_s$ to adenyl cyclase and activation of cAMP dependent protein kinase A (de Lucia et al. 2018).

Activation of pre-synaptic β_1-adrenoceptors increases noradrenaline release from sympathetic neurones (Heimburger et al. 1989). Pre-synaptic receptors coexist with β_2-adrenoceptors in both cat and rat hypothalamus (Misu and Kubo 1986). It has been suggested, pre-synaptic receptors may facilitate the development of essential hypertension and that anti-hypertensive effects of β-adrenoceptor antagonists may in part be due to antagonism of pre-synaptic receptors (Misu and Kubo 1986). However, this has not been further investigated.

In the vascular system, expression of β_1-adrenoceptors is somewhat limited (Tanaka et al. 2005). Their expression appears to be restricted to the coronary arteries, where they are the predominant receptor mediating epicardial vasodilation in numerous species including humans (Nakane et al. 1988). However, the importance of β_1-adrenoceptors in mediating vascular tone may have been overlooked. In isolated blood vessels from mice, the β_1-adrenoceptor is the prominent adrenoceptor involved in vasodilation (Chruscinski et al. 2001). Although, it is possible that this is a species dependent difference that is not conserved in higher mammals.

1.6.4 β_2-adrenoceptors

β_2-adrenoceptors are mainly post-synaptic, although like β_1-adrenceptors there is evidence of pre-synaptic activity (Misu and Kubo 1986). They are expressed in wide variety of tissues including skeletal muscle, liver, the gastrointestinal tract, vascular and airway smooth muscle (Bylund et al. 1994; Lands et al. 1967). Like all other β-adrenoceptor subtypes, β_2-adrenoceptors are coupled to cAMP production and smooth muscle relaxation (Guimaraes and Moura 2001).

It is now widely accepted that β_2-adrenoceptors are expressed on vascular endothelial cells (Vanhoutte 2001). Relaxation of a variety of arteries by β_2-adrenoceptor agonists can be reduced by removal of the endothelium or inhibitors of eNOS (Rubanyi and Vanhoutte 1985). Speculation that incomplete removal of endothelium may account for the remaining vasodilator response can be disregarded, since isoprenaline induced relaxation remained despite a lack of acetylcholine-evoked relaxant response (Brawley et al. 2000a). It is clear that β_2-adrenoceptor-mediated relaxation involves both endothelium-dependent and endothelium-independent components (Brawley et al. 2000b)

1.6.5 β_3-adrenoceptors

β_3-adrenoceptors differ from classical β_1- and β_2-adrenoceptors with regards to their pharmacological profile (Gauthier et al. 1996). They are selectively activated by pharmacological agents including, BRL-37344 and SR-58611, that lack any activity at β_1- and β_2-adrenoceptors (Bylund et al. 1994). β_3-adrenoceptors have demonstrated a variety of biological effects in several different tissues (Table 1.6.1).

In vascular tissues, expression of β_3-adrenoceptors has been confirmed in endothelial cells of rat thoracic aorta where it acts to stimulate eNOS (Trochu et al. 1999). The β_1-adrenoceptor antagonist nebivolol was later verified to dilate both human and rodent coronary arteries, an effect that was sensitive to eNOS inhibition and abolished in β_3-adrenoceptor deficient mice (Dessy et al. 2004; Dessy et al. 2005). These studies suggest agonists of β_3-adrenoceptors could be used as potential therapeutics to treat cardiac ischaemia (Conti et al. 2013; Dessy et al. 2004; Dessy et al. 2005).

Table 1.6.1. Expression and function of β_3-adrenoceptors.

Tissue	Function	Reference
White adipose tissue	Lipolysis	Lafontan 1994
Brown adipose tissue	Thermogenesis	Lafontan 1994
Gastrointestinal smooth muscle	Relaxation	Manara et al. 1995
Airway smooth muscle	Relaxation	Martin and Advenier 1995
Cardiac	Negative inotropy	Gauthier et al. 1996
Vascular endothelium	vasodilation	Trochu et al. 1999

1.7 Dopamine receptors

With regards to structure, synthesis and metabolism, dopamine is closely related to the trace amines (Grandy et al. 2016). Furthermore, dopamine is a known, albeit, weak agonist of TAAR1 (Bunzow et al. 2001). It is therefore possible that trace amines could mediate their effects through interactions with dopamine receptors. The physiological effects of dopamine are mediated by five subtypes of dopamine receptors (D_1 ,D_2, D_3, D_4, D_5, Beaulieu et al. 2015). Dopamine receptors are classified as D_1-class (D_1 and D_5) or D_2-class (D_2, D_3 and D_4) based on their ability to stimulate or inhibit production of cAMP through coupling to $G\alpha_s$ or $G\alpha_{i/o}$ respectively (Missale et al. 1998).

D_1-class receptors are exclusively found post-synaptically on dopamine receptive cells, whereas D_2-class receptors are located both pre- and post-synaptically on dopamine target cells and dopaminergic neurones (Rankin et al. 2010; Beaulieu and Gainetdinov 2011). In the vasculature, D_1-class receptors are expressed post-synaptically on vascular smooth muscle where they mediate vasodilation (Li et al. 2008). Unlike D_1-class receptors, vascular D_2-class receptors are located pre-synaptically, where they act to inhibit noradrenaline release promoting vasodilation (Murphy 2000). Therapeutically, the vasodilator effects of dopamine occur at low doses between 0.5 – 3.0 $\mu g/kg^{-1}/min^{-1}$. Although, it is well established that at higher doses (10.0 – 20.0 $\mu g/kg^{-1}/min^{-1}$) a vasoconstrictor response, mediated through binding to α_1-adrenoceptors, dominates (Overgaard and Dzavik 2008).

1.7.1 D_2-class receptor interactions with TAAR1

A functional-physical interaction between D_2-like receptors and TAAR1 has been reported in a number of studies both in-vitro and in-vivo. When co-expressed in human embryonic kidney 293 (HEK-293) cells, TAAR1 forms heterodimers with post synaptic D_2-class receptors (Espinoza et al. 2011). Although TAAR1 is predominately found intracellularly, co-expression with D_2-class receptors increases cell surface expression of TAAR1 (Espinoza et al. 2011; Harmeier et al. 2015). TAAR1-D_2 heterodimers have also been observed in midbrain and cortex membrane preparations of TAAR1 overexpressing rats through co-immunoprecipitation of TAAR1 and D_2-class receptors (Harmeier et al. 2015). Interestingly antagonists of D_2-class receptors enhanced TAAR1 signalling in the HEK293 cells (Espinoza et al. 2011).

It is well established that D_2-class receptor signalling decreases cAMP production through $G\alpha_{i/o}$ signalling (Missale et al. 1998). However, D_2-class receptors can simultaneously recruit β-arrestin 2 in a G-protein-independent manner (Beaulieu and Gainetdinov 2011). Recruitment of β-arrestin 2 leads to dephosphorylation of protein kinase B (AKT) and subsequent dephosphorylation of glycogen synthase kinase-3β (GSK-3β) thereby increasing GSK3β activity (Gainetdinov et al. 2018). Normally, TAAR1 interacts poorly with β-arrestin 2 (Grandy et al. 2016). However, TAAR1-D_2 receptor heterodimers have been shown to significantly enhance interaction between TAAR1 and β-arrestin 2, leading to a reduction in GSK3β activation (Harmeier et al. 2015). In the striatum of TAAR1 knock out mice, basal levels of phosphorylated AKT and GSK3β are significantly reduced (Espinoza et al. 2015).

As a result, GSK3β activity is significantly increased in TAAR1 knockout animals. Together with the findings of Harmeier et al. (2015), these results clearly demonstrate TAAR1-D_2 receptor heterodimerisation negatively regulates AKT/GSK3β signalling.

Efficient activation of eNOS has been reported to require phosphorylation of eNOS at Serine-1177 by AKT (Dimmeler et al. 1999; Fulton et al. 1999; Manning and Cantley 2007). Activation of AKT, via phosphatidylinositol 3-kinase (PI3-K), enhances eNOS activity resulting in increased nitric oxide production and subsequent vasodilation (Manning and Cantley 2007). D_2-like receptors are expressed in endothelial cells where they inhibit histamine-induced release of von Willebrand factor (Zarei et al. 2006). TAAR1-D_2 heterodimers may therefore act to inhibit endothelial nitric oxide release through dephosphorylation of AKT and subsequent absence of eNOS phosphorylation.

1.8 Monoamine transporters

The localisation of TAAR1 intracellularly (Miller et al. 2005; Xie et al. 2008) necessitates that trace amines must gain access to the intracellular compartment to exert their effects. As trace amines are characterised by a short half-life of less than 30 seconds (Durden and Philips 1980), mechanisms must exist to facilitate their rapid transport across membranes. As trace amines are relatively less polar than their corresponding neurotransmitter counterpart (Oldendorf 1971), it was suggested trace amines could freely diffuse across biological membranes (Berry 2004). Both tyramine and β-PEA have been shown to readily diffuse across synthetic lipid bilayers (Berry et al. 2013). However, previous work demonstrated tyramine and β-PEA release from synaptosomes was not increased by potassium-induced depolarisation (Dyck 1989), indicating trace amine release not to be mediated by exocytosis and therefore inconsistent with basic diffusion. Berry et al. (2016) suggested that the lack of increase of trace amines following depolarisation indicates the involvement of one or more transporters that are responsible for mediating synaptic trace amine levels.

A number of transporter proteins are reported to display affinity for the trace amines. These transporters can be classified as neuronal and extra-neuronal, which correspond to the uptake-1 and uptake-2 pathways described by Iversen (1973). Uptake-1 transporters include DAT, NET and SERT (5-HT transporter, Torres et al. 2003). These transporters display a high selectivity, but low capacity, for their corresponding neurotransmitters and are dependent on extracellular Na^+ (Rudnick et al. 2014). Although considered highly specific, the trace amines have all been recognised as substrates for these transporters (Raiteri et al. 1977; Danek Burgess and Justice 1999; Liang et al. 2009; Underhill et al. 2019). Although this has only been demonstrated at high micromolar to millimolar concentrations of trace amine, far beyond the normal physiological concentration of trace amines (Bunzow et al. 2001). In rat aortic rings, the non-selective uptake 1 pathway competitive antagonist cocaine (Torres et al. 2003) failed to abolish β-PEA-induced contractile responses (Broadley et al. 2013). Furthermore, in the perfused rat mesenteric vascular bed tryptamine responses were significantly potentiated by the presence of cocaine (Anwar et al. 2013). Considering the high micromolar to millimolar affinity of uptake-1 pathways and failure of cocaine to block vasoconstrictor responses to trace amines it is unlikely that uptake-1 pathways mediate plasma membrane transport of trace amines in the vasculature.

Uptake-2 transporters include the Organic Cation Transporter (OCT1-3; Slc22A1-3, Koepsell 2020) family of transporters and the Plasma Membrane Monoamine Transporter (PMAT; Slc29A4, Wang 2016a). These are considered polyspecific, low-sensitivity and high-capacity transporters, thought to be responsible for clearance of synaptic neurotransmitters following saturation of uptake-1 pathways (Courousse and Gautron 2015; Koepsell 2020). Trace amines have been reported as substrates of all four of these transporters (Engel and Wang 2005; Schomig et al. 2006; Chen et al. 2010; Berry et al. 2016). OCT1 and OCT2 display a nanomolar affinity for tyramine making them strong candidates for being the main transporter of trace amines (Schomig et al. 2006; Berry et al. 2016). OCT1-2 are primarily expressed in

the liver and kidney, where they are believed to be involved in systemic removal of organic cations (Gorboulev et al. 1997; Engel and Wang 2005), though OCT1 is also reportedly expressed in smooth muscle (Chen et al. 2010). By contrast OCT3 displays a broad expression and has been identified in many tissues including heart, skeletal and smooth muscle, placenta, kidney and brain (Wu et al. 2000; Chen et al. 2010). PMAT is most highly expressed in the brain and skeletal muscle, although transcripts have also been identified in the liver, kidney and heart (Engel et al. 2004). To date a role for uptake-2 pathways has not been investigated in vascular trace amine responses. Considering OCT1 and OCT2 have an affinity for the trace amines within their physiological range (Schomig et al. 2006; Berry et al. 2016) and OCT3 is highly expressed in smooth muscle (Chen et al. 2010), it is possible that transport of trace amines via one or all of these transporters is required to mediate the vascular effects of trace amines.

1.9 Amphetamines and amphetamine-like sympathomimetic amines

Amphetamine and methamphetamine are potent psychoactive drugs that cause wakefulness and euphoria which often leads to addiction (Sulzer et al. 2005). As illicit substances, amphetamines are second only to cannabis in global usage, with methamphetamine usage in particular, increasing in many regions including North America, Oceania and most parts of Asia (Drugs and Crime 2017). According to the Office for National Statistics, in the UK, use of the amphetamine-like illicit substance 3, 4-methylenedioxymethamphetmine (MDMA or ecstasy) has fallen over the last decade (Statistics 2017).The use of ecstasy can be attributed to its unusual prosocial and empathogenic effects (Bershad et al. 2016).

Mephedrone (4-methylmethcathinone) is a synthetic cathinone which became widely available as a recreational drug in Europe in 2008 (Dargan et al. 2010). The popularity of this drug was largely due to its widespread availability from internet suppliers and ability to elicit stimulant and empathogenic effects similar to amphetamine and cocaine (Schifano et al. 2011). However, it was not until 2010 that mephedrone joined the list of banned substances as a class B drug under the UK misuse of drugs act (Green et al. 2014).

(-)-S-cathinone is a sympathomimetic amine with similar properties to amphetamine and is the active constituent of Khat leaves (Kalix 1984). Chewing of Khat leaves is a social practice in East Africa and areas of the Middle East (Drake 1988). More recently this practice has spread with Ethnic communities worldwide, including the UK (Kassim and Croucher 2006). The practice of chewing Khat leaves leads to a euphoric experience which can be attributed to the amphetamine-like properties of (-)-S-cathinone (Brenneisen et al. 1990).

Amphetamine-like drugs are often used therapeutically. Methylphenidate (Ritalin^{TM}) is commonly used for the treatment of children with ADHD (Pelham et al. 1999). Other therapeutic amines include over the counter nasal decongestants, such as ephedrine and pseudoephedrine (Johnson and Hricik 1993). The former, is the precursor of all synthetic amphetamines (Broadley 2010) and is the active ingredient of the ephedra plant, also known as Ma huang in traditional Chinese medicine. Ma huang is one of the oldest medicinal herbs known to mankind and has been used as a stimulant and anti-asthmatic for over 5000 years (Chen and Schmidt 1926). Phenylpropanolamine is another sympathomimetic amine which is widely used as a dietary aid (Kernan et al. 2000). The weight loss effect of phenylpropanolamine can be attributed to suppression of appetite and increased thermogenic lipolytic activity of brown adipocytes (Wellman 1984; Wellman and Sellers 1986).

1.10 Adverse cardiovascular effects of trace amines and amphetamines

Excessive levels of circulating trace amines and amphetamines are associated with detrimental cardiovascular events (Broadley 2010). Elevated plasma trace amines have been reported in numerous clinical disorders including primary headaches (D'andrea et al. 2004). Excessive plasma trace amines are more prominent in patients with cluster headaches than migraine (Farooqui 2016). Dietary biogenic amines have long been considered triggers of migraine headaches (Hanington and Harper 1968; Peatfield et al. 1983). Ingestion of tyramine from cheese and β-PEA from chocolate are among the most frequently documented triggers in the literature (Salfield et al. 1987; Millichap and Yee 2003). Numerous studies support tyramine as a migraine trigger (Kohlenberg 1982; Vaughan 1994; Borkum 2016). However, others have concluded that there is no evidence of an association between dietary tyramine and onset of migraine headaches (Jansen et al. 2003). Furthermore, dietary β-PEA has not been confirmed as a definitive migraine trigger, indeed other constituents of chocolate and wine such as the flavonoids may be the true cause (Borkum 2016)

Elevated levels of circulating trace amines may also play a key role in hypertension. Increased levels of tyramine have been observed in the plasma of hypertensive patients (Andrew et al. 1993). Furthermore, infusion of tyramine causes a significantly larger increase in systolic blood pressure in hypertensive individuals compared with normotensives (Colombo et al. 1989). This evidence indicates that tyramine may have an important physiological role in blood pressure regulation in hypertensive individuals. Alternatively, hypertensives could be more sensitive to the vasoconstrictor actions of tyramine (Broadley 2010).

Negative cardiovascular effects are commonly associated with usage of illicit drugs. Abuse of amphetamines and MDMA is known to cause cardiovascular toxicity, through the release and accumulation of catecholamines and 5-HT into the synaptic cleft and circulation (Milroy et al. 1996; Carvalho et al. 2012). Strong links have been reported between acute myocardial infarction (AMI) and the chewing of Khat leaves (Al-Motarreb et al. 2002). This can be attributed to vasoconstrictor action of the active ingredient of Khat, (-)-S-cathinone, in the coronary vessels and aorta (Al-Motarreb and Broadley 2003).

Severe cardiovascular events have also been associated with therapeutic usage of sympathomimetic and amphetamine-like amines. Use of the nasal decongestant pseudoephedrine and the slimming aid phenylpropanolamine have been associated with AMI and acute myocardial ischaemia (Oosterbaan and Burns 2000; Pederson et al. 2001; Manini et al. 2005). More than 30 cases of intracranial haemorrhage have been reported following ingestion of phenylpropanolamine, which lead to the food and drug administration (FDA) declaring the drug unsafe for over-the-counter medications (Kernan et al. 2000; Yakoot 2012). Use of ephedrine and ephedrine containing products has been linked with increased incidence of ischaemic and haemorrhagic stroke (Wooten et al. 1983; Chen et al. 2004).

1.11 Vascular responses to trace amines

It is well established that an increase in blood pressure is observed following oral administration of tyramine (Peatfield et al. 1983), ephedrine (Berlin et al. 2001), Ma huang (Gurley et al. 1998), cathinone (Brenneisen et al. 1990), phenylpropanolamine (Biaggioni et al. 1987), methylphenidate (Wilens et al. 2005), amphetamine (Sprigg et al. 2007) and MDMA (Shenouda et al. 2010).

As a sympathomimetic, tyramine may be used as a pharmacological tool to assess noradrenaline release from sympathetic neurones when administered intravenously. Also referred to as the tyramine pressor test, this can be defined as the amount of tyramine

administered by bolus injection required to cause an increase of 30mm Hg in systolic blood pressure (Ghose 1984).

The increase in blood pressure, observed during the tyramine pressor test, has been attributed to an increase in cardiac output but not due to vasoconstriction (Meck et al. 2003). The tyramine-induced increase in blood pressure can be abolished using the β_1-adrenoceptor antagonist, bisoprolol, but is unaffected by α_1-adrenoceptor antagonist, doxazosin indicating the increase in blood pressure is purely of cardiac origin (Schäfers et al. 1997). Conversely tyramine causes a decrease in total peripheral resistance, which could indicate the involvement of a baroreceptor mediated reflex response to the initial increase in blood pressure (Meck et al. 2003). Infusion of tyramine in healthy individuals, causes an increase in systolic but not diastolic pressure (Jacob et al. 2003). Interestingly, this study also observed a paradoxical increase in forearm blood flow in response to tyramine despite causing local release of noradrenaline. However, this was later found to be a result of dopamine contamination, as dopamine-free preparations did not increase forearm blood flow (Jacob et al. 2005).

1.12 Mechanisms of vascular responses to trace amines

The recognised mechanism for the vascular effects of trace amines is that they act indirectly as sympathomimetic amines (Broadley 2010). This is achieved through promoting the efflux of noradrenaline from sympathetic neurones, which then interacts with adrenoceptors producing a sympathomimetic effect. However, this may be an oversimplification as there are several observations indicating that other noradrenaline independent mechanisms may also play a role. For example, during the tyramine pressor test, there is a dissociation between plasma noradrenaline levels and changes in blood pressure (Chalon et al. 2002).

Depletion of noradrenaline stores by reserpine has been shown to incompletely inhibit pressor responses to tyramine in spinal cats (Burn and Rand 1958). Furthermore, pre-treatment with reserpine fails to completely inhibit tyramine responses in rabbit (Hudgins and Fleming 1966) and rat aorta and atria (Maling et al. 1971; Rice et al. 1987). Cocaine, an inhibitor of monoamine transport into sympathetic neurones (Fleckenstein et al. 2000), fails to completely inhibit contractile responses to tyramine (Furchgott et al. 1963). Furthermore, this study demonstrated contractile responses, following reserpine treatment, were not affected by cocaine and indicating they were a direct response to tyramine. Trace amines demonstrate tachyphylaxis such that repeated dosage results in a declining response (Day 1967). This is most likely due to exhaustion of stored noradrenaline which is replaced in the vesicle by the less active trace amine. Generally, these responses are not completely abolished suggesting that any residual activity is independent of noradrenaline (Broadley 2010). However, between certain amines; including dexamphetamine and tyramine, there is a distinct lack of tachyphylaxis (Day 1967). Day suggested the lack of cross-tachyphylaxis is due to differential mechanisms of noradrenaline release. To date no clear explanation of the lack of cross tachyphylaxis has been provided (Broadley 1996). Despite these findings little research has been carried out into the underlying vascular mechanisms of amines.

Evidence suggests trace amines also exert their effects by activating alternative receptors to the adrenoceptors. Tryptamine causes vasoconstriction which can be inhibited by multiple antagonists of the 5-HT receptors (Stollak and Furchgott 1983). Other amines and amphetamines including tyramine, methylphenidate and amphetamine are also known to induce dopamine release (Jacob et al. 2003; Iversen 2006). Vasoconstriction of coronary arteries by cathinone, MDMA and β-PEA has been demonstrated to be independent of α_1-adrenoceptors (Al-Motarreb and Broadley 2003; Baker et al. 2007; Herbert et al. 2008; Broadley et al. 2009). It has been reported that tyramine, β -PEA, amphetamine, octopamine, cathinone, MDMA and methylphenidate induce concentration-dependent vasoconstriction of rat aortic rings (Broadley et al. 2013). The contractile responses of all, with the exception of octopamine, were shown to be resistant to blockade of α_1-adrenoceptors, β_2-adrenoceptors, inhibition of neuronal transport and inhibition of

monoamine oxidase B. Furthermore α_2-adrenoceptor mediated vasoconstriction in response to β-PEA was also ruled out through blockade of receptors using yohimbine (Broadley et al. 2013).

Octopamine was the only trace amine antagonised by the cocktail of inhibitors. Addition of inhibitors induced a rightward shift of the cumulative concentration response curve (CRC). This shift was concluded to be a result of α_1-adrenoceptor blockade, although was not further investigated (Broadley et al. 2013). Interestingly, in the presence of inhibitors, the octopamine contractile responses are significantly slower in onset than in the absence of inhibitors.

In rat aorta, tryptamine-induced vasoconstriction was resistant to 5-HT receptor antagonists (Fehler et al. 2010; Broadley et al. 2013). However, in the perfused rat mesentery tryptamine-induced vasoconstriction was completely abolished by antagonists of 5-HT receptors (Anwar et al. 2012). Furthermore, it was later demonstrated that in the presence of the eNOS inhibitor L-NAME, small vasoconstrictor responses to tryptamine can be restored during 5-HT receptor blockade, a result which was speculated to be a result of activation of TAARs (Anwar et al. 2013). A more recent investigation in rat gastric fundus demonstrated the contractile effects of β-PEA were significantly reduced by blockade of 5-HT receptors (Batista-Lima et al. 2018). Furthermore, the contractile effects of β-PEA were not inhibited by the TAAR1 selective antagonist EPPTB. The authors suggested that under resting tone, at high concentrations, β-PEA predominantly induces contraction through recruitment of 5-HT receptors (Batista-Lima et al. 2018).

The vascular action of trace amines is not limited to vasoconstriction. The trace amines were first reported as vasodilators by Varma and Chemtob (1993). A subsequent study demonstrated these vasodilator responses to be independent of the presence of a functional endothelium (Varma et al. 1995). Furthermore, these responses were not exerted by α_1-, α_2-, β_1- and β_2-adrenoceptors or receptors for 5-HT, histamine and adenosine (Varma et al. 1995). The authors hypothesised the vasodilator responses were mediated via novel tyramine receptors. Intriguingly the vasodilator potencies, were remarkably similar to those in rat mesenteric arteries (Anwar et al. 2012) and TAAR1 expressing cell lines (Bunzow et al. 2001). In the perfused rat mesenteric vascular bed pre-constricted with phenylephrine, tryptamine, β-PEA and tyramine have all demonstrated the ability to induce relaxation (Anwar et al. 2012). In contrast to vasodilation in the aorta, this was shown to be dependent on endothelium derived nitric oxide (Anwar et al. 2012). Based on the potency order of vasodilation (Table 1.4.2), the authors hypothesised that TAAR1 mediated the vasodilator response. More recently a relaxant effect of β-PEA was shown in rat gastric fundus (Batista-Lima et al. 2018). This study went further to demonstrate β-PEA induced relaxation of pre-constricted tissue that could be enhanced by blockade of 5-HT receptors and inhibited by EPPTB (Batista-Lima et al. 2018). The authors suggested a dual role of β-PEA as both a contractile, mediated by 5-HT receptors, and relaxant agent, mediated by TAAR1 (Batista-Lima et al. 2018)

Other possible vasoconstrictor mechanisms including angiotensin, histamine H_1, endothelin, prostacyclin and leukotrienes have also been ruled out in previous studies using guinea pig aorta and porcine coronary arteries (Baker and Broadley 2003; Herbert et al. 2008). However, it is unknown if these mechanisms play a role in trace amine mediated responses in the rat. Furthermore TAAR1 displays considerable sequence divergence across species (Lindemann et al. 2005), which could result in species dependent differences in trace amine-induced contractile responses. Trace amine-induced contractile responses may therefore be more complicated than originally thought. It is possible that trace amines may induce both smooth muscle contraction and relaxation through alternative mechanisms that are dependent on the tissue or vascular bed. It is currently unclear whether the vasoconstrictor or vasorelaxant effects of trace amines predominate in-vivo or in which vascular beds each mechanism predominates.

1.13 Hypothesis

Trace amines and amphetamines induce vasoconstriction and vasodilation through direct/indirect interaction with classical neurotransmitter receptors and activation of TAARs. TAAR mediated vasoconstriction is slower in onset and sustained. The TAAR pressor response and sympathomimetic pressor responses vary between different vascular beds. TAAR mediated vasodilation occurs via endothelial release of nitric oxide.

1.14 Aims

1. Determine the importance of the endothelium in trace amine-induced vascular responses.
2. Examine the time course of trace amine-induced vasoconstrictor responses.
3. Establish whether trace amines can induce vasodilator responses of rat aorta.
4. Confirm the involvement of TAARs in trace amine-induced vascular responses.
5. Investigate trace amine-induced vascular responses in conduit and resistance vessels.
6. Investigate the underlying mechanisms of in-vivo trace amine-induced vascular responses.

Chapter 2 Materials and Methods

This chapter details the materials and methods used for all experimental work described in this study. Subsequent chapters will provide a brief overview of the methods and details of specific protocols used.

2.1 Animals

Male and female Sprague Dawley rats (males 110-750g, females 290-350g) were obtained from Charles River (Harlow, United Kingdom). Lister hooded rats (males 200-350g) and female Sprague Dawley ex-breeders (300-450g) were a gift from the JBIOS unit at Cardiff University. Rats were maintained in conventional animal housing, subjected to a 12-hour light-dark cycle (8am-8pm) at a room temperature of 21^{o}C and humidity of 55 %. All experiments adhered strictly to the Animals (Scientific Procedures) Act 1986. Male animals above 350g and females above 250g, weights that correspond with the age of sexual maturity (Sengupta 2013), were considered as mature adults, below this threshold they were considered juvenile.

2.2 Rat aortic rings

Rats were killed by intraperitoneal injection of sodium pentobarbitone (Merial, Woking UK). Death was confirmed upon cessation of circulation. The thoracic aorta was removed cleaned of perivascular adipose tissue and cut into ring sections approximately 3 - 5 mm in length. One fixed and one mobile hook were then passed through the ring sections, the fixed hook was then secured inside a 20 mL organ bath. Organ baths were filled with prewarmed (37^{o}C) Krebs bicarbonate buffer which was continuously gassed with CO_2/O_2 (5%/95%) (BOC gasses, Guildford, United Kingdom). Krebs bicarbonate buffer was made up in distilled water and comprised of: NaCl (118 mM), KCl (4.7 mM), $NaHCO_3$ (25 mM), $MgSO_4 \cdot 7H_2O$ (1.2 mM), KH_2PO_4 (1.2 mM) $CaCl_2 \cdot 2H_2O$ (2.5 mM) and glucose (10 mM). The mobile hook was connected to an isometric transducer (Dynamometer UF1, 57g sensitivity range, Pioden Controls, Ltd Canterbury) and a resting tension of 1.5g was applied. Isometric tension was measured and displayed on a computer using Powerlab chart 5 software (AD instruments, Oxford, United Kingdom). In endothelium-denuded experiments, the endothelium was removed prior to mounting in organ baths by gentle rolling of the aortic ring around a metal wire.

2.2.1 Aortic ring experimental protocol

Following a 1-hour equilibration period, aortic rings were washed with prewarmed Krebs bicarbonate solution. Pre-warmed high potassium (60mM) Krebs bicarbonate solution was then added to organ baths to contract tissue and confirm viability. High potassium Krebs buffer was made by adjusting the NaCl concentration from 118mM to 62.7mM and the KCl to 60mM to maintain iso-osmolarity. At the peak of contraction, the presence of a functional endothelium was confirmed by relaxation to 100 μM carbachol. Rings were considered endothelium intact following a relaxation of ≥10% of the contractile response to 60mM KCl. After washout and equilibration, cumulative and non-cumulative concentration response curves (CRC's) were obtained in the absence or presence of antagonists. Antagonists were added 15 minutes prior to constructing CRCs or after each dose wash out in the case of non-cumulative CRC's. To construct CRCs, successive concentrations of agonist were added in half logarithmic increments with each response being allowed to plateau before subsequent bolus addition. Following drug washout, tissue viability was again confirmed using high potassium Krebs bicarbonate solution to contract tissue. At the peak of contraction, the presence of a functional endothelium was again confirmed by relaxation to 100μM carbachol.

2.2.2 5-HT desensitisation

G-protein coupled receptor (GPCR) desensitisation can be described as the loss of response following prolonged or repeated administration of an agonist due to uncoupling of the receptor from its signalling cascade (Rajagopal and Shenoy 2018). GPCR desensitisation can be classified as heterologous or homologous. Homologous desensitisation occurs following uncoupling of receptors specific to the agonist (Kelly et al. 2008). The alternative, heterologous desensitisation, results in the loss of response at multiple GPCRs even in the absence of ligand binding (Kelly et al. 2008).

In the current study, GPCR desensitisation was used as an alternative to multiple antagonists, to assess the role of all 5-HT receptors present in the rat aorta. Initial cumulative CRC's were generated for both 5-HT and β-PEA. Once the maximum response or concentration was reached, aortic rings were washed out and allowed to reach baseline tension. A concentration of 5-HT in excess of that required to elicit a maximal contractile response (100 µM) was then added and aortic rings incubated for 1-hour. Following incubation, aortic rings were washed and allowed to reach baseline tension. Aortic rings were then subjected to 3 1-hour challenges with a concentration of 5-HT that induced a sub-maximal contractile response (10 µM), with additional washes between each challenge. Contractile responses to 5-HT were considered desensitised following a decline in the response to the submaximal concentration of 5-HT (10 µM) to ≤ 50% of the initial response. After confirming desensitisation, a second cumulative CRC, to either 5-HT or β-PEA, was generated.

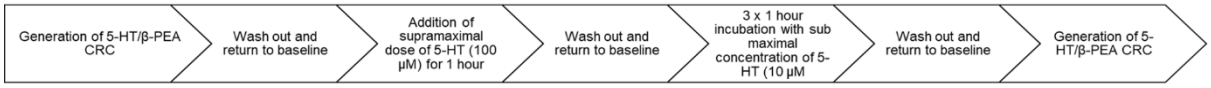

Figure 2.2.1. Method of attempted 5-HT receptor desensitisation in rat aortic rings.

2.3 Thin wire myography

Rats were killed by intraperitoneal injection of sodium pentobarbitone (Merial, Woking UK). Following confirmation of death by cessation of circulation, the entire mesenteric bed and attached small intestine was isolated and removed. The mesenteric bed was then pinned out in a petri dish in ice-cold Krebs bicarbonate solution. Third-order branches of the arterial tree were then identified (Figure 2.3.1) and cleared of connective tissue and fat under a dissection microscope. Using straight spring scissors (InterFocus Ltd, Linton) a small incision was made in the vessels through which the vessel was cannulated with a 40 µM stainless steel wire (Danish Myo Technologies, Denmark). The vessel and wire were then carefully removed and transferred to a petri-dish containing ice-cold Krebs bicarbonate solution. The vessel was measured to ensure it did not exceed 2 mm using a cm ruler. The vessel and wire were then transferred to the Mulvany-Halpern myograph chamber (Models 420A and 620M, Danish Myo Technologies, Denmark) containing pre-warmed (37ºC) Krebs bicarbonate solution. The vessel and wire were then secured between the myograph jaws (Figure 2.3.2.I). Following securing the first wire, a second wire was then passed carefully through the vessel and secured to the opposite myograph jaw (Figure 2.3.2,IV). The myograph jaws were adjusted until wires were just touching (Figure 2.3.2VI) prior to normalisation. For experiments in the absence of endothelium, the endothelium was removed following mounting by passing a human hair through the lumen of the vessel.

Once vessels were successfully mounted, they were allowed to equilibrate for 30 minutes prior to being normalised. All baths were maintained at 37ºC and continually supplied with a mixture of 95% O_2 and 5% CO_2. Vessels were normalised to a tension equivalent to that generated at 90% of the diameter of the vessel at 100 mmHg (Mulvany and Halpern 1977). The mean vessel diameter under these conditions was 279 ± 6.6 µm (n =59). Vessels were left for a further 30 minutes before experiments commenced.

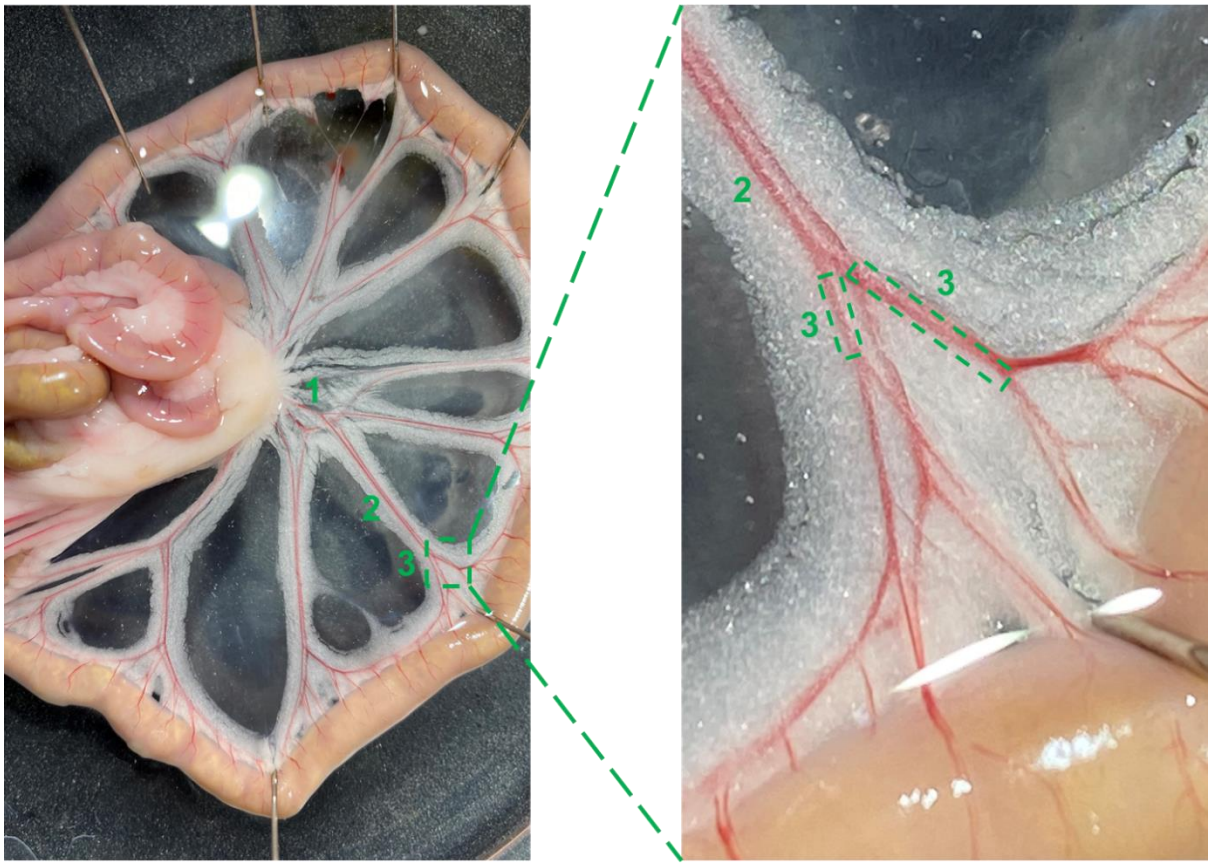

Figure 2.3.1. Identification of third-order branches of arterial tree. (Left) Identification of third-order (3) branches by following first-order (1) and second-order (2) arterial branches. (Right) magnified view of third-order branches.

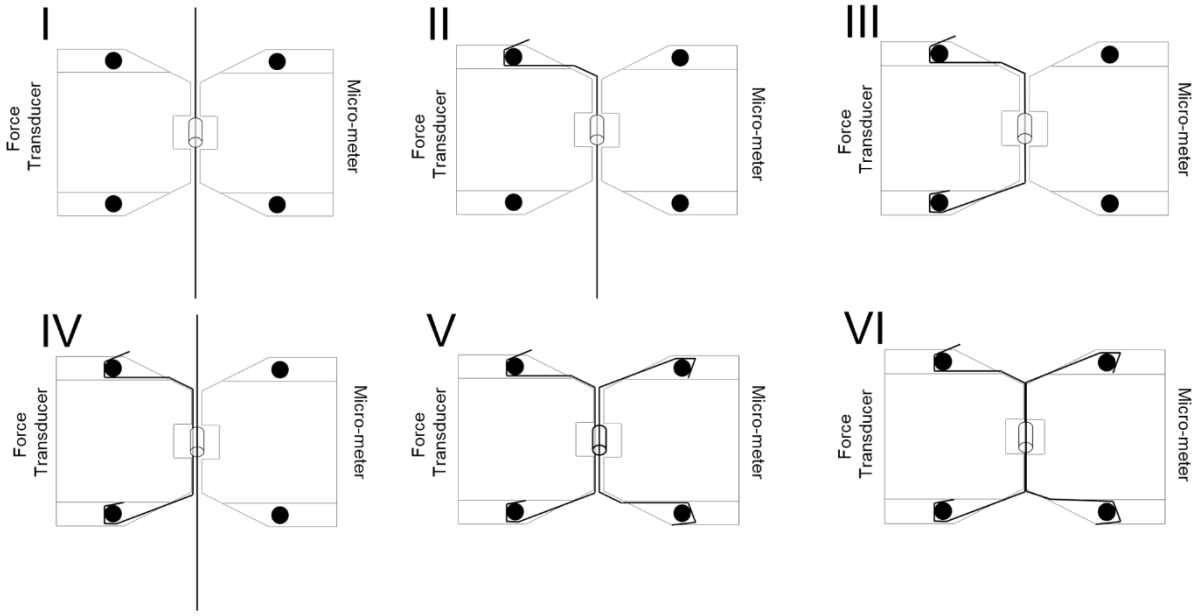

Figure 2.3.2 Step by step mounting of third-order mesenteric artery to Mulvany-Halpern myograph (not to scale). (I) Wire and vessel secured between jaws. (II) Wire secured to force transducer jaw via top left screw. (III) Vessel fully secured to force transducer jaw via bottom left screw. (IV) Second wire passed through vessel lumen and secured between jaws. (V) Vessel secured to micro-meter jaws. (VI) Jaws closed by adjusting micro-meter until wires are just touching.

2.3.1 Thin wire myography experimental protocol

Following a 30-minute incubation period, blood vessels were washed with pre-warmed Krebs bicarbonate solution. Pre-warmed high potassium (60mM) Krebs bicarbonate solution was then added to organ baths to contract tissue and confirm viability. Following wash out, the tissue was then pre-constricted with 10 μM phenylephrine. At the peak of contraction, the presence of a functional endothelium was confirmed by relaxation to 10 μM carbachol. Vessels were considered endothelium intact following a relaxation of ≥50% of the contractile response to phenylephrine. Following washout, cumulative and non-cumulative CRC's were obtained in the absence or presence of antagonists. Antagonists were added 15 minutes prior to constructing cumulative CRCs. To construct CRCs, successive concentrations of agonist were added in half logarithmic increments with each response being allowed to plateau before subsequent bolus addition.

2.4 Data analysis

The plateau for each contraction to each concentration of agonist was measured from the baseline prior to the CRC. Each contraction was then expressed as a percentage of the maximum contraction observed in response to high potassium (60mM KCl) Krebs bicarbonate solution. Dilator responses were expressed as a percentage of the initial contractile response to (3-10 μM) phenylephrine used to pre-constrict tissues. Contractions and dilations were plotted as the mean response ± standard error of the mean (S.E.M). Data was fitted to the following logistic equation:

$$Y = E_{Min} + \frac{(E_{Max} - E_{Min})}{(1 + 10^{(LogEC_{50} - [A]) \times Hill\ Slope)})}$$

Where Y is the response, E_{Min} is the expected response at a concentration of 0, E_{Max} is the expected response as the concentration goes to infinity and [A] is the concentration of agonist. E_{Max} values are reported as a percentage of the contractile responses to 60mM KCl ± S.E.M. Curve-fitting was carried out using Graphpad prism software (Graphpad software, San Diego). Negative $logEC_{50}$ values were obtained for each individual curve. EC_{50} values were calculated from the -$logEC_{50}$ and are reported as the mean value with 95% confidence

intervals. Mean EC_{50}, and E_{Max} values were compared by unpaired, two-tailed Student's t-test or one-way ANOVA with Dunnett's multiple comparison post-hoc test as appropriate. P-values ≤0.05 were considered statistically significant. n values represent the number of rats providing aortas. The equilibrium dissociation constant (K_B) of antagonists was calculated according to the following equation:

$$K_B = \frac{[B]}{(Dose\ Ratio - 1)}$$

where [B] is the concentration of antagonist. Dose ratio was calculated from ratio of the EC_{25}, EC_{50} or EC_{75} values in the presence or absence of antagonist. K_B was reported as a molar value ± SEM. The total area under the CRC (AUC) was calculated for agonist-responses using Microsoft Excel (Microsoft Corporation, Washington). The approximate AUC was calculated for each individual CRC using the following equation to plot the area between each increase in concentration:

$$\int_{[A]}^{[B]} f(x)\,dx \approx B - A \times \frac{f(A) + f(B)}{2}$$

Where B is the highest concentration and A is the lowest concentration. The mean AUC was reported as a % of the contraction to 60 mM KCl by Log[agonist(M)] (%.Log[M]).The means were compared by one-way ANOVA or unpaired, two-tailed Student's t-test as appropriate. Statistical significance was accepted for P ≤ 0.05.

2.5 Drugs and solutions

(R)- (-)-phenylephrine hydrochloride (PE), serotonin hydrochloride (5-hydroxytryptamine, 5-HT), ICI 118 551 hydrochloride, cinanserin hydrochloride, isoprenaline, carbamoylcholine chloride (carbachol), haloperidol hydrochloride, SCH 39166 hydrobromide and decynium 22 were purchased from Tocris Bioscience (Abingdon, United Kingdom). Tyramine hydrochloride, N_{ω}-Nitro-L-arginine methyl ester hydrochloride (L-NAME), prazosin hydrochloride, (S)-4-((S)-2-phenyl-butyl)-4,5-dihydro-oxazol-2-ylamine (RO5256390), N-(3-Ethoxy-phenyl)-4-pyrrolidin-1-yl-3-trifluoromethyl-benzamide (EPPTB), atropine, dopamine hydrochloride and β-phenylethylamine hydrochloride (β-PEA) were purchased from Sigma Aldrich (Poole, United Kingdom). The tyramine analogues, Tyramine A1 and Tyramine A2, were designed and synthesised by Dr Youcef Mehellou. All chemicals for Krebs bicarbonate buffer were of analytical grade and purchased from Fischer Scientific (Loughborough, United Kingdom). All drugs were dissolved in Krebs bicarbonate buffer with the exception of atropine, prazosin hydrochloride, RO5256390, haloperidol hydrochloride, SCH 39166, Tyramine A1, Tyramine A2 and EPPTB which were dissolved in dimethyl sulfoxide (DMSO; Fischer Scientific, United Kingdom) then diluted to desired concentration with Krebs bicarbonate buffer. The concentration of DMSO in organ baths did not exceed 0.25%.

Chapter 3 Variability of trace amine-induced vasoconstrictor responses

3.1 Introduction

Of all TAARs, TAAR1 is the most highly characterised, being regarded as the primary receptor for both tyramine and β-PEA (Lewin 2006). Although TAAR1 is evolutionarily conserved, there is considerable sequence divergence across species (Lindemann et al. 2005). This could suggest, trace amine-induced vascular responses at TAAR1 could vary significantly across species. Furthermore, genetic and hormonal influences are well established to have a significant effect on blood pressure (Sandberg and Ji 2012), for example across mammals males consistently display a higher blood pressure through much of their life (Maris et al. 2005). Consequently, trace amine-induced vascular responses may display different levels of sensitivity across gender and animal strain.

Whilst investigating trace amine-induced vasoconstrictor responses, large differences were observed in the contractile profiles of rats of different ages. The study of the trace amine-induced vasoconstrictor responses were therefore extended to investigate the influence of strain, weight/age, gender and pregnancy. Considering the sequence diversity of TAAR1 across species, it is possible that trace amine-induced vasoconstrictor responses could be influenced by hormonal and genetic differences. This study therefore aimed to identify the experimental conditions on which subsequent chapters are based.

3.2 Hypothesis

Variability in trace amine induced vasoconstrictor responses are a result of differences in age/weight, gender, strain and pregnancy.

3.3 Aims

1. Evaluate any weight/age related differences in vasoconstrictor responses to TAAR1 agonists.
2. Evaluate the effects of gender on TAAR1 agonist-induced vasoconstrictor responses.
3. Evaluate the variability of TAAR1 agonist-induced vasoconstriction post-partum.
4. Evaluate any strain-dependent differences in vasoconstrictor responses to TAAR1 agonists.

3.4 Materials and Methods in Brief

3.4.1 Animals

Male and female Sprague Dawley rats (males 110-750g, females 290-350g) were obtained from Charles River (Harlow, United Kingdom). Lister hooded rats (males 200-350g) and female Sprague Dawley ex-breeders (300-450g) were obtained from the JBIOS unit at Cardiff University. All animals were housed as described above. All experiments adhered strictly to the Animals (Scientific Procedures) Act 1986. Male animals above 350g and females above 250g were considered as mature adults, animals below this threshold were considered juvenile.

3.4.2 Rat Aortic rings

Tissue viability and the presence or absence of a functional endothelium were confirmed as described above. Cumulative-concentration response curves (CRC's) were obtained for phenylephrine, β-phenylethylamine (β-PEA), RO5256390 and tyramine.

3.4.3 Drugs and solutions

All drugs were dissolved in Krebs bicarbonate buffer unless otherwise stated. RO5256390 was dissolved in dimethyl sulfoxide (DMSO) prior to dilution with Krebs bicarbonate buffer. The concentration of DMSO did not exceed 0.25% of the total volume. The source of all drugs used in this chapter and the composition of Krebs bicarbonate buffer is detailed in above.

3.4.4 Data analysis

Contractile responses were expressed as a percentage of the contractile response to high potassium Krebs bicarbonate solution as described above. Responses were plotted as the mean response ± the standard error of the mean (S.E.M) where n represents the number of rats. Curve fitting was applied as described in chapter 2. EC_{50} values were calculated from the -logEC_{50} and are reported as the mean value with 95% confidence intervals. Mean EC_{50} values were compared by unpaired Student's t-test. P-values ≤0.05 were considered statistically significant. Area under the curve (AUC) was calculated for specified concentration ranges from individual CRCs. The mean AUC was reported as a % of contraction to 60 mM KCl by Log[agonist(M)] (%.Log[M]). AUC's were compared by one-way ANOVA or unpaired, two-tailed Student's t-test as appropriate.

3.5 Results

3.5.1 Age dependent variability of trace amine-induced vasoconstrictor responses

In endothelium-intact aortic rings from adult animals, 60 mM KCl induced a mean contractile response of 1.7 ± 0.1 g (n = 47). This was significantly greater (P ≤ 0.001, unpaired Students T-test) than response to 60 mM KCl in juvenile animals (1.2 ± 0.1 g, n = 51). The α_1-adrenoceptor agonist, phenylephrine, induced concentration-dependent contraction of both adult and juvenile rat aortic rings (Figure 3.5.2A, B). In endothelium-intact aortic rings from adult animals, phenylephrine had an EC_{50} of 34 nM (16 – 70 nM, Table 3.5.1). This was not different to the EC_{50} obtained in aortic rings from juvenile animals of 91 nM (49 – 167 nM, Table 3.5.1). Phenylephrine had an E_{Max} of 96 ± 4 % in aortic rings from adult animals. This was not different to the E_{Max} obtained in aortic rings from juvenile animals (87 ± 6 %).

In endothelium-denuded aortic rings from adult animals, 60 mM KCl induced a mean contractile response of 1.4 ± 0.1 g (n = 39). This was significantly greater (P ≤ 0.05, unpaired Students T-test) than response to 60 mM KCl in juvenile animals (1.1 ± 0.1 g, n = 28). In endothelium-denuded aortic rings from adult animals, phenylephrine had an EC_{50} of 15 nM (8 – 27 nM, Table 3.5.1). This was not different to the EC_{50} obtained in aortic rings from juvenile animals of 7 nM (6 – 9 nM, Table 3.5.1). The E_{Max} of phenylephrine in aortic rings from adult animals of 95 ± 5 % was not different to that obtained in juvenile aortic rings (109 ± 11 %).

β-PEA induced concentration dependent contraction of both adult and juvenile aortic rings (Figure 3.5.2C, D). In endothelium-intact tissues, the AUC (between 1 µM and 1000 µM) of the β-PEA CRC from adult aortic rings (23 ± 2 %.Log[M]) was not different to that obtained in juvenile aortic rings (37 ± 3 %.Log[M]). A complete CRC was not obtained in endothelium-intact aortic rings, therefore neither an EC_{50} nor E_{Max} were calculated.

In the endothelium-denuded tissues, β-PEA contractile responses were generally weaker in aortic rings from adult animals (

Figure 3.5.1D). The AUC of the β-PEA CRC from adult aortic rings (105 ± 6 %.Log[M]) was significantly lower (P ≤ 0.05, one-way ANOVA) than the AUC obtained in juvenile aortic rings (147 ± 8 %.Log[M]). The EC_{50} of β-PEA in aortic rings from adult animals of 58 µM (34 – 98 µM, Table 3.5.1) was not different to the EC_{50} obtained in juvenile aortic rings of 23 µM (8 – 70 µM, Table 3.5.1). The E_{Max} of β-PEA in adult aortic rings (88 ± 6 %) was not different to the E_{Max} obtained in juvenile aortic rings (103 ± 10 %).

Interestingly whilst investigating β-PEA-induced contractile responses, a clear difference in the profile of the contractile responses to β-PEA was observed between aortic rings from juvenile and adult animals. In adult aortic rings β-PEA-induced contractile responses were characterised by a slow and sustainable contraction that was independent of the presence of a functional endothelium (

Figure 3.5.1A, B). Contractile responses in aortic rings from juvenile animals were characterised by a rapid onset and rapid decline to baseline after reaching a plateau (

Figure 3.5.1C, D). Furthermore, secondary contractile responses were often observed following the decline to baseline that were greater than or equal to the initial response. Addition of an increased concentration of β-PEA during or immediately after the decline to baseline failed to induce an immediate contractile response.

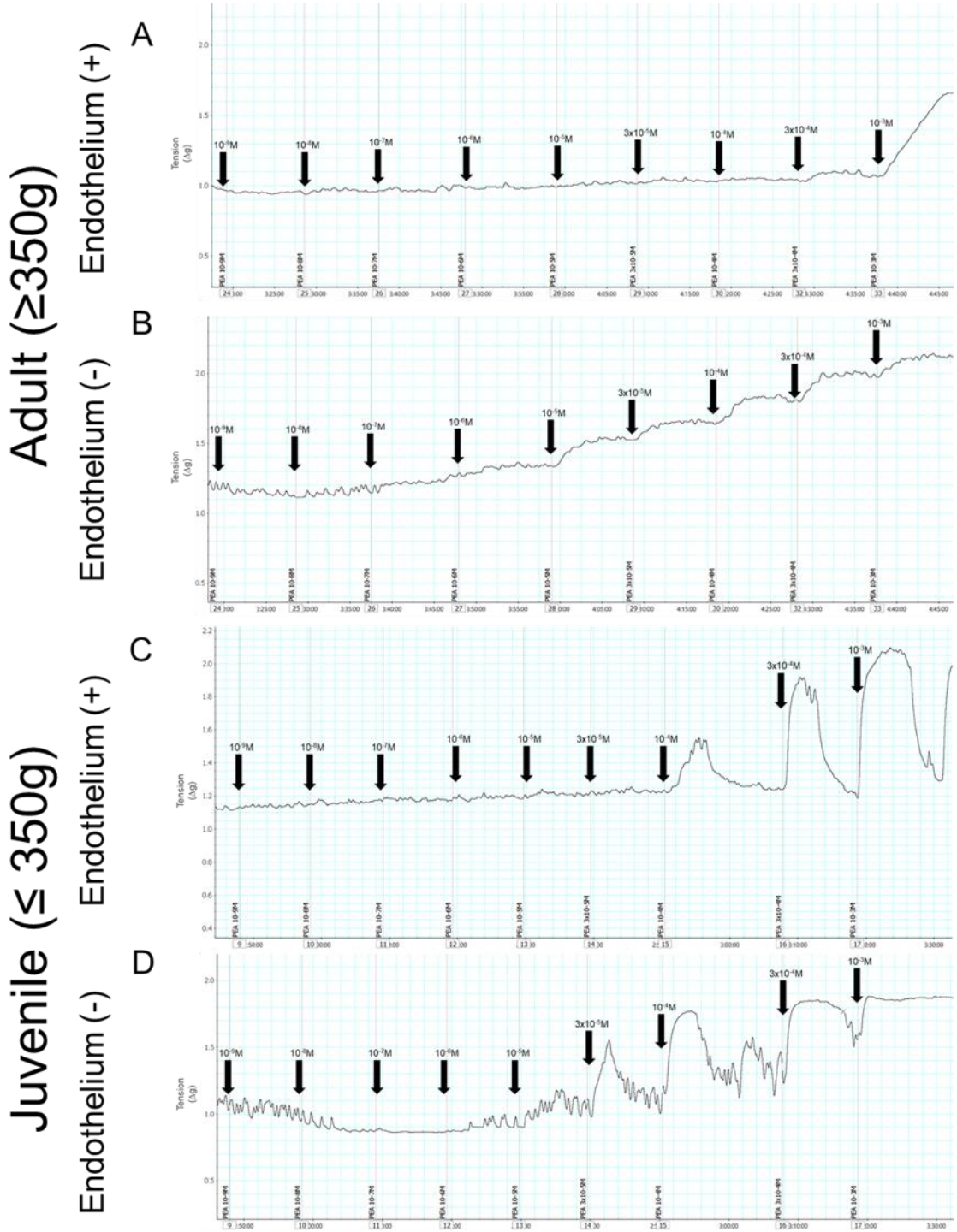

Figure 3.5.1 Representative traces of cumulative β-PEA CRCs in Adult (A-B) and juvenile (C-D) Sprague Dawley rats. Representative β-PEA trace in endothelium-intact (A) and endothelium denuded (B) aortic rings from adult animals. Representative β-PEA trace in endothelium-intact (C) and endothelium denuded (D) aortic rings from juvenile animals.

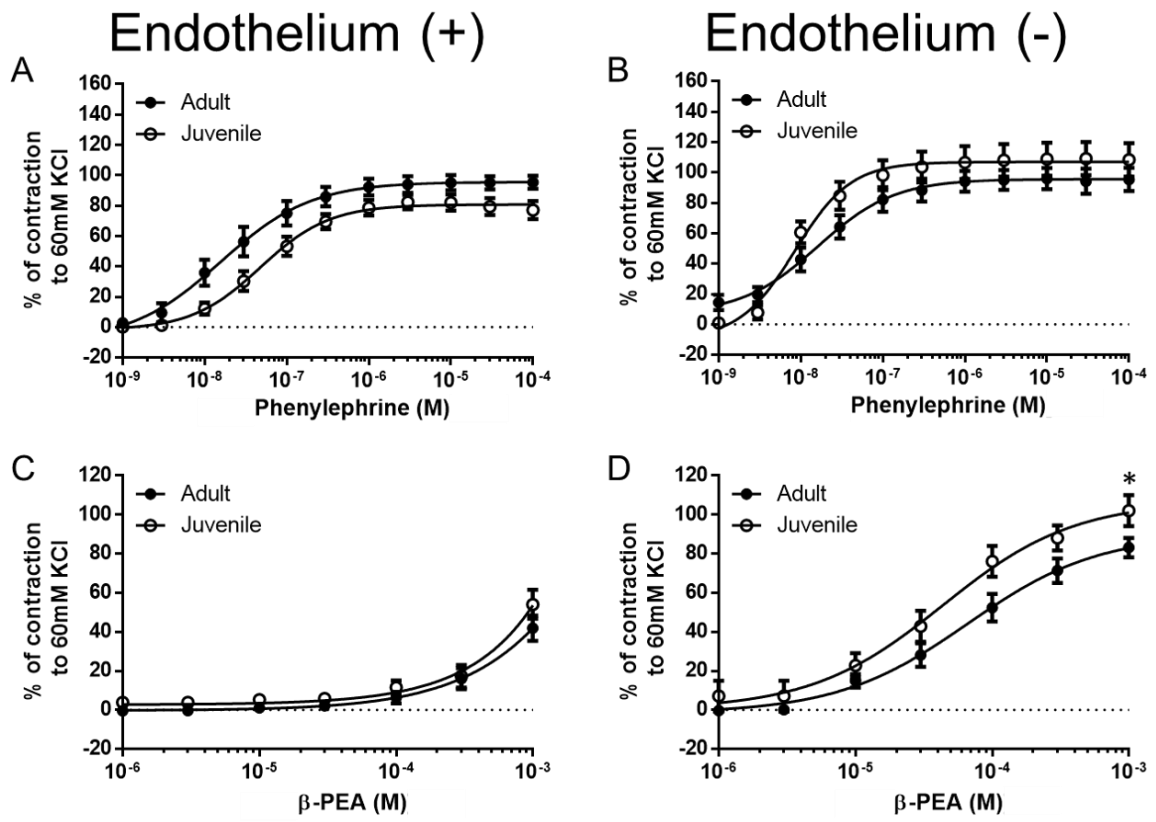

*Figure 3.5.2. Mean cumulative CRC's of phenylephrine (A-B) and β-PEA (C-D) in adult (●) and juvenile (○) Sprague Dawley rats. Cumulative CRCs for phenylephrine were obtained in endothelium-intact aortic rings (A) isolated from adult (● n = 10) and juvenile rats (○ n = 16). Cumulative CRCs for phenylephrine were obtained in endothelium-denuded aortic rings (B) isolated from adult (● n = 9) and juvenile rats (○ n = 9) and -denuded (B, ● n = 9, ○ n = 9) aortic rings. Cumulative CRCs for β-PEA were obtained in endothelium-intact aortic rings (C) isolated from adult (● n = 17) and juvenile rats (○ n = 10). Cumulative CRCs for β-PEA were obtained in endothelium-intact aortic rings (D) isolated from adult (● n = 14) and juvenile rats (○ n = 8). Contractile responses are reported as the mean percentage (±SEM) of the maximum contractile response to 60mM KCl. * indicates a significant difference (P ≤ 0.05) in AUC.*

These characteristics were not only limited to β-PEA. The selective TAAR1 agonist RO5256390 also displayed differential contractile profiles in adult and juvenile animals (Figure 3.5.3A-D). In the presence of a functional endothelium, RO5256390-induced contractile responses were minimal in both adult and juvenile aortic rings (Figure 3.5.3A, B, E). The AUC (between 0.1 µM and 30 µM) of the CRC to RO5256390 in aortic rings from adult animals (12 ± 1 %.Log[M]) was not different to the AUC obtained in juvenile aortic rings (7 ± 1 %.Log[M]). A complete CRC was not obtained in endothelium-intact aortic rings, therefore neither an EC_{50} nor E_{Max} were calculated.

In the absence of a functional endothelium, the AUC of the CRC to RO5256390 in aortic rings from adult animals (72 ± 4 %.Log[M]) was not different to the AUC obtained in juvenile aortic rings (85 ± 4 %.Log[M]). The EC_{50} of RO5256390 in adult aortic rings of 3 µM (2 – 4 µM, Table 3.5.1) was not significantly different from the EC_{50} obtained in juvenile aortic rings of 3 µM (2 – 5 µM, Table 3.5.1). The E_{Max} of RO5256390 in aortic rings from adult animals (62 ± 6 %) was found to be significantly lower ($P \leq 0.01$, unpaired, two-tailed, T-test) than the E_{Max} obtained in juvenile aortic rings (90 ± 6 %).

Like β-PEA, contractile responses in adult aortic rings were sustainable and slow in onset. In juvenile aortic rings, RO5256390-induced contractile responses mimicked those seen for β-PEA. Responses were rapid in onset, followed by a rapid decline to baseline following plateau of the response. Secondary contractile responses to RO5256390 were generally more prevalent than β-PEA (Figure 3.5.3C, D). Like β-PEA, addition of an increased concentration of RO5256390 would not induce further contraction during the declining phase.

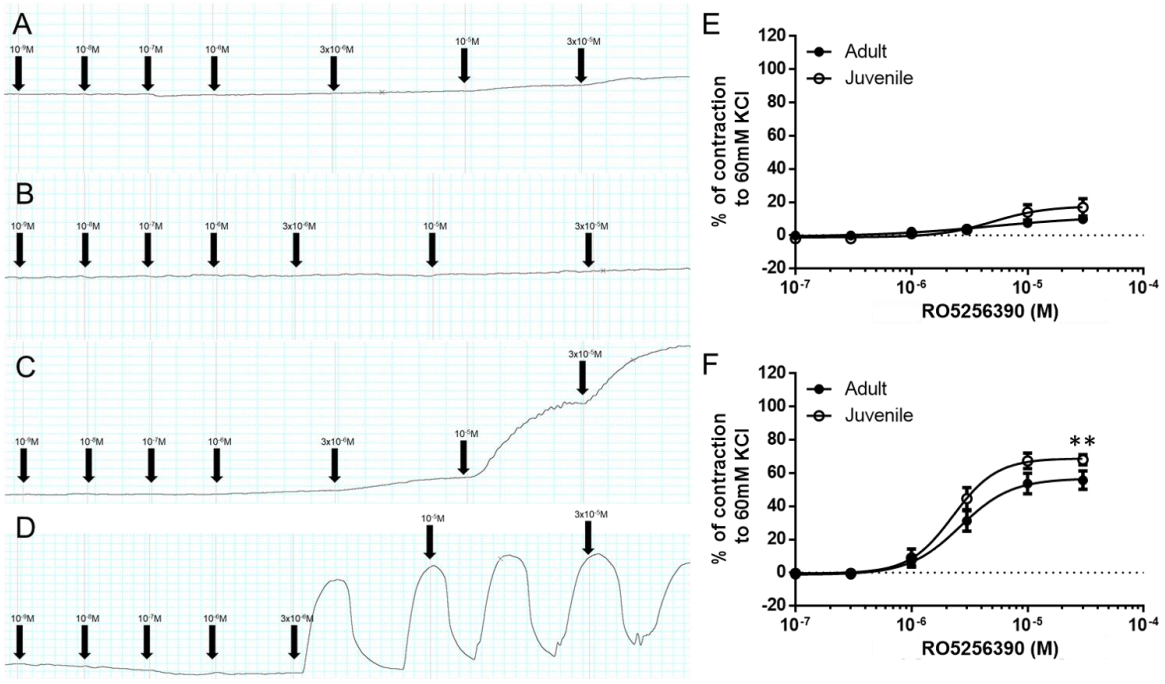

*Figure 3.5.3. RO5256390-induced contractile responses in adult and juvenile rat aortic rings. Representative RO5256390 trace in endothelium-intact aortic rings from adult (A) and juvenile (B) animals. Representative RO5256390 trace in endothelium-denuded aortic rings from adult (C) and juvenile (D) animals. RO5256390 CRCs were obtained in endothelium-intact aortic rings (E) from adult (● n = 20) and juvenile (○ n = 15). RO5256390 CRCs were obtained in the endothelium-denuded aortic rings (F) from adult (● n = 17) and juvenile (○ n = 10). Contractile responses are reported as the mean percentage (±SEM) of the maximum contractile response to 60mM KCl. ** indicates a significant ($P \leq 0.01$) difference in E_{Max}.*

3.5.2 Effect of gender on trace amine-induced vasoconstriction

In endothelium-intact aortic rings from male animals, 60 mM KCl induced a mean contractile response of 1.8 ± 0.1 g (n = 27). This was not different from the response to 60 mM KCl in female animals (2.1 ± 0.1 g, n = 12). No significant differences in the contractile response to phenylephrine were observed between aortic rings from adult male and adult female rats (Figure 3.5.4A, B). Phenylephrine had an EC_{50} of 40 nM (26 – 61 nM, Table 3.5.1) in aortic rings from female animals. This was not significantly different from the EC_{50} obtained in aortic rings from male animals of 34 nM (16 – 70 nM, Table 3.5.1). In aortic rings from female animals, the E_{max} of phenylephrine (81 ± 5 %) was not significantly different from the E_{max} obtained in aortic rings from male animals (96 ± 4 %).

In endothelium-denuded aortic rings from male animals, 60 mM KCl induced a mean contractile response of 1.3 ± 0.1 g (n = 23). This was not different from the response to 60 mM KCl in female animals (1.8 ± 0.2 g, n = 11). In endothelium-denuded tissues, phenylephrine had an EC_{50} of 8 nM (4 – 16 nM, Table 3.5.1) in female aortic rings. This was not different from the EC_{50} obtained in male aortic rings of 15 nM (8 – 27 nM, Table 3.5.1). Phenylephrine had an E_{Max} of 105 ± 4 % in aortic rings from female rats. This was not different from the E_{Max} of 95 ± 5 % obtained in male aortic rings.

In endothelium-intact tissues, there was no significant difference in β-PEA-induced contractile responses between male and female aortic rings (Figure 3.5.4C). The AUC (between 1 µM and 1000 µM) of the CRC to β-PEA in female aortic rings was 24 ± 2 %.Log[M]. This was not different from the AUC of β-PEA in male aortic rings (23 ± 2 %.Log[M]). A complete CRC was not generated in endothelium-intact aortic rings. Therefore, neither an EC_{50} nor E_{max} were obtained.

In the absence of endothelium, the CRC to β-PEA was shifted rightwards in female aortic rings (Figure 3.5.4D). The AUC of the CRC to β-PEA (75 ± 5%.Log[M]) was significantly ($P \leq 0.05$, one-way ANOVA) lower than the AUC of the β-PEA CRC in male aortic rings (105 ± 6 %.Log[M]). β-PEA had an EC_{50} of 352 µM (102 µM – 1215 µM, Table 1.4.1) in female aortic rings. This was significantly greater ($P \leq 0.05$, one-way ANOVA) than the EC_{50} obtained in male aortic rings of 58 µM (34 – 98 µM, Table 1.4.1). The observed shift between male and female aortic rings was approximately 6-fold. The E_{Max} of β-PEA was 126 ± 24 % in female aortic rings. This was found to be significantly ($P \leq 0.05$) greater than the E_{Max} of β-PEA in male aortic rings of 88 ± 6%.

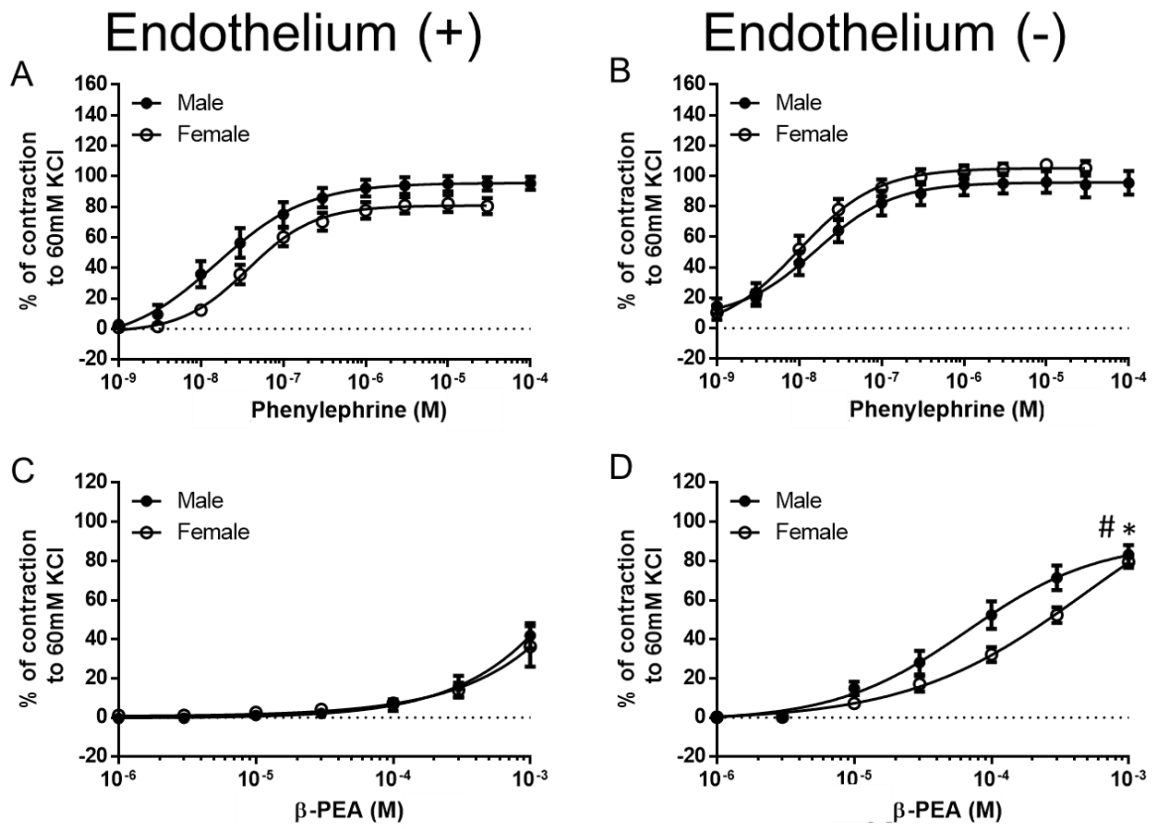

*Figure 3.5.4 The effect of gender on phenylephrine- (A-B) and β-PEA- (C-D) induced contractile responses in Sprague Dawley rat aortic rings. Phenylephrine CRCs were obtained in endothelium-intact aortic rings (A) from male (● n = 10) and female (○ n = 6) rats. Phenylephrine CRCs were obtained in endothelium-denuded aortic rings (B) from male (● n = 9) and female (○ n = 6) rats. β-PEA CRCs were obtained in endothelium-intact aortic rings (C) from male (● n = 17) and female (○ n = 6) rats. β-PEA CRCs were obtained in endothelium-denuded aortic rings (D) from male (● n = 14) and female (○ n = 5) rats. Contractile responses are reported as the mean percentage (±SEM) of the maximum contractile response to 60mM KCl. # indicates a significant (P ≤ 0.05) in EC_{50}. * indicates a significant (P ≤ 0.05) in E_{Max}.*

3.5.3 Post-partum differences in trace amine-induced vasoconstrictor responses

In endothelium-intact aortic rings from female non-breeder animals, 60 mM KCl induced a mean contractile response of 2.1 ± 0.1 g (n = 12). This was not different from the response to 60 mM KCl in female ex-breeder animals (1.9 ± 0.1 g, n = 11). No significant difference in the contractile response to phenylephrine was observed between female non-breeders and female ex-breeder aortic rings (Figure 3.5.5A, B). In endothelium-intact aortic rings from ex-breeders, phenylephrine had an EC_{50} of 67 nM (43 – 107 nM, Table 3.5.1). This was not different from the EC_{50} of phenylephrine in non-breeder aortic rings of 40 nM (26 – 61 nM, Table 3.5.1). Phenylephrine had an E_{Max} of 65 ± 5% in aortic rings from ex-breeder females. This was not different from the E_{Max} of phenylephrine in non-breeder aortic rings of 81 ± 5%.

In endothelium-denuded aortic rings from female non-breeder animals, 60 mM KCl induced a mean contractile response of 1.8 ± 0.2 g (n = 11). This was not different from the response to 60 mM KCl in female ex-breeder animals (1.5 ± 0.2 g, n = 11). In endothelium-denuded tissues, phenylephrine had an EC_{50} of 25 nM (6 – 100 nM, Table 3.5.1) in ex-breeder aortic rings. This was not different from the EC_{50} of phenylephrine in non-breeder aortic rings of 8 nM (4 – 16 nM, Table 3.5.1). The E_{Max} of phenylephrine (90 ± 7 %) in aortic rings from ex-breeder females was not different from the EC_{50} of phenylephrine obtained in non-breeder aortic rings (105 ± 4 %).

No significant difference was found between non-breeder and ex-breeder β-PEA CRCs (Figure 3.5.5C, D). In the presence of a functional endothelium the AUC (between 1 µM and 1000 µM) of the β-PEA CRC from ex-breeder aortic rings (17 ± 1 %.Log[M]) was not different from the AUC of β-PEA obtained in non-breeder aortic rings (24 ± 2 %.Log[M]). A complete CRC was not obtained in endothelium-intact aortic rings, therefore neither an EC_{50} nor E_{Max} were obtained.

In endothelium-denuded tissues, the AUC of the β-PEA CRC from ex-breeder aortic rings (64 ± 5 %.Log[M]) was not different from that obtained in non-breeder aortic rings (75 ± 5%.Log[M]). β-PEA had an EC_{50} of 261 µM (72 – 947 µM, Table 3.5.1) in aortic rings from ex-breeders. This was not different from the EC_{50} of β-PEA obtained in non-breeder aortic rings of 352 µM (102 µM – 1215 µM, Table 3.5.1). In ex-breeder aortic rings, the E_{Max} of β-PEA (124 ± 9 %) was not different from the E_{Max} obtained in non-breeder aortic rings (126 ± 24 %).

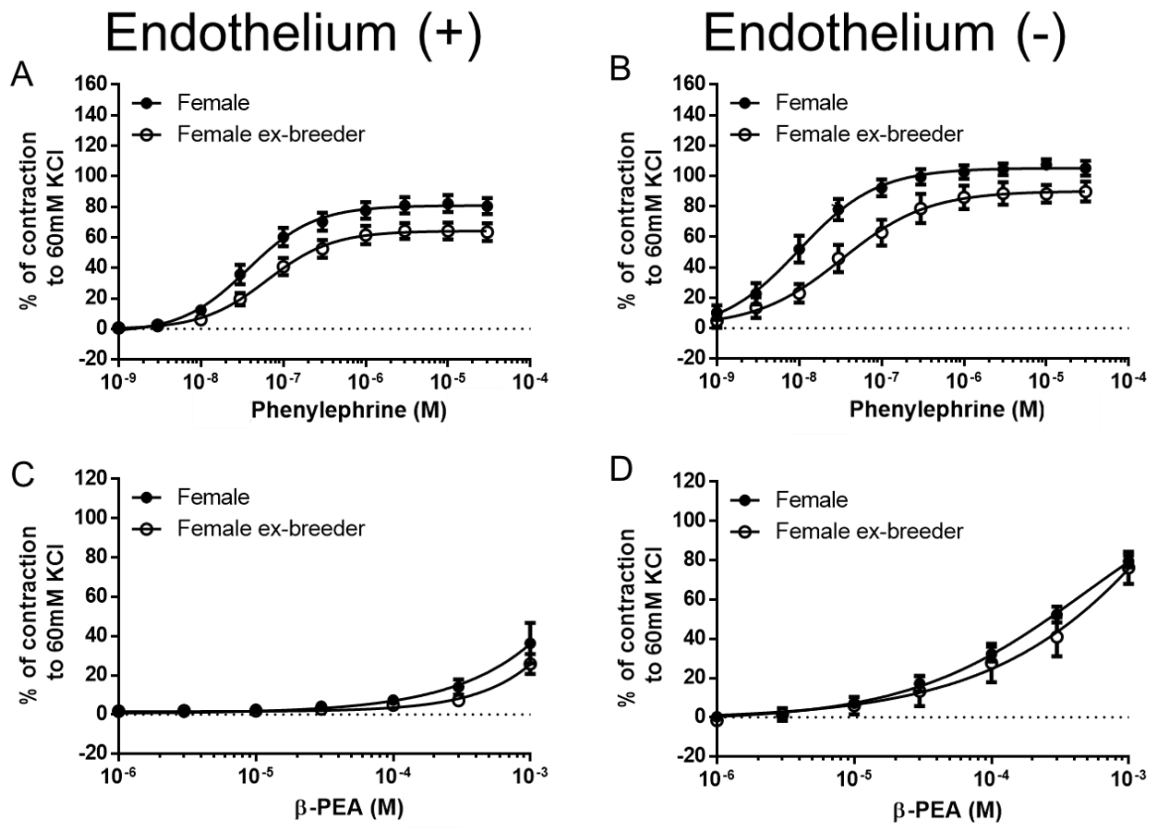

Figure 3.5.5 Post-partum effects on phenylephrine (A-B) and β-PEA (C-D) CRCs in female Sprague Dawley rat aortic rings. Phenylephrine CRCs were obtained in endothelium-intact aortic rings (A) isolated from female non-breeder (● n = 6) and female ex-breeder rats (○ n = 5). Phenylephrine CRCs were obtained in endothelium-denuded aortic rings (B) isolated from female non-breeder (● n = 6) and female ex-breeder rats (○ n = 5). β-PEA CRCs were obtained in endothelium-intact aortic rings (C) isolated from female non-breeder (● n = 6) and female ex-breeder rats (○ n = 6). β-PEA CRCs were obtained in endothelium-denuded aortic rings (D) isolated from female non-breeder (● n = 5) and female ex-breeder rats (○ n = 6). Contractile responses are reported as the mean percentage (±SEM) of the maximum contractile response to 60mM KCl.

3.5.4 Strain dependent differences in trace amine-induced vasoconstrictor responses

In endothelium-intact aortic rings from juvenile male Sprague Dawley rats, 60 mM KCl induced a mean contractile response of 1.1 ± 0.1 g (n = 26). This was not different from the response to 60 mM KCl in juvenile Lister Hooded rats (0.8 ± 0.1 g, n = 11). No significant difference was found between phenylephrine CRCs obtained in juvenile male Sprague Dawley and juvenile male Lister Hooded rats (Figure 3.5.7A, B). In endothelium-intact aortic rings, from Sprague Dawley rats, phenylephrine had an EC_{50} of 91 nM (49 – 167 nM, Table 3.5.1). This was not different from the EC_{50} obtained in aortic rings from Lister Hooded rats of 19 nM (12 – 30 nM, Table 3.5.1). In Sprague Dawley aortic rings, the E_{Max} of phenylephrine was 87 ± 6 %. This was not different from the E_{Max} of phenylephrine obtained in Lister Hooded aortic rings of 90 ± 8 %.

In endothelium-denuded aortic rings from juvenile male Sprague Dawley rats, 60 mM KCl induced a mean contractile response of 0.9 ± 0.1 g (n = 15). This was significantly ($P \leq 0.05$, unpaired Students T-test) greater than the response to 60 mM KCl in juvenile Lister Hooded rats (0.5 ± 0.1 g, n = 10). In endothelium-denuded tissues, phenylephrine had an EC_{50} of 7 nM (6 – 9 nM, Table 3.5.1) in aortic rings from Sprague Dawley rats. This was not different from the EC_{50} obtained in aortic rings from Lister Hooded rats of 11 nM (4 – 30 nM, Table 3.5.1). Phenylephrine had an E_{Max} of 109 ± 11 % in Sprague Dawley aortic rings. This was not different from than the E_{Max} of phenylephrine obtained in Lister Hooded aortic rings of 97 ± 11 %.

No significant difference was observed between tyramine CRCs in endothelium-intact aortic rings from Sprague Dawley and Lister Hooded rats (Figure 3.5.7C). In endothelium-intact tissues, the AUC of the tyramine CRC (between 1 µM and 1000 µM) in aortic rings from Sprague Dawley rats (18 ± 1 %.Log[M]) was not different from the AUC of tyramine obtained in aortic rings from Lister Hooded rats (13 ± 2 %.Log[M]). A complete CRC to tyramine was not obtained in the presence of a functional endothelium, therefore neither an EC_{50} nor E_{Max} were obtained.

In endothelium-denuded tissues, tyramine-induced contractile responses were generally weaker in aortic rings from Sprague Dawley rats (Figure 3.5.7D). The AUC (between 1 µM and 1000 µM) of the tyramine CRC in Sprague Dawley aortic rings was 105 ± 5 %.Log[M]. This was not different from the AUC of the tyramine CRC obtained in Lister Hooded aortic rings (155 ± 10 %.Log[M]). In aortic rings obtained from Sprague Dawley rats, tyramine had an EC_{50} of 84 µM (17 – 424 µM, Table 3.5.1). This was not different from the EC_{50} of tyramine obtained in Lister Hooded aortic rings of 28 µM (17 – 465 µM, Table 3.5.1). The E_{Max} values for tyramine-induced contraction did not significantly differ between aortic rings taken from Sprague Dawley and Lister Hooded rats (99 ± 21 % and 124 ± 20 %, respectively).

Like β-PEA and RO5256390, tyramine responses in both juvenile Sprague Dawley and Lister Hooded aortae were characterised by phasic contractions consisting of a rapid onset of contraction followed by a relaxation to baseline (

Figure 3.5.6). Secondary contractile responses were not observed in either Sprague Dawley or Lister Hooded aortae. However, a delay in onset, of up to 10-minutes, following addition of the next concentration of tyramine was observed in both strains.

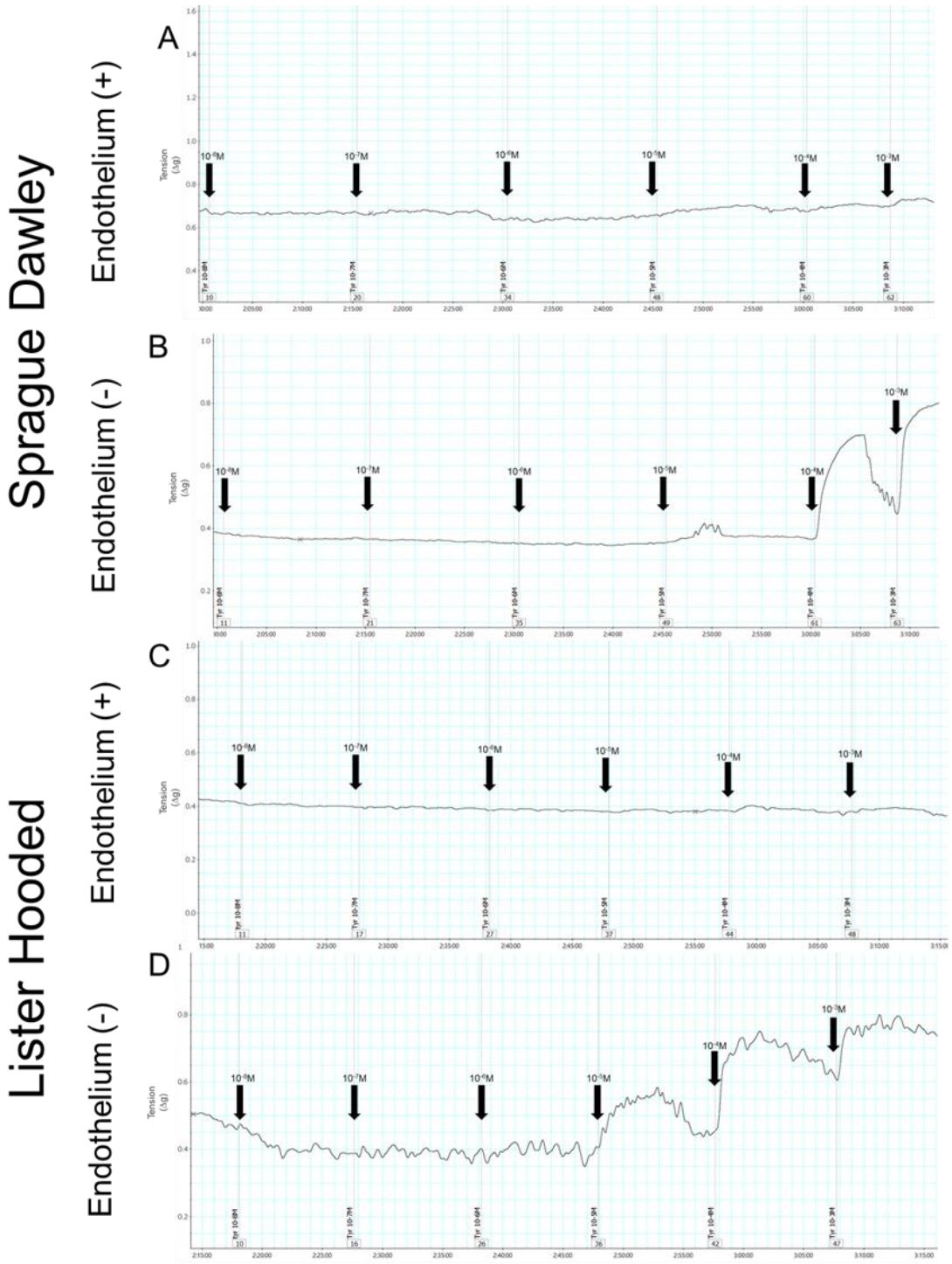

Figure 3.5.6. Representative traces of cumulative tyramine CRCs in Sprague Dawley (A-B) and Lister Hooded (C-D) rats. Representative tyramine trace in endothelium-intact (A) and endothelium denuded (B) aortic rings from Sprague Dawley rats. Representative tyramine trace in endothelium-intact (C) and endothelium denuded (D) aortic rings from Lister Hooded rats.

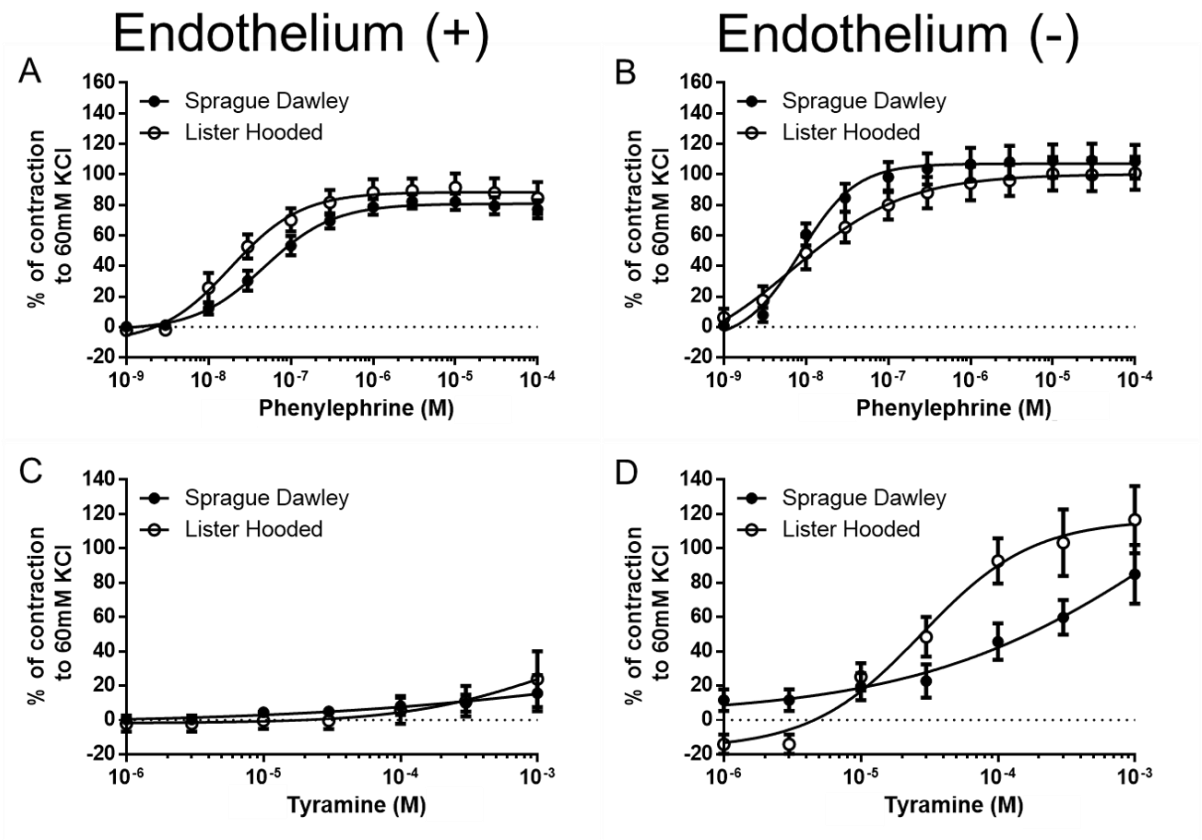

Figure 3.5.7. Strain dependent differences in phenylephrine (A-B) and tyramine (C-D) CRCs in juvenile male Sprague Dawley and juvenile male Lister Hooded rat aortic rings. Phenylephrine CRCs were obtained in endothelium-intact aortic rings (A) isolated from Sprague Dawley (● n = 16) and Lister Hooded rats (○ n = 6). Phenylephrine CRCs were obtained in endothelium-denuded aortic rings (B) isolated from Sprague Dawley (● n = 9) and Lister Hooded rats (○ n = 5). Tyramine CRCs were obtained in endothelium-intact aortic rings (C) isolated from Sprague Dawley (● n = 9) and Lister Hooded rats (○ n = 5). Tyramine CRCs were obtained in endothelium-denuded aortic rings (D) isolated from Sprague Dawley (● n = 6) and female Lister Hooded rats (○ n = 5). Contractile responses are reported as the mean percentage (±SEM) of the maximum contractile response to 60mM KCl.

Table 3.5.1. Variation of EC_{50} values of contractile agonists in endothelium intact and denuded aortic rings across strain, gender, age and pregnancy.

Agonist	Strain	Gender (M/F)	Adult/Juvenile	Endothelium +/-	EC50 CI95
β-PEA	Sprague Dawley	M	Adult	+	ND
				-	58 µM (34 – 98 µM)
			Juvenile	+	ND
				-	23 µM (8 – 70 µM)
		F	Adult	+	ND
				-	352 µM (102 µM – 1215 µM)
		F ex-breeder	Adult	+	ND
				-	261 µM (72 – 947 µM)
Tyramine	Sprague Dawley	M	Juvenile	+	ND
				-	84 µM (17 – 424 µM)
	Lister Hooded	M	Juvenile	+	ND
				-	28 µM (17 – 465 µM)
RO5256390	Sprague Dawley	M	Adult	+	ND
				-	3 µM (2 – 4 µM)
			Juvenile	+	ND
				-	3 µM (2 – 5 µM)
Phenylephrine	Sprague Dawley	M	Adult	+	34 nM (16 – 70 nM)
				-	15 nM (8 – 27 nM)
			Juvenile	+	91 nM (49 – 167 nM)
				-	7 nM (6 – 9 nM)
		F	Adult	+	40 nM (26 – 61 nM)
				-	8 nM (4 – 16 nM)
		F ex-breeder	Adult	+	67 nM (43 – 107 nM)
				-	25 nM (6 – 100 nM)
	Lister Hooded	M	Juvenile	+	19 µM (12 – 30 µM)
				-	11 nM (4 – 30 nM)

3.6 Discussion

3.6.1 Age dependent differences in trace amine-induced vasoconstrictor responses

The α_1-adrenoceptor agonist, phenylephrine, induced concentration-dependent contraction of aortic rings across a similar concentration range in mature and juvenile Sprague Dawley rats (Figure 3.5.2). In endothelium-intact aortic rings from mature rats, contractile responses to phenylephrine were generally greater than those from juvenile animals. However, there was no difference in the potency of phenylephrine, as determined by the EC_{50} values between adult and juvenile animals. In the absence of endothelium, no difference was observed between the two groups. It is possible that the endothelium of juvenile animals is more susceptible to phenylephrine-induced release of nitric oxide (Dora et al. 2000). An increase in nitric oxide would oppose the vasoconstrictor actions of phenylephrine (Zhao et al. 2015), thus explaining the weaker contractile response in aortae from juvenile animals.

The TAAR1 agonists, β-PEA and RO5256390 induced contractile responses in both mature and juvenile aortae (Figure 3.5.2 and

Figure 3.5.1). No differences were observed with regards to efficacy or potency of either agonist. However, whilst investigating β-PEA and RO5256390-induced vasoconstrictor responses, distinct differences in the onset and sustainability of vasoconstrictor responses were observed between juvenile and mature animals (

Figure 3.5.1 and Figure 3.5.3). Aortic rings from mature animals (≥350g), were characterised by a slow and sustainable contractile response to both β-PEA and RO5256390. These results are similar to the contractile profile of octopamine in the presence of prazosin previously described by Broadley and Richards (2015). The aforementioned study suggested that the slow onset of vasoconstriction of octopamine in the presence of α_1-adrenoceptor blockade could represent TAAR1 mediated vasoconstriction. Interestingly in the current study both β-PEA and RO5256390 contractile responses displayed rapid onset and decline in aortic rings from juvenile (≤350g) animals. Furthermore, in many cases following the rapid decline to baseline, secondary contractile responses were observed for both TAAR1 agonists, however, were more prevalent for RO5256390 (

Figure 3.5.1 and Figure 3.5.3). These differences were limited to agonists of TAAR1 as no difference in the contractile profile of phenylephrine was observed between mature and juvenile animals.

The differences between the contractile profile of juvenile and adult animals have not been previously reported. In humans, it is well established that during the period of sexual maturity, the rate of change in blood pressure is vastly increased (Shankar et al. 2005). However, little investigation has been carried out into the effects of sexual maturity on blood pressure in rat models and most blood pressure studies involving animal models utilise young adult rats (Doggrell and Brown 1998). In the current study adult rats were defined as weighing ≥350g, which corresponds with the age of a sexually mature young adult male rat (Sengupta 2013). It is plausible that the period of sexual maturity influences the contractile responses to trace amines. Since TAAR1 was first identified, it has proven a challenging receptor to characterise as it is mostly expressed intracellularly (Berry et al. 2017). In juvenile animals, TAAR1 may be more highly localised at the cell surface and with sexual maturity gradually becomes an intracellular receptor, leading to slower onset in contractile responses. However, Chapter 6 demonstrated blockade of intracellular transport of trace amines failed to abolish β-PEA induced contractile responses in adult animals, suggesting it is unlikely that the contractile response is due to an intracellular receptor. Furthermore, a rapid decline in response is observed in juvenile animals. This could represent activation of the vasodilator response to the trace amines demonstrated in Chapter 4. It is unlikely that the declining phase of contraction represents a rapid desensitisation of receptors as this

would not explain the secondary contractile responses observed in many cases. Without the development of a selective TAAR1 antagonist, it may be impossible to establish the mechanism by which trace amines induce vasoconstriction and understand the changes that occur during sexual maturity.

3.6.2 Gender related difference in trace amine-induced vasoconstriction

It is well established that, at least until the age of menopause, women have a lower blood pressure than men of similar age (Dubey et al. 2002; Maris et al. 2005). This sexual dimorphism is not restricted to humans and several animal studies have reported sex-dependent differences in blood pressure (Cambotti et al. 1984; Ganten et al. 1989; Calhoun et al. 1994). The current study investigated sex-dependent differences in trace amine-induced vasoconstrictor responses in Sprague Dawley rats. Aortic rings from male and female animals displayed no difference in the potency or efficacy of α_1-adrenoceptor mediated contractile responses. Previous studies have suggested male androgens modulate the expression levels of α_1-adrenoceptors (McConnaughey and Iams 1993). It could be expected that in male aortic rings that the α_1-adrenoceptor agonist phenylephrine may be more potent than aortic rings from female animals. However, in the current study the mean EC_{50} of phenylephrine calculated for male and female animals were not significantly different. Furthermore, no difference was observed in the E_{Max} of phenylephrine between genders. These results indicate that gender has little if any effect on α_1-adrenceptor mediated vasoconstriction in rat aorta.

In the absence of endothelium, the EC_{50} and E_{max} of β-PEA in female aortic rings were significantly higher than in male aortic rings. A distinct rightward shift of the β-PEA CRC was observed in female animals. These results indicate that female animals are less susceptible to β-PEA induced vasoconstriction. Vasoconstrictor responses to β-PEA, were comparable in terms of onset, duration and sustainability. These results suggest that sex hormones do not influence the contractile profile of the trace amines. However, it is likely that female sex hormones influence the ability of trace amines to induce a contractile response. Further research will be required to establish whether this sexual dimorphism is due to a decrease in the number of receptors, receptor sensitivity or an increased vasodilator response to trace amines.

3.6.3 Post-partum variation in vasoconstrictor responses

It is well established that the increased oestrogen levels during pregnancy are associated with a substantial reduction in blood pressure (Dubey et al. 2002). However, progesterone has been reported to, independently of oestrogen, exert a protective influence on the vasculature (Rylance et al. 1985). Bolus injections of progesterone have previously been demonstrated to blunt the pressor response to noradrenaline in anesthetised rats (Barbagallo et al. 2001). The effects of pregnancy on α_1-adrenoceptor and trace amine mediated vasoconstrictor responses were assessed using ex-breeder female rats. No difference in phenylephrine response was observed between non-breeder and ex-breeder rats. Although phenylephrine-induced contractile responses were generally weaker in ex-breeder rats, this was not found to be significantly different as evidenced by the AUC.

β-PEA induced contractile responses in female ex-breeders were not found to be significantly different from non-breeder rats. Similarly, no difference in the EC_{50} or E_{Max} of β-PEA was observed between ex-breeders and non-breeder animals. Contractile responses of ex-breeder females mimicked those of non-breeder females being slow in onset, duration and were sustained. These results indicate an increased level of sex hormones has little effect on trace amine induced contractile responses. However, as responses were not analysed during the gestational period, the effects of sex hormones on trace amine-induced contractile responses cannot be fully evaluated. Further experiments in the presence of

bolus doses of oestrogen and progesterone will be required to establish whether sex hormones influence trace amine-induced vasoconstriction.

3.6.4 Strain dependent differences in trace amine-induced vasoconstrictor responses

To assess strain-dependent differences in trace amine-induce contraction, tyramine and phenylephrine-induced vasoconstrictor responses were assessed in juvenile male Sprague Dawley rats and juvenile Lister Hooded rats. Contractile responses to phenylephrine were observed across a similar concentration range between the two strains. In the presence and absence of endothelium, no differences in phenylephrine-induced contractile responses were observed between Sprague Dawley and Lister Hooded aortic rings.

Tyramine induced concentration dependent contractile responses in both Sprague Dawley and Lister Hooded aortic rings. In the presence of a functional endothelium, contractile responses were weak and observed across a similar concentration range in both strains. In the absence of endothelium stronger contractile responses were observed across a similar concentration range in both strains. Although contractile responses in endothelium-denuded tissues were generally greater in aortic rings from Lister Hooded rats, these were not found to be significantly different across strains. Both the EC_{50} and E_{Max} of tyramine were not significantly different across strains. The contractile profile of tyramine in juvenile animals mimicked that of β-PEA (

Figure 3.5.1 and Figure 3.5.6). Contractile responses were characterised by a rapid onset followed by a rapid decline to baseline in both strains. Although this was only observed in the absence of endothelium as only weak tyramine-induced contractile responses were observed in the presence of endothelium. As the characteristics of tyramine-induced contractile responses are the same as β-PEA, it suggests the same mechanism is responsible for mediating contractile responses for the two trace amines. Furthermore, as the characteristics of tyramine-induced contraction were identical between Sprague Dawley and Lister Hooded aortic rings, this suggests that the change in contractile response over time may be conserved across strains.

3.7 Conclusion

The results of this study indicate a clear influence of age and gender on the contractile profile of trace amines. Contractile responses are generally slower in onset and are more sustainable with increasing age. In younger animals' contractile responses are rapid in onset and characterised by a rapid declining phase. Aortic rings from female animals were less susceptible to β-PEA-induced vasoconstriction than male aortic rings. However, the mechanisms underlying these differences have yet to be established. As only the sexual maturity of animals had any effect on trace amine-induced vasoconstrictor responses all future chapters utilised sexually mature male adult rats.

Chapter 4 Involvement of the endothelium in trace amine-induced vascular responses

4.1 Introduction

Previous investigations of trace amine-induced vascular responses in rat aortic rings have reported conflicting views on the importance of the endothelium. Studies by Fehler et al. (2010) and Broadley et al. (2013) evidenced that trace amines, including *P*-tyramine, β-PEA, tryptamine and amphetamine, induce vasoconstriction independently of the presence of a functional endothelium. In contrast, Khwanchuea et al. (2008) demonstrated tyramine-induced vasoconstriction is potentiated by endothelium removal or inhibition of nitric oxide synthase suggesting the endothelium may act to suppress trace amine induced vasoconstriction through activation of eNOS. Further evidence of an opposing nitric oxide dependent dilator response has been observed in guinea pig aortic rings, where inhibition of eNOS potentiated β-PEA-induced vasoconstrictor responses (Broadley and Broadley 2017).

A vasodilator response to the trace amines was first reported in rat aorta by Varma and Chemtob (1993) and Varma et al. (1995). Similar to the vasoconstrictor actions of trace amines described by Fehler et al. (2010) and Broadley et al. (2013), these trace amine-induced vasodilator responses were shown to be independent of the presence of a functional endothelium (Varma and Chemtob 1993; Varma et al. 1995). Later investigations by Anwar et al. (2012) demonstrated that β-phenylethylamine (β-PEA), tyramine, tryptamine and 5-HT elicit vasodilator responses in the perfused mesenteric vascular bed of rats. In contrast to the findings of Varma and Chemtob (1993) and Varma et al. (1995), these vasodilator responses could be abolished by the nitric oxide synthase inhibitor L-NAME, indicating the involvement of a functional endothelium (Anwar et al. 2012).

The current views of the importance of the endothelium in mediating vascular responses to the trace amines are therefore conflicting. It is likely trace amine-induced vasodilator responses are comprised of endothelium-dependent and -independent components. This chapter aims to clarify the importance of the endothelium during trace amine-induced vasoconstrictor and vasodilator responses.

4.2 Hypothesis

Trace amine-induced vasoconstriction is functionally opposed by an endothelium-dependent vasodilator response.

4.3 Aims

1. Establish the role of the endothelium in mediating TAAR1 agonist-induced vasoconstrictor and vasodilator responses.
2. Determine whether endothelium-derived nitric oxide is involved in trace amine-induced vascular responses.

4.4 Materials and methods

4.4.1 Animals

Male Sprague Dawley, rats (350-750g) were obtained and housed as described in Chapter 2. All experiments adhered strictly to the Animals (Scientific Procedures) Act 1986.

4.4.2 Rat Aortic Rings

Tissue viability and the presence or absence of a functional endothelium were confirmed as described in Chapter 2. Cumulative CRCs were obtained for the contractile responses to β-PEA and RO5256390 under resting tension. Cumulative CRCs were also obtained for the vasodilator responses to β-PEA following pre-constriction with phenylephrine (0.3 μM). Where used, the eNOS inhibitor, L-NAME (100 μM), was added to organ baths 15 minutes prior to carrying out CRCs. To assess the effects of nitric oxide in the absence of endothelium, L-NAME was also added to endothelium denuded preparations as a control as described in Chapter 2.

4.4.3 Drugs and solutions

All drugs were dissolved in Krebs bicarbonate buffer unless otherwise stated. RO5256390, was dissolved in dimethyl sulfoxide (DMSO) prior to dilution with Krebs bicarbonate buffer. The concentration of DMSO did not exceed 0.25% of the total volume in the tissue bath. The composition of Krebs bicarbonate buffer is detailed in Chapter 2.

4.4.4 Data analysis

Contractile responses are expressed as a percentage of the contractile response to high potassium (60mM) Krebs bicarbonate solution (as described in Chapter 2). Vasodilator responses were expressed as a percentage of the contractile response to phenylephrine (0.3 μM) used to pre-constrict tissues. Responses were plotted as the mean response ± the standard error of the mean (S.E.M) where n represents the number of rats. Curve fitting was applied as described in Chapter 2. E_{Max} values are reported as a percentage of the contractile responses to 60 mM KCl or phenylephrine ± S.E.M as appropriate. Negative $logEC_{50}$ values were obtained for each individual curve. EC_{50} values were calculated from the $-logEC_{50}$ and are reported as the mean value with 95% confidence intervals. Mean EC_{50} values were compared by unpaired/paired, two-tailed Student's t-tests or one-way ANOVA with Dunnett's multiple comparison post-hoc test as appropriate. P-values ≤0.05 were considered statistically significant. Area under the curve (AUC) was calculated for specified concentration ranges from individual CRCs. The mean AUC for contractile CRCs was reported as a % of contraction to 60 mM KCl by Log[agonist(M)] (%.Log[M]). For relaxant CRCs AUC was reported as a % of the initial contraction to 0.3 μM phenylephrine by Log[agonist(M)] (%P.Log[M]). AUC's were compared by one-way ANOVA or unpaired, two-tailed Student's t-test as appropriate.

4.5 Results

4.6 Sensitivity of carbachol-induced vasodilation to endothelium removal or inhibition of eNOS

To confirm endothelium removal and complete inhibition of eNOS, the ability of carbachol to induce a vasodilator response of tissue pre-constricted with phenylephrine was assessed following endothelium removal or inhibition of eNOS. Addition of the eNOS inhibitor L-NAME (100 µM) or endothelium removal completely abolished carbachol-induced vasodilator responses in rat aortic rings (Figure 4.6.1). In the presence of a functional endothelium, carbachol had an EC_{50} of 947.2 nM (691.7 nM – 1.3 µM). In endothelium-intact aortic rings the mean contractile response to 0.3 µM phenylephrine was 1.58 ± 0.16 g. This was not significantly different from the response to phenylephrine in the absence of endothelium (1.57 ± 0.20 g) or presence of L-NAME (1.95 g ± 0.23), respectively.

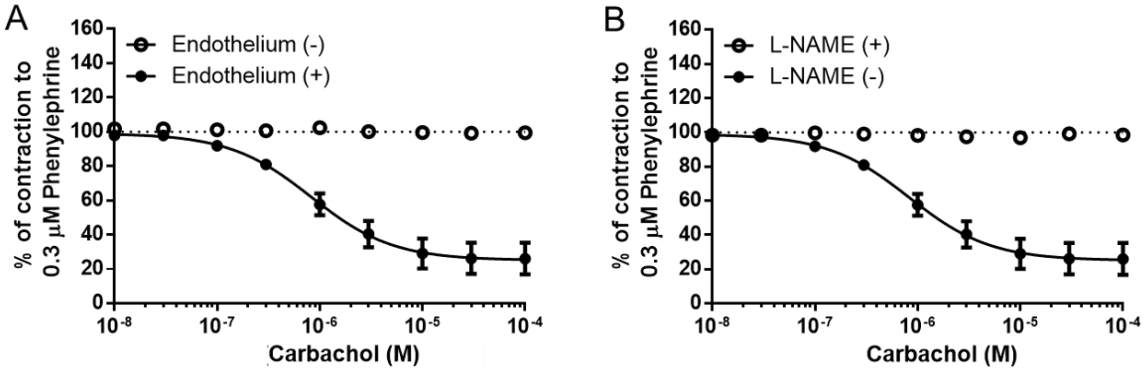

Figure 4.6.1. Mean carbachol cumulative CRCs. Carbachol-induced vasodilator responses of pre-constricted adult Sprague Dawley rat aortic rings in the presence (● n =14) or absence (○ n = 8) of endothelium (A). Carbachol-induced vasodilator responses in pre-constricted endothelium intact aortic rings the presence (● n =14) or absence (○ n = 7) of 100 µM L-NAME (B). Vasodilator responses are reported as the mean contraction (±SEM) expressed as a percentage of the contractile response to 0.3 µM phenylephrine.

4.6.1 The effects of endothelium removal on β-PEA induced vasoconstriction

In endothelium-intact aortic rings, 60 mM KCl induced a mean contractile response of 1.35 ± 0.11 g (n = 17). This was not different from the response to 60 mM KCl in endothelium-denuded aortic rings (1.21 ± 0.11 g, n = 14). β-PEA-induced concentration-dependent contraction of rat aortic rings (Figure 4.6.2). In endothelium-intact aortic rings, contractile responses were only observed at high concentrations (≥100 µM) of β-PEA (Figure 4.6.2A, C). As a maximum contractile response could not be obtained in the presence of a functional endothelium, EC_{50} and E_{Max} values could not be calculated.

Removal of the endothelium induced a leftward shift of the β-PEA CRC (Figure 4.6.2C). In endothelium-denuded aortic rings, contractile responses to β-PEA were observed from 10 µM to 1000 µM (Figure 4.6.2B, C). The area under the β-PEA CRC (AUC, between 1 µM and 1000 µM), in endothelium-denuded aortic rings was found to be significantly greater than that of the β-PEA CRC in endothelium-intact aortic rings (103.7 ± 6.7 %.Log[M] vs 23 ± 2 %.Log[M],respectively, P ≤ 0.001, Figure 4.6.2C). In endothelium-denuded aortic rings, an apparent maximal response to β-PEA was observed and therefore an EC_{50} value of 58 µM (34 – 98 µM, Table 4.6.1) and E_{Max} value of 88 ± 6% were calculated.

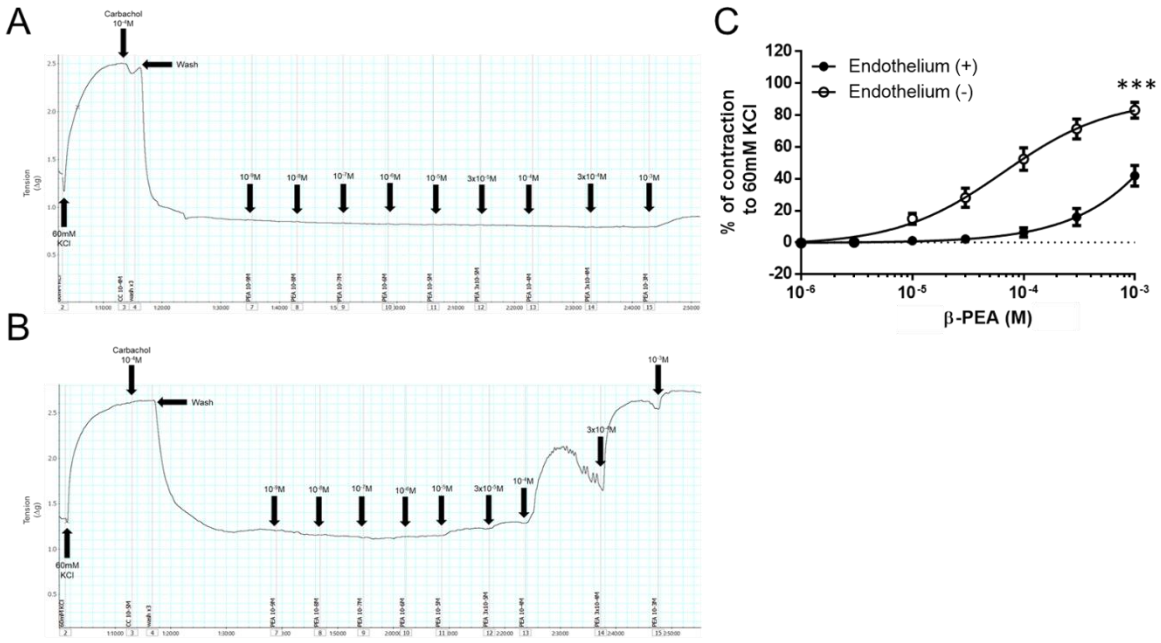

*Figure 4.6.2. β-PEA-induced contractile responses in rat aortic rings. Representative trace of β-PEA-induced contractile responses in the presence (A) or absence (B) of endothelium. Cumulative concentration response curves (CRCs) for β-PEA (C) were generated in the presence (● n = 17) or absence (○ n = 14) of endothelium. Contractile responses are reported as the mean percentage (±SEM) of the maximum contractile response to 60mM KCl. Cumulative CRCs in (C) are generated from the same data used in Figure 3.5.2C-D. *** indicates a significant (P ≤ 0.001) difference in AUC.*

4.6.2 The effects of endothelium removal on theTAAR1 selective agonist RO5256390-induced vasoconstriction

In endothelium-intact aortic rings, 60 mM KCl induced a mean contractile response of 1.26 ± 0.11 g (n = 20). This was not different from the response to 60 mM KCl in endothelium-denuded aortic rings (1.22 ± 0.10 g, n = 16). The TAAR1 selective agonist RO5256390 induced concentration-dependent contractions of rat aortic rings (Figure 4.6.3). In the endothelium-intact aortic rings, small contractile responses were observed at concentrations above 3 µM (Figure 4.6.3A, C). In the presence of a functional endothelium, a full RO5256390 CRC could not be generated and therefore EC_{50} and E_{Max} values were not calculated.

In endothelium-denuded aortic rings, contractile responses to RO5256390 were observed across a similar concentration range to endothelium-intact aortic rings. The area under the RO5256390 CRC (from 1 µM to 30 µM), in endothelium-denuded aortic rings was found to be significantly greater than that of the RO5256390 CRC in the presence of a functional endothelium (59 ± 6 %.Log[M] vs 9 ± 1 %.Log[M],respectively, P ≤ 0.001, Figure 4.6.3C). A full concentration response curve to RO5256390 was apparent in endothelium-denuded aortic rings and therefore an EC_{50} value of 3 µM (2 – 4 µM, Table 4.6.1) and E_{Max} of 62 ± 6 % were estimated.

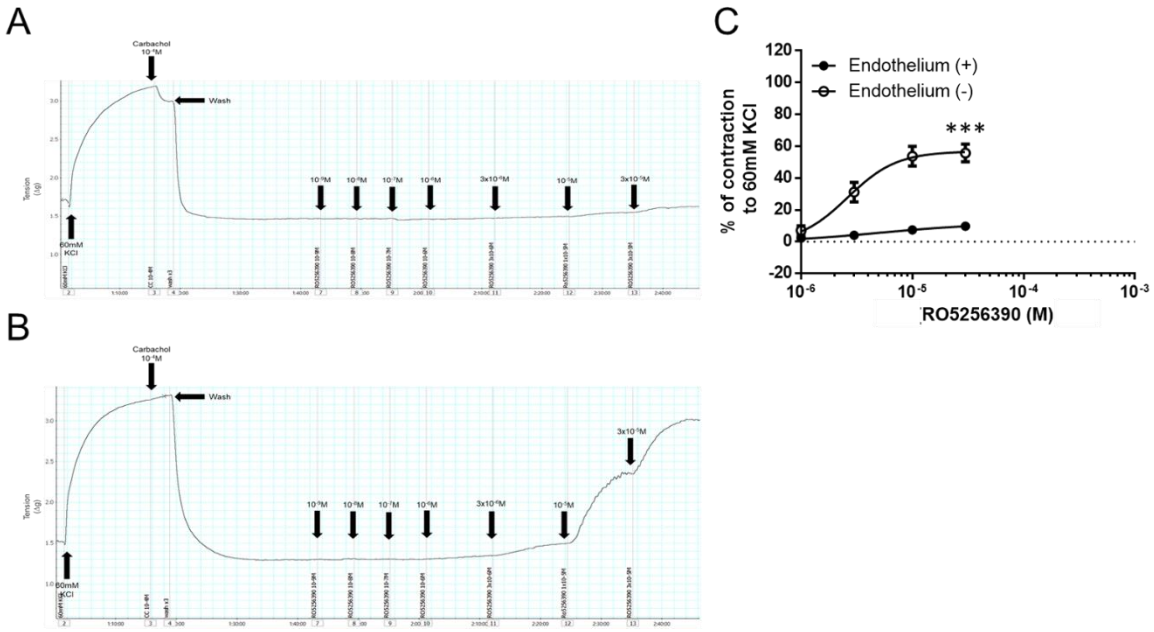

*Figure 4.6.3. RO5256390-induced contractile responses in rat aortic rings. Representative trace of RO5256390-induced contractile responses in the presence (A) or absence (B) of endothelium. Cumulative concentration response curves (CRCs) for RO5256390 (C) were generated in the presence (● n =20) or absence (○ n = 16) of endothelium. Contractile responses are reported as the mean percentage (±SEM) of the maximum contractile response to 60mM KCl. Cumulative CRCs in (C) are generated from the same data used in Figure 3.5.3E-F. *** indicates a significant (P ≤ 0.001) difference in AUC.*

4.6.3 The effects of endothelium-derived nitric oxide on β-PEA- and RO5256390-induced vasoconstriction

In endothelium-intact aortic rings in the presence of L-NAME, 60 mM KCl induced a mean contractile response of 1.62 ± 0.11 g (n = 6). This was not different from the response to 60 mM KCl in the absence of L-NAME (1.35 ± 0.11 g, n = 17). The eNOS inhibitor, L-NAME (100 µM), induced a leftward shift of the β-PEA CRC in endothelium-intact aortic rings (Figure 4.6.4A). The AUC (between 1 µM and 1 mM) in endothelium-intact aortic rings in the presence of L-NAME (78 ± 7 %.Log[M]) was found to be significantly greater (P ≤ 0.01, one-way ANOVA) than the AUC in the absence of L-NAME (23 ± 2 %.Log[M],). In endothelium-intact aortic rings incubated with L-NAME, β-PEA had an EC_{50} value of 534 µM (279 – 1023 µM, Table 4.6.1) and E_{Max} value of 94 ± 3 %.

In the presence of L-NAME and endothelium, β-PEA-induced contractile responses were observed across a similar concentration range to those following endothelium removal (Figure 4.6.4B). In endothelium-intact aortic rings in the presence of L-NAME the EC_{50} (534 µM, 279 – 1023 µM) was not significantly different (one-way ANOVA) from the EC_{50} of β-PEA in endothelium-denuded aortic rings in the absence of L-NAME (58 µM, 34 – 98 µM). Likewise, the E_{Max} of β-PEA in endothelium-intact aortic rings in the presence of L-NAME (94 ± 3 %) was not significantly different (one-way ANOVA) from the E_{Max} of β-PEA in endothelium-denuded aortic rings in the absence of L-NAME (88 ± 6%).

In endothelium-intact aortic rings in the presence of L-NAME, 60 mM KCl induced a mean contractile response of 1.52 ± 0.14 g (n = 7). This was not different from the response to 60 mM KCl in the absence of L-NAME (1.27 ± 0.11 g, n = 20). eNOS inhibition enhanced RO5256390-induced contractile responses in endothelium intact aortic rings (Figure 4.6.4C). Unlike β-PEA, no statistically significant shift in the CRC was observed for RO5256390 in the presence of L-NAME and a functional endothelium. Contractile responses were observed from 10 – 30 µM. The AUC (between 1 µM and 30 µM) in endothelium-intact aortic rings in the presence of L-NAME (16 ± 2 %.Log[M]) was not found to be significantly greater

(one-way ANOVA) than the AUC in the absence of L-NAME (9 ± 1 %.Log[M]). However, the AUC in in endothelium-intact aortic rings in the presence of L-NAME was found to be significantly lower (P ≤ 0.05, one-way ANOVA) than the AUC of endothelium denuded aortic rings in the absence of L-NAME (59 ± 6 %.Log[M]). Despite inhibition of eNOS, a complete CRC to RO5256390 was not observed in the presence of a functional endothelium therefore EC_{50} and E_{Max} values were not calculated.

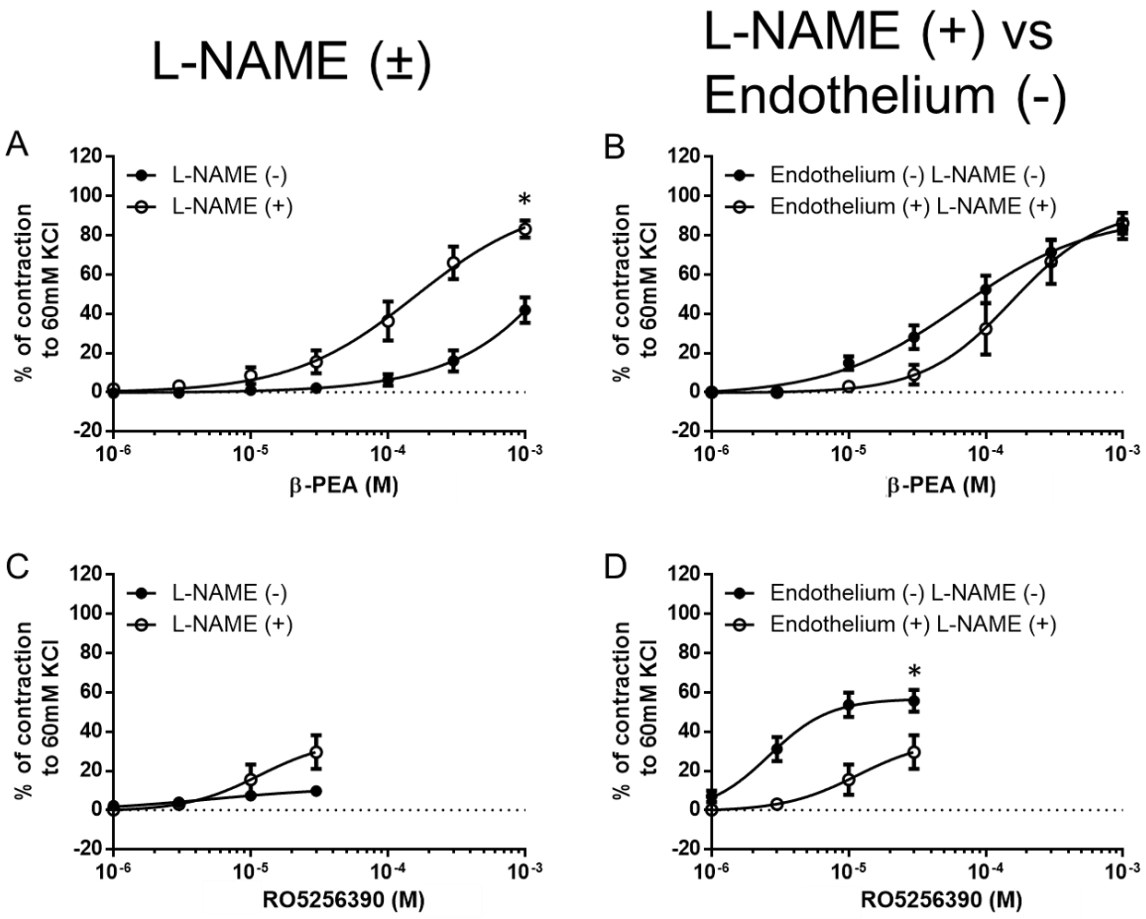

*Figure 4.6.4. The effect of L-NAME 100 μM on β-PEA (A-B) and RO525390 (C-D) cumulative CRCs in rat aortic rings. β-PEA CRCs were obtained (A) in the presence (○ n = 6) or absence (● n = 17) of L-NAME in endothelium-intact aortic rings. A comparison of β-PEA CRCs (B) from endothelium-intact aortic rings (○ in A) in the presence of L-NAME (○ n = 6) with endothelium-denuded aortic rings in the absence of L-NAME (● n = 14). RO5256390 CRCs were generated (C) in the presence (○ n = 7) or absence (● n = 20) of L-NAME in endothelium-intact aortic rings. A comparison of RO5256390 CRCs (D) in endothelium intact aortic rings (○ in C) in the presence of L-NAME (○ n = 7) with endothelium-denuded aortic rings in the absence of L-NAME (● n = 16). Contractile responses are reported as the mean percentage (±SEM) of the maximum contractile response to 60mM KCl. * and ** indicates a significant difference (P ≤ 0.05 or P ≤ 0.01, respectively) in AUC.*

Table 4.6.1. EC_{50} values of β-PEA and RO5256390 contractile responses in the presence or absence of L-NAME in endothelium-intact and -denuded aortic rings

Agonist	Endothelium +/-	L-NAME +/-	EC50 (CI95)
β-PEA	+	-	N/D
		+	534 µM (279 – 1023 µM)
	-	-	58µM (34 – 98 µM)
		+	43 µM (19 – 100 µM)
RO5256390	+	-	N/D
		+	ND
	-	-	3 µM (2 – 4 µM)
		+	3 µM (2 – 5 µM)

4.6.4 β-PEA-induced vasodilator responses in endothelium-intact and -denuded aortic rings

To assess β-PEA-induced vasodilator responses, aortic rings were pre-constricted with a concentration of phenylephrine (0.3 µM) that induced a sub-maximal contractile response. β-PEA induced concentration dependent relaxation of pre-constricted rat aortic rings (Figure 4.6.5). In the presence of a functional endothelium, vasodilator responses were observed from 3 µM to 1000 µM (Figure 4.6.5A, C). The AUC (between 1 µM and 1000 µM) of the β-PEA CRC was 248 ± 4 %.Log[M]. In the presence of a functional endothelium the CRC for β-PEA had an EC_{50} of 128 µM (7 – 144 µM,Table 4.6.2) and an E_{Max} of 47 ± 5 % of the contraction to phenylephrine. In endothelium-intact aortic rings the mean contractile response to 0.3 µM phenylephrine was 1.47 ± 0.07 g. This was not significantly different from the response to phenylephrine in the absence of endothelium of 1.52 ± 0.15 g.

Endothelium removal significantly reduced but did not abolish β-PEA-induced vasodilator responses (Figure 4.6.5B, C). In the absence of endothelium, the AUC (between 1 µM and 1000 µM) of the CRC to β-PEA (286 ± 1 %.Log[M]) was significantly ($P \leq 0.001$, one-way ANOVA) greater than in the presence of endothelium. In the absence of endothelium, the EC_{50} of β-PEA (255 µM , 10 – 460 µM, Table 4.6.2) was significantly ($P \leq 0.01$) increased compared to endothelium intact aortic rings. Similarly, the E_{max} (80 ± 3%) was significantly ($P \leq 0.001$) lower than the E_{max} in the presence of a functional endothelium.

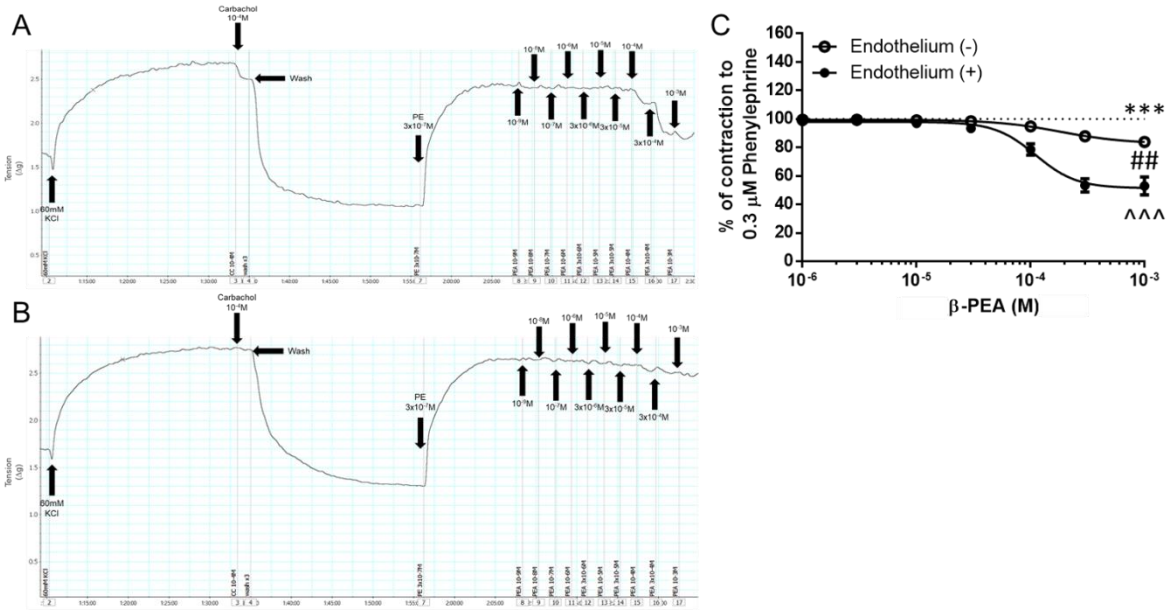

*Figure 4.6.5. β-PEA-induced vasodilator responses in rat aortic rings. Representative traces of β-PEA-induced relaxation in the (A) presence or (B) absence of endothelium. Cumulative CRC's for β-PEA were obtained (C) in the presence (● n = 17) and absence (○ n = 11) of endothelium. Vasodilator responses are reported as the mean response (±SEM) expressed as a percentage of the contractile response to 0.3 µM phenylephrine. *** and ^^^ represent a significant difference (P ≤ 0.001) in the AUC and E_{Max}, respectively. ## indicates a significant (P ≤ 0.01) difference in EC_{50}.*

4.6.5 Involvement of endothelium-derived nitric oxide in β-PEA-induced vasodilation

In endothelium-intact aortic rings in the presence of L-NAME (100 µM) the mean contractile response to 0.3 µM phenylephrine was 2.01 ± 0.18 g. This was significantly (P ≤ 0.05) greater than the response to phenylephrine in the absence of L-NAME of 1.47 ± 0.07 g. L-NAME (100 µM) significantly reduced β-PEA-induced vasodilator responses in the presence of a functional endothelium to a similar degree as endothelium removal (Figure 4.6.6). In the presence of L-NAME the AUC (between 1 µM and 1000 µM) of the CRC to β-PEA was 285 ± 2 %.Log[M]. This was significantly (P≤0.01, one-way ANOVA) increased when compared with the absence of L-NAME, although was not significantly different from endothelium-denuded aortic rings in the absence of L-NAME (Figure 4.6.6C). L-NAME did not significantly (one-way ANOVA) increase the EC_{50} (226 µM ,10 – 367 µM, Table 4.6.2) of β-PEA in the presence of a functional endothelium. However, the E_{Max} (78 ± 4 %) was significantly (P ≤ 0.01, one-way ANOVA) reduced by the presence of L-NAME.

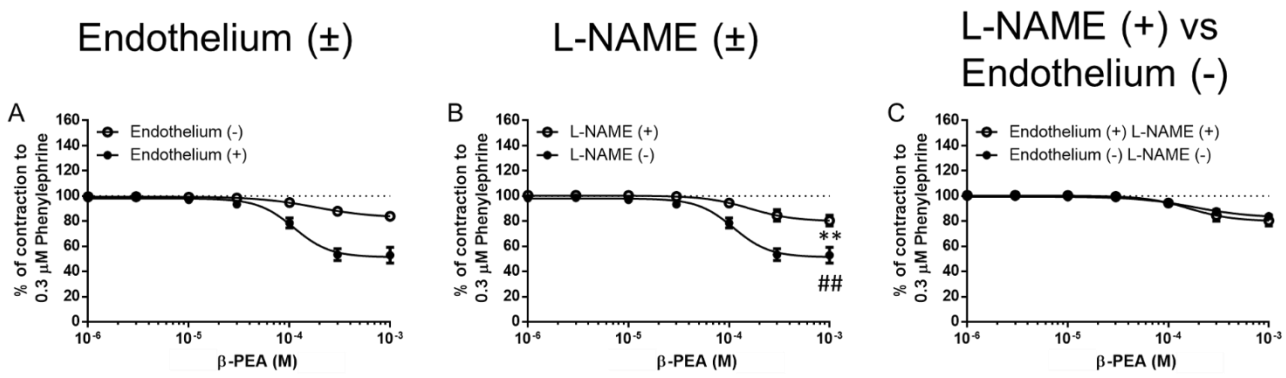

*Figure 4.6.6. The effect of endothelium-derived nitric oxide on β-PEA cumulative CRCs. Cumulative CRCs were obtained for β-PEA in (A) the presence (● n = 17) and absence (○ n = 11) of endothelium. Cumulative CRCs were obtained for β-PEA in (B) the presence (○ n = 7) or absence (● n = 17) of L-NAME (100 µM) in endothelium-intact aortic rings. A comparison of β-PEA-induced vasodilator responses (C) in endothelium-intact aortic rings in the presence of L-NAME (○ n = 7) with endothelium-denuded aortic rings in the absence of L-NAME (● n = 11). Vasodilator responses are reported as the mean contraction (±SEM) expressed as a percentage of the contractile response to 0.3 µM phenylephrine. ** and ## represent a significant difference (P ≤ 0.01) in the AUC and E_{Max}, respectively.*

4.6.6 Effects of nitric oxide in endothelium-denuded tissues

To confirm that inhibition of nitric oxide synthase had no effect on TAAR1 agonist-induced vascular responses in the absence of endothelium, L-NAME (100 µM) was also added to endothelium-denuded preparations as a control. In endothelium-denuded aortic rings, under resting tension, incubation with L-NAME had no significant effect on either β-PEA or RO5256390 CRCs (Figure 4.6.7A, B). The EC_{50} (42.9 µM,18.5 – 99.5 µM) and E_{Max} (78.8 ± 7.7 % of the contractile response to 60 mM KCl) of β-PEA in the presence of L-NAME were not significantly different (one-way ANOVA) from those of in the absence of L-NAME (EC_{50} = 57.9 µM, 34.2 - 98.2 µM, E_{Max} = 88.1 ± 5.6 % of the contraction to 60 mM KCl).

In the absence of a functional endothelium, L-NAME had no significant effect on the CRC to RO5256390. The EC_{50} (2.8, 1.6 – 5.1 µM, Figure 4.6.7) and E_{Max} (58.2 ± 6.9%) of RO5256390 obtained in the presence of L-NAME were not significantly different (one-way ANOVA) from those in its absence (EC_{50} = 2.7 µM, 1.8 – 4.1 µM, E_{Max} = 61.7 ± 5.5 %).

In endothelium-denuded aortic rings pre-constricted with phenylephrine (0.3 µM), inhibition of eNOS, had no significant effect on β-PEA-induced vasodilator responses (Figure 4.6.8). The EC_{50} and E_{max} values of 141.2 µM (7.4 – 175.3 µM) and 75.1 ± 6.3 % of the initial contractile response to phenylephrine, respectively, were not significantly different (one-way ANOVA) from the absence of L-NAME (EC_{50} = 255.3 µM, 9.8 – 459.7 µM, E_{Max} = 79.8 ± 2.9 %).

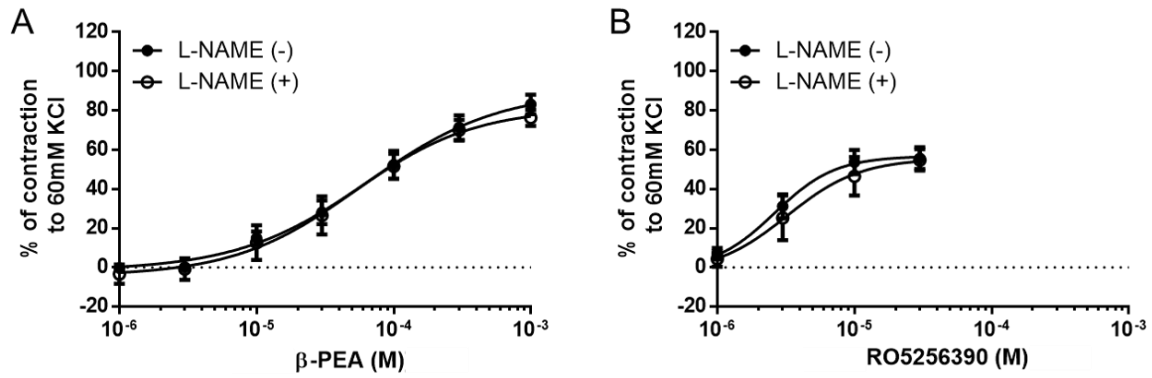

Figure 4.6.7 Mean cumulative CRCs in endothelium-denuded aortic rings from male adult Sprague Dawley rats in the presence of L-NAME (100 µM). (A) β-PEA CRCs in the presence (○ n = 5) or absence (● n = 14) of L-NAME. (B) RO5256390 CRCs in the presence (○ n = 7) or absence (● n = 16) of L-NAME. Contractile responses are reported as the mean percentage (±SEM) of the maximum contractile response to 60mM KCl.

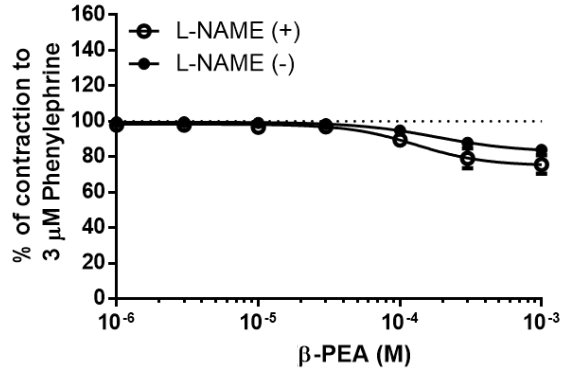

Figure 4.6.8. β-PEA-induced vasodilator responses in the presence (○ n = 6) or absence (● n = 11) of L-NAME (100 µM) in endothelium-denuded aortic rings from adult male Sprague Dawley rats. Vasodilator responses are reported as the mean contraction (±SEM) expressed as a percentage of the contractile response to 3 µM phenylephrine.

Table 4.6.2. EC_{50} values of β-PEA in the presence or absence of L-NAME in endothelium-intact and -denuded aortic rings

Agonist	Endothelium +/-	L-NAME +/-	EC50 (CI95)
β-PEA	+	-	128 µM (7 - 144 µM)
		+	226 µM (10 – 367 µM)
	-	-	255 µM (10 – 460 µM)
		+	141 µM (7 – 175 µM)

4.7 Discussion

4.7.1 β-PEA-induced vasoconstriction is potentiated by endothelium removal

β-PEA caused concentration-dependent contractions of rat aortic rings at concentrations consistent with the previous investigations of Fehler et al. (2010) and Broadley et al. (2013). Interestingly, in the current investigation contractile responses to β-PEA were significantly enhanced by endothelium removal (Figure 4.6.2). This contrasts with the findings of Fehler et al. (2010), who previously reported that β-PEA-induced contractile responses were unaffected by the endothelium removal. As a result, in the subsequent study by Broadley et al. (2013), experiments were carried out in endothelium-denuded aortic rings. Another trace amine, tyramine, also induces concentration dependent contraction of rat aortic rings (Broadley et al. 2013; Khwanchuea et al. 2008). Khwanchuea et al. (2008), found that endothelium removal or inhibition of eNOS, significantly enhanced tyramine-induced contraction of rat aortic rings. β-PEA-induced vasoconstriction of guinea pig aortic rings has also been found to be enhanced following inhibition of eNOS (Broadley and Broadley 2017). Although species differences are likely to exist, these findings and those of Khwanchuea et al. (2008), support those of the current investigation, in that trace amine-induced vasoconstriction is functionally opposed by an endothelium-dependent dilator response.

4.7.2 TAAR1 selective agonist-induced vasoconstriction requires endothelium removal

The TAAR1 selective agonist RO5256390 induced concentration-dependent contractions of rat aortic rings (Figure 4.6.3). This is the first time a selective TAAR1-agonist has been shown to stimulate a contractile response in rat aortic rings. Like β-PEA, contractile responses to RO5256390 were significantly enhanced by removal of the endothelium, supporting the hypothesis that the endothelium suppresses trace amine and RO5256390-induced vasoconstriction. RO5256390 was shown to be approximately 20-fold more potent than β-PEA with an EC_{50} value of 3 µM compared with 58 µM for β-PEA. However, the mean E_{Max} of RO5256390 (62 ± 6 %) was significantly ($P \leq 0.01$) smaller than the E_{Max} of β-PEA (88 ± 6%). This could indicate that RO5256390 may be a partial agonist whereas β-PEA is a full agonist.

4.7.3 Nitric oxide release suppresses β-PEA and RO5256390-induced vasoconstriction

As removal of the endothelium significantly enhanced contractile responses to both β-PEA and RO5256390, it is clear that an endothelium derived factor opposes the vasoconstrictor response. A strong candidate, nitric oxide, is an endothelium derived vasodilator (Palmer et al. 1987), that has previously been shown to be released in response to tyramine and β-PEA (Khwanchuea et al. 2008; Anwar et al. 2012). To determine if nitric oxide was the responsible mediator opposing trace amine and RO5256390-induced vasoconstriction, the sensitivity of contractile responses to the eNOS inhibitor, L-NAME, was assessed.

Inhibition of eNOS, by L-NAME, significantly potentiated both β-PEA and RO5256390-induced vasoconstriction (Figure 4.6.4). eNOS inhibition enhanced β-PEA-induced vasoconstriction to the same degree as endothelium removal whereas RO5256390-induced contractile responses were only partially enhanced by eNOS inhibition. These results suggest that β-PEA and RO5256390 both activate endothelial nitric oxide synthesis. eNOS inhibition completely abrogated the endothelium-dependent functional antagonism of β-PEA-induced vasoconstriction, indicating that nitric oxide release from the endothelium is the sole mediator. Contractile responses to RO5256390 were only partially increased by eNOS inhibition compared with endothelium removal. As the AUC of the RO5256390 CRC was only assessed between 3 µM and 30 µM, which represents the start of the CRC, the AUC was not significantly affected by eNOS inhibition. It would be expected that at higher concentrations, eNOS inhibition would induce a statistically significant separation of the

CRC's, similar to that seen with β-PEA (Figure 4.6.4A) resulting in an increase of the AUC of the RO5256390 CRC. As eNOS inhibition did not potentiate RO5256390 contractile responses to the same degree as β-PEA, it can be hypothesised that RO5256390 might induce release of additional endothelium-derived relaxant factors such as prostacyclin and/or endothelium derived hyperpolarisation factor (EDHF, Jiang et al. 2000). Although structurally very different to β-PEA, RO5256390 actives rat TAAR1 with comparable efficacy to β-PEA in TAAR1 expressing cell lines (Revel et al. 2013). Despite being a TAAR1-selective agonist, RO5256390 clearly activates additional endothelium-dependent vasodilator pathways, whereas β-PEA is entirely dependent on activation of eNOS. At concentrations in excess of 3 μM, RO5256390 is known to show selectivity for alternative receptors including 5-$HT_{2A/B}$, opiod, imdazoline and muscarinic M_3 acetylcholine receptors (Revel et al. 2013). It is likely that RO5256390, unlike β-PEA, has the ability to activate additional endothelium-dependent vasodilator pathways through activation of multiple receptors other than TAAR1.

Collectively with the findings of Khwanchuea et al. (2008) and Anwar et al. (2012), the results of the current study demonstrate that a functional vasodilator response opposes trace amine-induced contraction. This response appears to be dependent on the presence of a functional endothelium and raises the question of whether activation of TAAR1 localised to the endothelium is responsible.

4.7.4 β-PEA induces vasodilator responses in rat aorta

Vasodilator responses to trace amines, including β-phenylethylamine, were first reported in rat aorta by Varma and Chemtob (1993). These vasodilator effects were found to be independent of the presence of a functional endothelium and are discussed in more detail in Chapter 5 (Varma and Chemtob 1993; Varma et al. 1995). In contrast to the findings of the aforementioned studies, in the current study, β-PEA-induced vasodilator responses were significantly more potent in the presence of a functional endothelium (Figure 4.6.5). This indicates that β-PEA can induce vasodilation through both endothelium-dependent and -independent pathways. Studies in the perfused mesenteric vascular bed of rats have also indicated the endothelium plays an important role in trace amine-induced vasodilation (Anwar et al. 2012; Anwar et al. 2013). However, it is possible that β-PEA-induced vasodilator responses vary between different vascular beds and the mesenteric vessels may lack the required receptor at the smooth muscle level. Alternatively, utilising a whole vascular bed may not be sensitive enough to identify the weaker vasodilator responses in the absence of endothelium. Outside of the vasculature a dual relaxant and contractile role of β-PEA was identified in the smooth muscle of rat gastric fundus (Batista-Lima et al. 2018). Although gastric tissues lack an endothelial layer, these dual contractile and relaxant action of β-PEA are possibly mediated by a similar mechanism to those mediating endothelium-independent vasodilation and vasoconstriction identified in the current study.

The findings of previous studies (Varma and Chemtob 1993; Varma et al. 1995; Anwar et al. 2012; Anwar et al. 2013; Batista-Lima et al. 2018) support those of the current study. It is evident that trace amines induce opposing vasoconstrictor and vasodilator response. Importantly, vasodilator responses to the trace amines are comprised of endothelium-dependent and -independent components.

4.7.5 Nitric oxide mediates endothelium dependent β-PEA-induced vasodilation

Endothelium removal significantly attenuated β-PEA induced vasodilator responses, although interestingly a weak vasodilator response remained. It is unlikely that these weak vasodilator responses are due to residual endothelium as endothelium removal completely abolished carbachol induced vasodilation (as demonstrated in Chapter 4). Inhibition of eNOS only partially attenuated β-PEA-induced vasodilator responses in endothelium-intact aortic rings (Figure 4.6.6). Furthermore, inhibition of eNOS did not further inhibit the vasodilator response in endothelium-denuded aortic rings (Figure 4.6.8). This confirms that any residual

vasodilator response to β-PEA cannot be due to endothelium-derived nitric oxide. The findings of this study support the work of Anwar et al. (2012), demonstrating a clear role for nitric oxide in mediating endothelium-dependent vasodilator responses to β-PEA. However, this study clearly demonstrates that β-PEA can also induce vasodilation independently of the endothelium and nitric oxide, supporting the previous work of Varma and Chemtob (1993).

4.8 Conclusion

β-PEA acts as both a vasoconstrictor and vasodilator in rat aortic rings. The vasodilator actions of β-PEA are comprised of endothelium-dependent and -independent components. At the level of the endothelium, β-PEA induces the release of nitric oxide leading to a vasodilator response. Likewise, the TAAR1 selective agonist, RO5256390 also appears to induce both vasoconstrictor and vasodilator responses as evidenced by the enhancement of contractile responses by endothelium removal. Although the vasodilator response to RO525690 remains to be fully elucidated, it seems likely that any vasodilator response would also involve other endothelium-derived vasodilators such as EDHF and/or prostacyclin in addition to nitric oxide.

Chapter 5 Receptors involved in trace amine-induced vasoconstriction

5.1 Introduction

The trace amines include *p*-tyramine, tryptamine, β-PEA and *p*-octopamine (Broadley 2010). In terms of their structure, synthesis and catabolism, they are closely related to the classic aminergic neurotransmitters including, dopamine, noradrenaline and 5-HT (Lindemann and Hoener 2005). Vasoconstrictor responses to the trace amines have typically been attributed to a sympathomimetic effect, through release of noradrenaline from sympathetic neurones (Broadley 2010). However, considering the structural similarities between the trace amines and the other aminergic neurotransmitters, the vasoconstrictor effects of the trace amines could be a result of direct interaction with these receptors (Broadley et al. 2013). Alternatively, evidence suggests the trace amines may also mediate their vascular effects through direct interaction with TAAR1 (Broadley et al. 2013; Fehler et al. 2010)

Expression of TAAR1 at both the mRNA and protein levels has previously been confirmed in rat aorta by Fehler et al. (2010). Numerous agonists of TAAR1 including; *p*-tyramine, tryptamine, β-PEA and amphetamine, induce concentration-dependent contraction of rat aortic rings (Broadley et al. 2013; Fehler et al. 2010). The aforementioned studies by Fehler et al. (2010) and Broadley et al. (2013) demonstrated that trace amine-induced contractile responses are resistant to a cocktail of receptor antagonists and inhibitors of α_1 and β_2-adrenoceptors, monoamine transport and monoamine oxidases. The authors subsequently suggested that TAAR1 mediates the contractile responses. The major limitation of these investigations is the lack of TAAR1 selective reagents. Consequently, the authors resorted to comparing the potency order of vasoconstrictor responses with published values from TAAR1 transfected cell lines (Broadley et al. 2013; Fehler et al. 2010). Although a viable method, the potency order for vasoconstriction did not recapitulate that of cell lines (Fehler et al. 2010). This could indicate that TAAR1 does not mediate trace amine induced vasoconstriction. However, the overexpression and forced coupling of TAAR1 to adenyl cyclase in cell lines (Borowsky et al. 2001; Lindemann et al. 2005), may introduce differences in the relative affinity of the trace amines for TAAR1.

In recent years, a number of selective full and partial TAAR1 agonists and a single TAAR1 antagonist have been developed (Bradaia et al. 2009; Revel et al. 2011; Revel et al. 2012; Revel et al. 2013). The aim of this chapter is to confirm a role for TAAR1 in mediating vasoconstrictor responses to trace amines by utilising both the TAAR1 selective agonist RO5256390 (Revel et al. 2013) and murine selective TAAR1 antagonist, EPPTB (Bradaia et al. 2009).

5.2 Hypothesis

Trace amine-induced vasoconstrictor responses are mediated by TAAR1.

5.3 Aims

1. Confirm the involvement of TAAR1 in mediating TAAR1 agonist-induced contractile responses.
2. Assess the ability of TAAR1 agonists to induced-contractile responses via classic aminergic neurotransmitter receptors.

5.4 Materials and methods

5.4.1 Animals

Male Sprague Dawley, rats (350-750g) were obtained and housed as described in Chapter 2. All experiments adhered strictly to the Animals (Scientific Procedures) Act 1986.

5.4.2 Rat aortic rings

Endothelium removal potentiates both β-PEA- and RO5256390-induced contractile responses (see Chapter 4). Unless otherwise stated, all experiments in this chapter were carried out in the absence of endothelium. Tissue viability and the presence or absence of a functional endothelium were confirmed as described in Chapter 2. Cumulative CRCs were obtained for phenylephrine, dopamine, 5-HT, β-PEA, carbachol and RO5256390. Non-cumulative CRC's were also obtained for 5-HT. Where used, antagonists were added to organ baths 15 minutes prior to carrying out CRCs.

5.4.3 Cumulative and non-cumulative 5-HT concentration response curves

To establish a method of 5-HT receptor desensitisation, it was first necessary to assess the sensitivity of 5-HT responses to cumulative or non-cumulative addition of 5-HT. 5-HT induced concentration-dependent contraction of rat aortic rings when added cumulatively or non-cumulatively (Figure 5.4.1). Endothelium removal induced a significant leftward shift of both cumulative and non-cumulative 5-HT CRCs (Figure 5.4.2). There was no significant difference (one-way ANOVA) in the AUC (for the concentration range of 0.1 µM to 100 µM) between cumulative (142 ± 7 %.Log[M]) and non-cumulative (150 ± 7 %.Log[M]) addition of 5-HT, in the presence of a functional endothelium. Similarly, in the absence of endothelium, there was no significant difference (one-way ANOVA) in the AUC (for the concentration range of 0.1 µM to 100 µM) between cumulative (244 ± 7 %.Log[M]) and non-cumulative (230 ± 6 %.Log[M]) addition of 5-HT. However, the AUC in the absence of endothelium were found to be significantly greater ($P \leq 0.001$, one-way ANOVA) than the corresponding AUCs obtained in endothelium-intact aortic rings. In the presence of a functional endothelium, the EC_{50} for the cumulative addition of 5-HT (3.7 µM, 2.3 – 5.9 µM) was not significantly different from that obtained for non-cumulative addition (2.4 µM, 1.5 – 3.7 µM, one-way ANOVA, Table 5.4.1). In the absence of a functional endothelium, the EC_{50} for the cumulative addition of 5-HT (0.6 µM, 0.4 µM – 1.2 µM) was not significantly different from that obtained for non-cumulative addition (0.5 µM, 0.3 – 0.8 µM, one-way ANOVA, Table 5.4.1). However, with both cumulative and non-cumulative addition of 5-HT, the EC_{50} value for 5-HT CRCs were significantly decreased ($P \leq 0.001$) by endothelium removal. As no differences were observed between cumulative and non-cumulative addition of 5-HT, cumulative addition was selected for desensitisation of 5-HT responses.

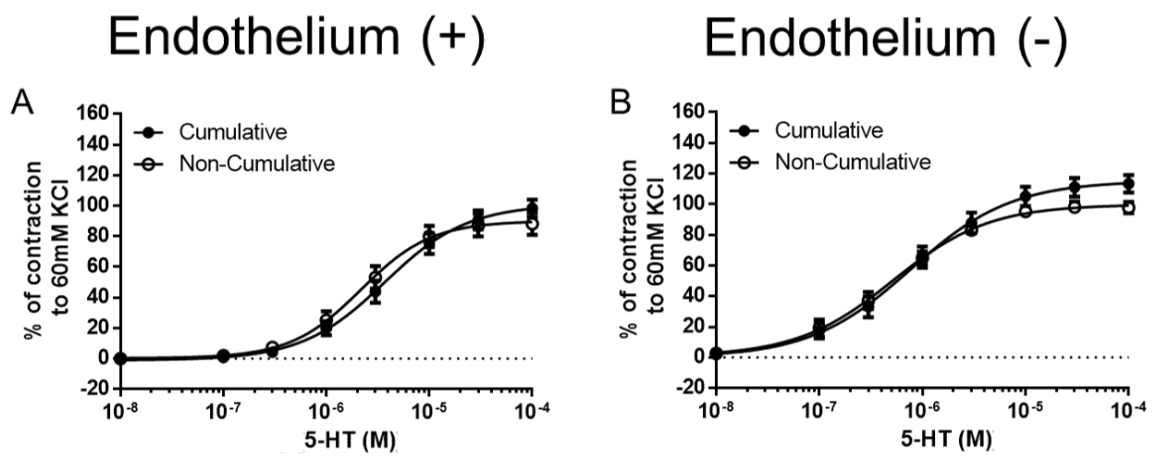

Figure 5.4.1. CRCs for cumulative (●) or non-cumulative (○) addition of 5-HT in rat aortic rings. Data is reported for (A) endothelium-intact aortic rings (● n = 10, ○ n = 12) and (B) endothelium-denuded aortic rings (● n = 10, ○ n = 12). Contractile responses are reported as the mean percentage (±SEM) of the maximum contractile response to 60mM KCl.

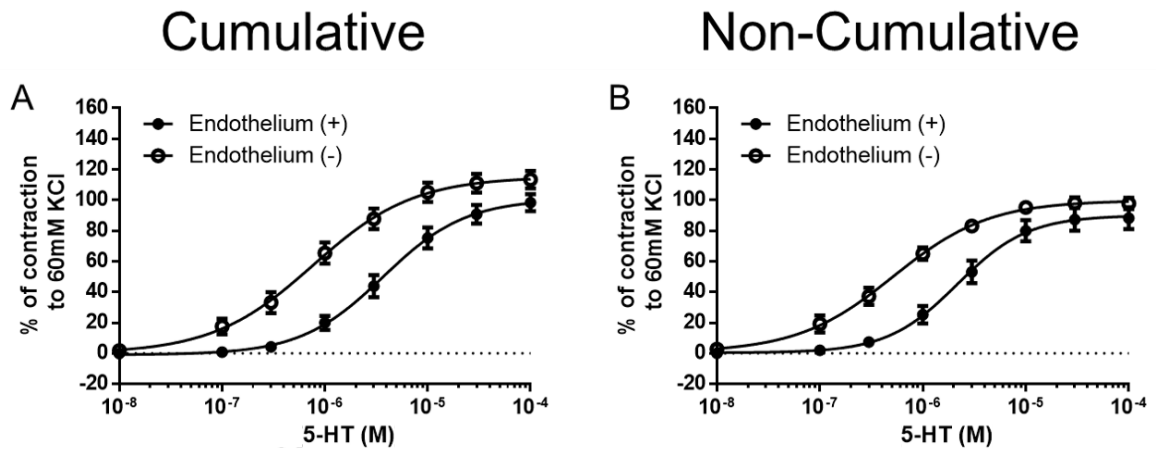

Figure 5.4.2. The effect of endothelium removal on cumulative (A) and non-cumulative (B) CRCs for 5-HT in rat aortic rings. Cumulative CRCs (A) were obtained in the presence (● n = 10) or absence (○ n = 10) of endothelium. Non-cumulative CRCs (B) were obtained in the presence (● n = 12) or absence (○ n = 12) of endothelium. Contractile responses are reported as the mean percentage (±SEM) of the maximum contractile response to 60mM KCl.

Table 5.4.1. EC_{50} of 5-HT added cumulatively or non-cumulatively in the presence or absence of endothelium

Agonist	Cumulative/ non-cumulative	Endothelium (+/-)	EC_{50} (CI95)
5-HT	Cumulative	+	3.7 µM (2.3 – 5.9 µM)
		-	0.6 µM (0.4 µM – 1.2 µM)
	Non-cumulative	+	2.4 µM (1.5 – 3.7 µM)
		-	0.5 µM (0.3 – 0.8 µM)

5.4.4 5-HT desensitisation

A detailed description of the protocol used for desensitisation of 5-HT responses is provided in Chapter 2. In brief, initial cumulative CRC's were obtained for both β-PEA and 5-HT after confirming tissue viability. Following drug washout and return to baseline tension, a concentration of 5-HT in excess of that required to elicit a maximal contractile response (100 µM) was added for a 1-hour incubation period. Tissues were washed and allowed to reach baseline tension prior to bring subjected to 3x 1-hour incubations with a submaximal concentration of 5-HT (10 µM). Contractile responses to 5-HT were considered desensitised following a decline in the response to single 10 µM bolus additions of 5-HT to ≤ 50% of the initial response. After confirming 5-HT desensitisation, a second cumulative CRC to either β-PEA or 5-HT was generated.

5.4.5 Drugs and solutions

All drugs were dissolved in Krebs bicarbonate buffer unless otherwise stated. RO5256390, haloperidol, SCH 39166 and prazosin were dissolved in dimethyl sulfoxide (DMSO) prior to dilution with Krebs bicarbonate buffer. Due to the insoluble nature of EPPTB, this was dissolved in DMSO but not further diluted with Krebs bicarbonate buffer. To control for any potential effect of DMSO, an equivalent concentration of DMSO was added to organ baths without antagonists. The concentration of DMSO did not exceed 0.25% of the total volume. The source of all drugs used in this chapter and the composition of Krebs bicarbonate buffer are detailed in Chapter 2.

5.4.6 Data analysis

Contractile responses are expressed as a percentage of the contractile response to high potassium (60mM) Krebs bicarbonate solution as described in Chapter 2. Responses are plotted as the mean response ± S.E.M where n represents the number of rats. Curve fitting was applied as described in Chapter 2. E_{Max} values are reported as a percentage of the contractile responses to 60mM KCl ± S.E.M. Negative logEC_{50} values were obtained for each individual curve. EC_{50} values were calculated from the -logEC_{50} obtained from individual CRCs and are reported as the mean value with 95% confidence intervals. Mean EC_{50} and E_{max} values were compared by unpaired/paired, two-tailed Student's t-tests or one-way ANOVA with Dunnett's multiple comparison post-hoc test as appropriate. P-values ≤0.05 were considered statistically significant. The apparent equilibrium dissociation constant (K_B) for antagonists was calculated as described in Chapter 2 and reported as the molar value ± S.E.M. AUC was calculated for specified concentration ranges from individual CRCs. The mean AUC was reported as a % of contraction to 60 mM KCl by Log[agonist(M)] (%.Log[M]). AUC's were compared by unpaired, two-tailed Student's T-test or one-way ANOVA as appropriate.

5.5 Results

5.5.1 The effect of the murine selective TAAR1 antagonist EPPTB on β-PEA- and RO5256390-induced contractile responses

In endothelium-denuded aortic rings, 60 mM KCl induced a mean contractile response of 1.21 ± 0.11 g (n = 14). This was not different from the response to 60 mM KCl in endothelium-denuded aortic rings used for the DMSO (0.25% vol/vol) vehicle control (1.15 ± 0.18 g, n = 6) or EPPTB (1.12 ± 0.14 g, n = 7).DMSO, used as a vehicle control for EPPTB, appeared to reduce the vasoconstrictor response to β-PEA at the high affinity end of the CRC to β-PEA (Figure 5.5.1, A). In the presence of DMSO, the AUC, (for the concentration range 1 µM to 1000 µM) of the β-PEA CRC (55 ± 5%.Log[M]), was not significantly different (one-way ANOVA) than the AUC in the absence of DMSO (104 ± 7%.Log[M]). The EC_{50} of β-PEA in the presence of DMSO (130 µM,1 – 631 µM) was not significantly different (one-way ANOVA) from that obtained in the absence of DMSO (58 µM, 34 – 98 µM, Table 5.5.1). The E_{Max} of β-PEA in the presence of DMSO (63 ± 7 %) was not significantly different from that obtained in the absence of DMSO (88 ± 6 %, one-way ANOVA).

Addition of the TAAR1-selective antagonist EPPTB had no significant effect on the CRC for β-PEA compared with the vehicle control (Figure 5.5.1B). EPPTB had no significant effect (unpaired, two-tailed Students T-Test) on the AUC for β-PEA (101 ± 4 %.Log[M]) compared with the vehicle control (55 ± 3 %.Log[M]). The EC_{50} for the CRC to β-PEA in the presence of DMSO (130 µM,1 – 631 µM) was not significantly different (unpaired, two-tailed Students T-Test) from the EC_{50} in the presence of EPPTB (197 µM,11 µM – 9000 µM Table 5.5.1). The E_{Max} of β-PEA in the presence of EPPTB (62 ± 12%) was not significantly different from that obtained in the absence of EPPTB (62 ± 7 %, one-way ANOVA).

In endothelium-denuded aortic rings used for RO5256390, 60 mM KCl induced a mean contractile response of 1.22 ± 0.10 g (n = 16). This was not significantly different from the mean contractile response induced by 60 mM KCl in endothelium-denuded aortic rings used with RO5256390 and EPPTB (0.81 ± 0.1, n = 6). EPPTB had no significant effect on the TAAR1 selective agonist RO5256390 CRCs (Figure 5.5.2). The AUC (between 3 µM and 30 µM) for RO5256390 in the presence of EPPTB (46 ± 4 %.Log[M]) was not significantly different (one-way ANOVA) from the AUC obtained in the absence of EPPTB (59 ± 6 %.Log[M]). In the presence of EPPTB, the EC_{50} (4 µM, 2 – 8 µM) and E_{Max} (48 ± 8%) were not significantly different (one-way ANOVA) from the EC_{50} (3 µM, 2 – 4 µM) or E_{max} (62 ± 6%) obtained in the absence of EPPTB (Table 5.5.1).

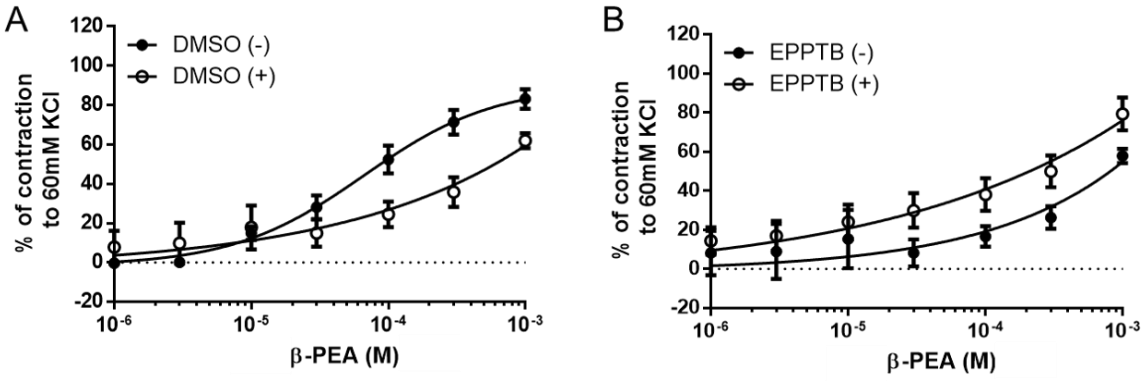

Figure 5.5.1. The effect of EPPTB (5 µM) and DMSO (0.25%) on β-PEA cumulative CRCs in endothelium-denuded aortic rings. β-PEA CRCs were generated (A) in the absence (● n=14, curve the same as endothelium-denuded in Figure 4.6.2C) or presence (○ n = 7) of DMSO or (B) in the absence (● n = 7, same data from A) or presence of EPPTB (○ n = 6). Contractile responses are reported as the mean percentage (±SEM) of the maximum contractile response to 60mM KCl.

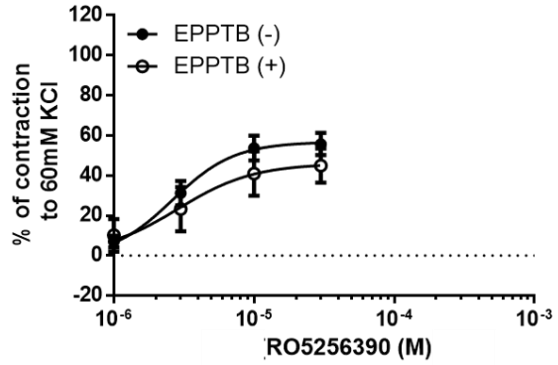

Figure 5.5.2. The effect of EPPTB (5 μM) on RO5256390 cumulative CRCs in endothelium-denuded aortic rings. CRCs were generated in the absence (● n = 16) or presence (○ n = 6) of EPPTB. Contractile responses are reported as the mean percentage (±SEM) of the maximum contractile response to 60mM KCl.

Table 5.5.1. EC_{50} of β-PEA and RO5256390 in endothelium-denuded aortic rings in the presence or absence of 5 μM EPPTB

Agonist	Antagonist	EC_{50} (CI95)
β-PEA	None	58 μM (34 – 98 μM)
	DMSO (0.25% vol/vol)	130 (1 – 631 μM)
	EPPTB (5 μM)	197 μM (11 μM – 9000 μM)
RO5256390	Control	3 μM (2 – 4 μM)
	EPPTB (5 μM)	4 μM (2 – 8 μM)

5.5.2 Sensitivity of β-PEA- and RO5256390-induced vasoconstriction to α_1-adrenoceptor antagonism

In endothelium-denuded aortic rings used for phenylephrine, 60 mM KCl induced a mean contractile response of 1.22 ± 0.10 g (n = 16). This was not significantly different from the mean contractile response induced by 60 mM KCl in endothelium-denuded aortic rings used for prazosin experiments of 1.34 ± 0.05 g (n = 5). The α_1-adrenceptor antagonist prazosin (1μM) induced a significant rightward shift of the phenylephrine CRC (Figure 5.5.3A). In the absence of prazosin, the EC_{50} of the phenylephrine CRC was 15 nM (8 – 27 nM, Table 5.5.2). As a complete CRC was not obtained in the presence of prazosin, an EC_{50} was not estimated. To calculate the observed degree of shift the EC_{20} (20% of the contractile response to 60mM KCl, (Table 5.5.3) was used. Prazosin (1 μM) induced an approximately 10,000-fold rightward degree of shift of the phenylephrine CRC.

In endothelium-denuded aortic rings used for β-PEA experiments in the presence of prazosin, 60 mM KCl induced a mean contractile response of 1.38 ± 0.12 g (n = 7). This was not significantly different from the mean contractile response induced by 60 mM KCl in endothelium-denuded aortic rings used experiments in the absence of prazosin of 1.21 ± 0.11 g (n = 14). Prazosin induced an approximately 3-fold rightward shift of the β-PEA CRC (Figure 5.5.3B). The EC_{50} for the CRC to β-PEA in the presence of prazosin (322 μM, 219 – 473 μM, Table 5.5.2) was significantly greater ($P \leq 0.01$, paired, two-tailed, Students T-test) than the EC_{50} in the absence of prazosin (58 μM, 34 – 98 μM, Table 5.5.2). The shift of the β-PEA CRC induced by prazosin (~3-fold), was found to be significantly ($P \leq 0.05$, one-way ANOVA) less than that observed for the phenylephrine CRC(~10,000-fold).

In endothelium-denuded aortic rings used for RO5256390 experiments in the presence of prazosin, 60 mM KCl induced a mean contractile response of 1.37 ± 0.19 g (n = 6). This was

not significantly different from the mean contractile response induced by 60 mM KCl in endothelium-denuded aortic rings used experiments in the absence of prazosin of 1.22 ± 0.10 g (n = 16). Prazosin had no significant effect on the CRC to RO5256390 (Figure 5.5.3C). The EC_{50} value calculated in the presence of prazosin (3 µM, 1 – 11 µM, Table 5.5.2) was not significantly different (one-way ANOVA) from the EC_{50} obtained in the absence of prazosin (3 µM, 2 – 4 µM, Table 5.5.2).

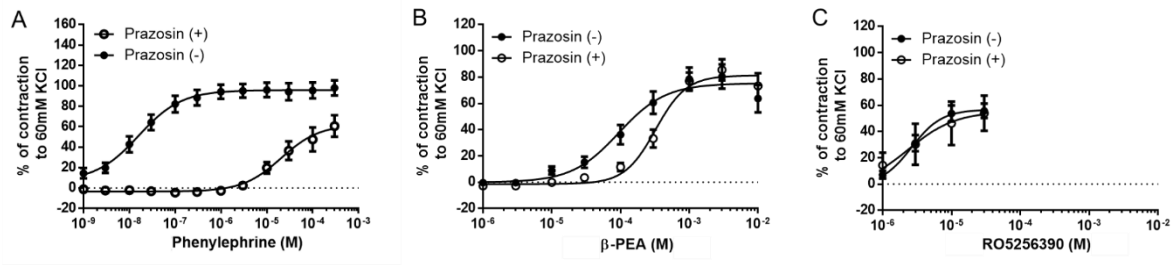

Figure 5.5.3. The effects of 1 µM prazosin on CRCs to phenylephrine (A), β-PEA (B) and RO5256390 (C) in endothelium-denuded aortic rings. CRCs for (A) phenylephrine were obtained in the absence (● n = 9) or presence (○ n = 5) of prazosin. CRCs for (B) β-PEA were obtained in the absence (● n = 7) or presence (○ n = 7) of prazosin. CRCs for (C) RO5256390 were obtained in the absence (● n = 6) or presence (○ n = 6) of prazosin. Contractile responses are reported as the mean percentage (±SEM) of the maximum contractile response to 60mM KCl.

Table 5.5.2. EC_{50} of phenylephrine, β-PEA and RO5256390 in endothelium denuded aortic rings in the presence or absence of 1 µM Prazosin

Agonist	Antagonist	EC_{50} (CI95)
Phenylephrine	Control	15 nM (8 – 27 nM)
	Prazosin (1 µM)	ND
β-PEA	None	58µM (34 – 98 µM)
	Prazosin (1 µM)	322 µM (219 – 473 µM)
RO5256390	Control	3 µM (2 – 4 µM)
	Prazosin (1 µM)	3 µM (1 – 11 µM)

Table 5.5.3. EC_{20} of phenylephrine in the presence or absence of prazosin in endothelium-denuded aortic rings.

Agonist	Antagonist	EC_{20} (CI95)
Phenylephrine	None	0.8 nM (0.2 – 2.8 nM)
	Prazosin (1 µM)	6.1 µM (3.7 – 10.0 µM)

5.5.3 Sensitivity of β-PEA vasoconstrictor responses to 5-HT-receptor desensitisation

In endothelium-denuded aortic rings used for 5-HT desensitisation experiments, 60 mM KCl induced a mean contractile response of 1.53 ± 0.14 g (n = 11). The mean contractile response to 10 µM 5-HT was reduced to 41 ± 7% of the initial contractile response, following desensitisation. Desensitisation of 5-HT receptors, significantly reduced the AUC (for the concentration range 0.01 µM to 100 µM) from 213 ± 8 %.Log[M] to 41 ± 3 %.Log[M] ($P \leq 0.01$, paired, two-tailed, Students T-test). Receptor desensitisation significantly increased the EC_{50} from 1.1 µM (0.5 – 2.4 µM, Table 5.5.4) to 6.8 µM (3.1 – 15.3 µM, $P \leq 0.001$, paired, two-tailed, Students T-test Table 5.5.4). Desensitisation induced a significant 6.3-fold rightward degree of shift of the 5-HT CRC (Figure 5.5.4A).

Prior desensitisation of the response to 5-HT had no significant effect on the β-PEA EC_{50} (Figure 5.5.4B and Table 5.5.4). However 5-HT receptor desensitisation significantly reduced the AUC (for the concentration range 1 µM to 1000 µM) for the β-PEA CRC from 46 ± 4 %.Log[M] to 8 ± 3% ($P \leq 0.05$).

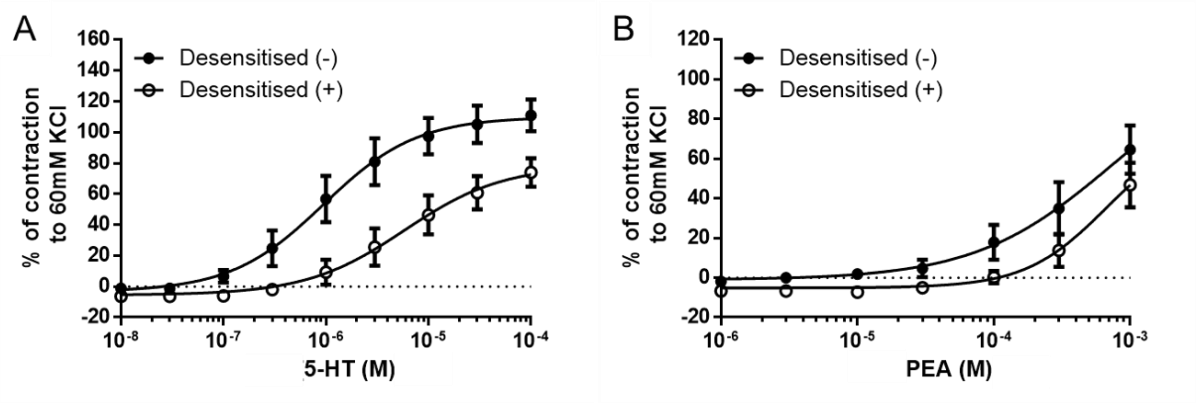

Figure 5.5.4 The effects of 5-HT receptor desensitisation on mean cumulative (A) 5-HT and (B) β-PEA CRCs in endothelium-denuded aortic rings. Mean cumulative CRCS were obtained for (A) 5-HT prior (● n = 6) and post (○ n = 6) desensitisation of 5-HT responses. Mean cumulative CRCs were obtained for (B) β-PEA CRCs prior (● n = 5) and post (○ n = 5) desensitisation of 5-HT responses. Contractile responses are reported as the mean percentage (±SEM) of the maximum contractile response to 60mM KCl.

Table 5.5.4. EC_{50} of 5-HT and β-PEA in endothelium denuded aortic rings before and after 5-HT receptor desensitisation.

Agonist	Desensitisation	EC_{50} (CI95)
5-HT	None	1.1 µM (0.5 – 2.4 µM)
	Desensitised	6.8 µM (3.1 – 15.3 µM
β-PEA	None	31 µM (9 – 108 µM)
	Desensitisation	49 µM (16 – 154 µM)

5.5.4 Sensitivity of β-PEA-induced contraction to 5-HT_2 receptor antagonism

In endothelium-denuded aortic rings used for 5-HT experiments, 60 mM KCl induced a mean contractile response of 1.68 ± 0.14 g (n = 10). This was not different from the mean contractile response induced by 60 mM KCl in endothelium-denuded aortic rings used for 5-HT experiments in the presence of cinanserin of 1.37 ± 0.11 (n = 6). The 5-HT_2 receptor antagonist, cinanserin (100 nM), induced a significant rightward shift of 5-HT-induced CRC (Figure 5.5.5A). Cinanserin significantly (P≤0.001) increased the EC_{50} from 0.6 µM (0.4 - 1.2 µM) to 5.1µM (2.8 – 9.4 µM, Table 5.5.5).The observed degree of shift in the EC_{50} was approximately 10.0-fold. The calculated K_B for cinanserin, based on the apparent shift of 5-HT CRCs, was 13 ± 2 nM. Cinanserin, significantly reduced the AUC (between 0.01 µM and 100 µM) of the 5-HT CRC from 244 ± 7 %.Log[M] to 138 ± 7 %.Log[M] (P ≤ 0.001, one-way ANOVA).

In endothelium-denuded aortic rings used for β-PEA experiments in the presence of cinanserin, 60 mM KCl induced a mean contractile response of 1.03 ± 0.15 g (n = 5). This was not different from the mean contractile response induced by 60 mM KCl in endothelium-denuded aortic rings used the absence of cinanserin of 1.21 ± 0.11 (n = 14). Cinanserin had no effect on the CRC for β-PEA (Figure 5.5.5B). In the presence of cinanserin, the EC_{50} for the CRC to β-PEA in endothelium-denuded aortic rings was 37 µM (0.2 – 200 µM, Table 5.5.5). This was not significantly different from the EC_{50} (58µM, 34 – 98 µM, Table 5.5.5) obtained in the absence of cinanserin. The AUC (between 1 µM and 1000 µM) in the presence of cinanserin (135 ± 5 %.Log[M]) was not significantly different from the AUC in the absence of cinanserin (104 ± 7 %.Log[M]).

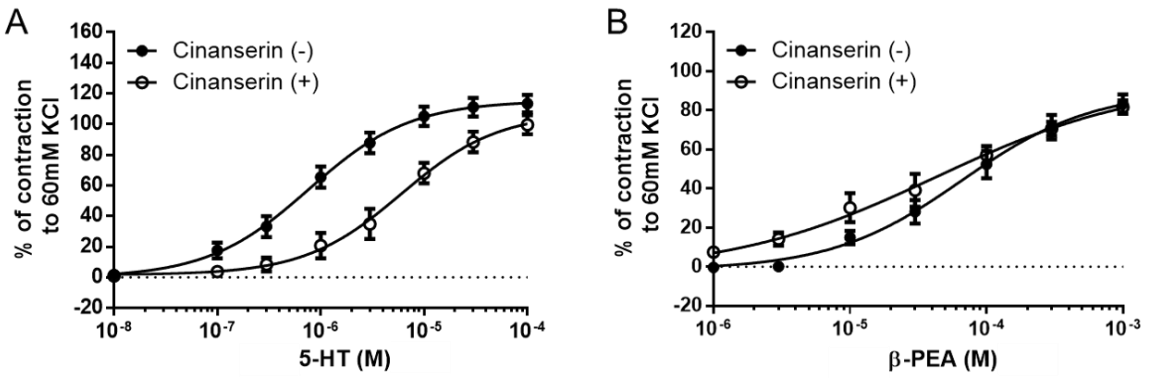

Figure 5.5.5. The effects of 100 nM cinanserin on (A) 5-HT and (B) β-PEA CRCs in endothelium-denuded aortic rings. CRCs were generated for (A) 5-HT CRCs in the absence (● n = 10) or presence (○ n = 6) of cinanserin. CRCs for β-PEA (B) were generated in the absence (● n = 14) or presence (○ n = 5) of cinanserin. Contractile responses are reported as the mean percentage (±SEM) of the maximum contractile response to 60mM KCl.

Table 5.5.5. EC_{50} of 5-HT and β-PEA in endothelium denuded aortic rings in the presence or absence of cinanserin.

Agonist	Antagonist	EC_{50} (CI95)
5-HT	None	0.6 µM (0.4 - 1.2 µM)
	Cinanserin 100 nM	5.1µM (2.8 – 9.4 µM)
β-PEA	None	58µM, 34 – 98 µM
	Cinanserin 100 nM	37 µM (0.2 – 200 µM)

5.5.5 Involvement of dopamine receptors in β-PEA-induced vasoconstriction

In endothelium-intact aortic rings used for dopamine experiments, 60 mM KCl induced a mean contractile response of 2.36 ± 0.10 g (n = 5). This was not different from the mean contractile response induced by 60 mM KCl in endothelium-intact aortic rings used in the presence of DMSO (2.16 ± 0.1, n = 8) or endothelium-denuded aortic rings (1.69 ± 0.34, n = 5). In endothelium-denuded aortic rings used for dopamine experiments in the presence of DMSO, 60 mM KCl induced a mean contractile response of 1.68 ± 0.11 g (n = 8). This was not different from the endothelium-denuded aortic rings used in the absence of DMSO (1.69 ± 0.34, n = 5). Dopamine induced concentration-dependent contraction of rat aortic rings (Figure 5.5.6A). In the presence of a functional endothelium, the AUC (between 0.01 µM and 1000 µM,177 ± 5 %.Log[M]) was significantly ($P \leq 0.01$, one-way ANOVA) lower than in the absence of endothelium (284 ± 7 %.Log[M]). DMSO, used as a vehicle for the antagonists (0.05% of total volume), did not have any significant effect on dopamine responses (Figure 5.5.6B, C). The EC_{50} for the dopamine CRC in the presence of 0.05% DMSO (1.0 µM, 0.7 µM – 1.4 µM, Table 5.5.6) was not significantly different from that in the absence of DMSO (2.5 µM, 1.3 -5.0 µM, Table 5.5.6).

In endothelium-denuded aortic rings used for dopamine experiments in the presence of haloperidol or SCH 39166, 60 mM KCl induced a mean contractile response of 1.55 ± 0.22 g (n = 5) and 1.88 ± 0.2 g (n = 5), respectively. These were not different from the endothelium-denuded aortic rings used in the presence of DMSO (1.68 ± 0.11 g, n = 8). The dopamine D_2 receptor antagonist, haloperidol (1 µM) and the $D_{1/5}$ receptor antagonist, SCH 39166 (3 µM) caused a significant rightward shift of the dopamine CRC (Figure 5.5.7A-B). The EC_{50} for the dopamine CRC in the presence of DMSO (1.0 µM, 0.7 µM – 1.4 µM, Table 5.5.6), was

significantly (P ≤ 0.001. one-way ANOVA) increased by both haloperidol (5.7 µM, 3.4 – 9.3 µM, Table 5.5.6) and SCH 39166 (5.5 µM, 4.1 – 7.3 µM, Table 5.5.6). Haloperidol and SCH 39166 induced a shift of approximately 4.0-fold and 6.3-fold, respectively. The E_{Max} for the dopamine CRC in the presence of DMSO (94 ± 4%) was not significantly different from that obtained in the presence of haloperidol (85 ± 7%) or SCH 39166 (89 ± 7%).

In endothelium-denuded aortic rings used for β-PEA experiments in the presence of DMSO, 60 mM KCl induced a mean contractile response of 1.30 ± 0.20 g (n = 9). This was not different from the contractile response to 60 mM KCl in aortic rings used for -PEA experiments in the presence of haloperidol (1.07 ± 0.10 g, n = 7) or SCH 39166 (1.58 ± 0.19 g, n = 6). DMSO (0.05% vol/vol) had no significant effect on the β-PEA CRC (Figure 5.5.8A). Neither haloperidol nor SCH 39166 had a significant effect on the β-PEA CRC (Figure 5.5.8B-C). The EC_{50} values for the β-PEA CRC in the presence of haloperidol and SCH 39166 were 114 µM (67 – 194 µM) and 89 µM (31 – 251 µM), respectively (Table 5.5.6). These were not significantly different (one-way ANOVA) from the EC_{50} value for the CRC to β-PEA obtained in the presence of DMSO alone (64 µM, 2 – 100 µM, Table 5.5.6).

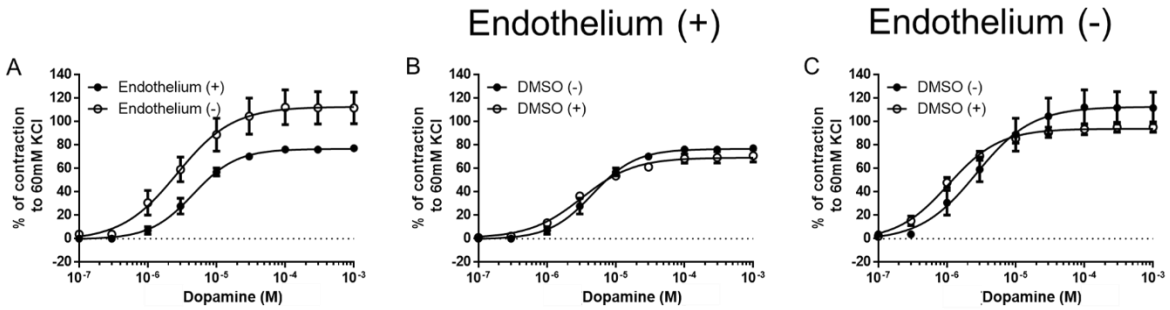

Figure 5.5.6. The effects of endothelium removal and 0.05% DMSO on dopamine CRCs in rat aortic rings. Cumulative CRCs for dopamine were obtained (A) in presence (● n = 5) or absence (○ n = 5) of endothelium. In endothelium-intact aortic rings (B), cumulative CRCs for dopamine were obtained in the absence (● n = 5) or presence (○ n = 8) of DMSO. In the absence of endothelium (C), cumulative CRCs for dopamine were obtained in the absence (● n = 8) or presence (○ n = 5) of DMSO. Contractile responses are reported as the mean percentage (±SEM) of the maximum contractile response to 60mM KCl.

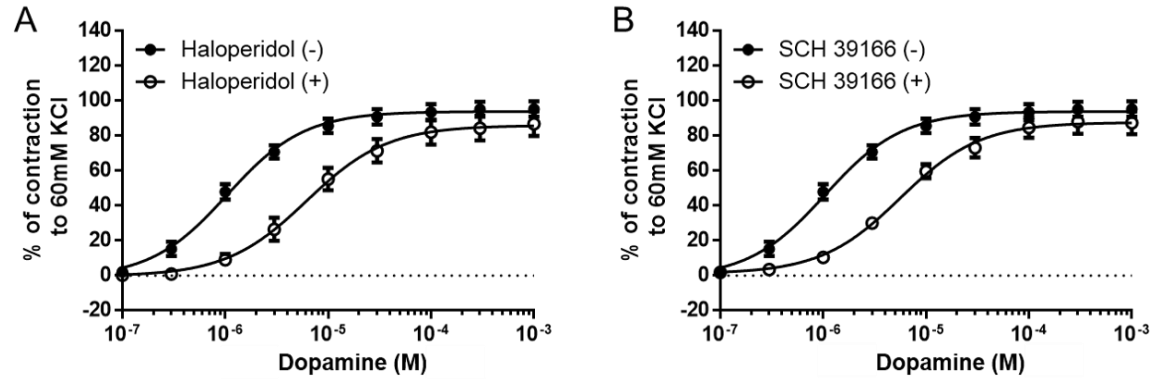

Figure 5.5.7. The effects of 1 µM haloperidol (A) and 3 µM SCH 39166 (B) on dopamine CRCs in endothelium-denuded aortic rings. CRCs for dopamine were obtained (A) in the absence (DMSO vehicle control, ●, n = 8) or presence (○ n = 6) of 1 µM haloperidol. CRCs for dopamine were obtained (B) in the absence (DMSO vehicle control, ●, n = 8) or presence (○ n = 5) of 3 µM SCH 39166. Contractile responses are reported as the mean percentage (±SEM) of the maximum contractile response to 60mM KCl.

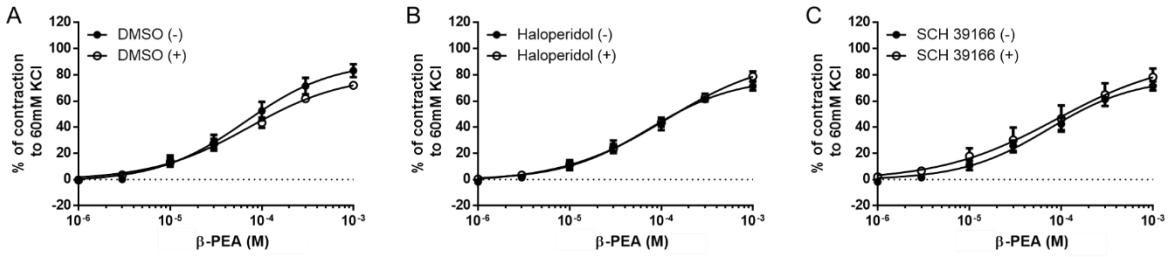

Figure 5.5.8. The effects of (A) 0.05% DMSO, (B) 1 μM haloperidol and (C) 3 μM SCH 39166 on β-PEA CRCs in endothelium-denuded aortic rings. Cumulative β-PEA CRCs obtained in (A) the absence (● n = 14) or presence (○ n = 9) of 0.05% DMSO. Cumulative β-PEA CRCs obtained (B) in the absence (● n = 9) or presence (○ n = 7) of 1 μM haloperidol. Cumulative β-PEA CRC obtained (C) in the absence (● n = 9) or presence (○ n = 6) of 3 μM SCH 39166. Contractile responses are reported as the mean percentage (±SEM) of the maximum contractile response to 60mM KCl.

Table 5.5.6. EC_{50} of dopamine and β-PEA in endothelium denuded aortic rings in the presence or absence of haloperidol or SCH 31966.

Agonist	Antagonist	EC_{50} (CI95)
Dopamine	Control	2.5 μM (1.3 -5.0 μM)
	DMSO 0.05% (Vol/Vol)	1.0 μM (743.7 nM – 1.4 μM)
	Haloperidol 1 μM	5.7 μM (3.4 – 9.3 μM)
	SCH 31966 3 μM	5.5 μM (4.1 – 7.3 μM)
β-PEA	Control	58 μM (34 – 98 μM)
	DMSO 0.05% (Vol/Vol)	64 μM (2 – 100 μM)
	Haloperidol 1 μM	114 μM (67 – 194 μM
	SCH 31966 3 μM	89 μM (31 – 251μM

5.6 Discussion

5.6.1 TAAR1 involvement in β-PEA- and RO5256390-induced vasoconstriction

Many endogenous agonists of TAAR1 have already been identified including the trace amines themselves and the thyronamines (Borowsky et al. 2001; Bunzow et al. 2001; Scanlan et al. 2004). Considerable efforts have also led to the development of several full and partial synthetic agonists of TAAR1 (Revel et al. 2011; Revel et al. 2012; Revel et al. 2013). To date, only a single TAAR1 antagonist, EPPTB has been discovered (Bradaia et al. 2009). The current investigation is the first time that a selective TAAR1 antagonist has been used to investigate trace amine-induced vasoconstriction in rats.

EPPTB (5 μM) had no significant effect on the β-PEA CRC (Figure 5.5.1). EPPTB also failed to block contractile responses to the selective TAAR1 agonist RO5256390 (Figure 5.5.2). EPPTB has a low affinity for rat TAAR1 with an IC_{50} of 4539 nM (Bradaia et al. 2009) and it is therefore possible that the concentration used in this study (5 μM) may be too low to observe a shift in CRC's. However, based on the published inhibition constant (K_I) of EPPTB for rat TAAR1 (0.942 μM, Bradaia et al. 2009; Stalder et al. 2011), at the concentration used in the current study the expected shift of a response mediated by TAAR1 would be 6.3-fold. This indicates that the concentration of EPPTB should be sufficient. However, a 6.3-fold shift may be difficult to observe without a larger sample size to overcome the natural variability. Batista-Lima et al. (2018) previously demonstrated that 50 μM EPPTB was sufficient to block β-PEA-induced relaxation of rat gastric fundus. Furthermore at 100 μM, EPPTB was shown to significantly reduce tyramine-, synephrine- and octopamine-induced contractile responses in porcine coronary arteries (Koh et al. 2019). However, the limit of solubility in the current study was found to be 5 μM for EPPTB when added to organ baths. Considering the poor solubility and selectivity of EPPTB for rat TAAR1, EPPTB appears to be unsuitable to use as a tool for the study of vascular trace amine responses in rats. The development of more species-specific and soluble TAAR1 antagonists will be required to definitively confirm a role for TAAR1 in mediating the vasoconstrictor responses to the trace amines in a species other than mice.

5.6.2 β-PEA and RO5256390 contractile responses are not mediated by α_1-adrenoceptors

Historically trace amine-induced vasoconstriction has been attributed to their ability to induce noradrenaline release from sympathetic neurones (Broadley 2010). As trace amines are structurally similar to adrenaline and noradrenaline (McGeer 2013), it is also possible that trace amines could directly activate adrenoceptors (Broadley et al. 2013). The current study investigated the ability of β-PEA and the TAAR1 selective agonist RO5256390 to induce vasoconstriction through direct and indirect activation of α1-adrenoceptors. This was achieved by testing the sensitivity of β-PEA- and RO5256390-induced contractile responses to the α_1-adrenoceptor antagonist, prazosin.

Blockade of α-adrenoceptors failed to antagonise β-PEA and RO5256390 induced contractile responses. However, the concentration of prazosin (1 μM) used in this investigation induced an ~10,000-fold shift of the phenylephrine CRC (Figure 5.5.3A). This is higher than the expected shift of approximately 2800-fold calculated from the published pA_2 value for prazosin antagonising an α_1-adrenoceptor response (Aboud et al. 1993). Although, prazosin induced a slight rightward shift of the β-PEA CRC (Figure 5.5.3B), this degree of shift was many orders of magnitude less than the shift observed with phenylephrine. This is strong evidence that α_1-adrenoceptors do not mediate β-PEA-induced vasoconstriction. However, the small rightward shift indicates that at high concentrations prazosin may have some affinity for the receptor through which β-PEA mediates a contractile response. This slight rightward shift of the β-PEA CRC was not seen in the previous observations of Fehler et al. (2010) and Broadley et al. (2013). However, octopamine induced vasoconstriction was

shown to also be partially antagonised by prazosin (Broadley et al. 2013; Broadley and Richards 2015) Interestingly the rate of onset of octopamine induced-contraction was significantly reduced in the presence of prazosin (Broadley and Richards 2015). However, the onset of β-PEA induced-vasoconstriction in the presence of α1-adrenoceptor blockade was not investigated in the current study.

5.6.3 5-HT receptors do not mediate β-PEA-induced vasoconstrictor responses

In addition to noradrenaline, the trace amines are structurally related to 5-HT (McGeer 2013). Furthermore, the discovery of TAAR1 by Borowsky et al. (2001) was achieved using oligonucleotides based on the 6th and 7th transmembrane domains of 5-HT receptors. This highlights the potential for trace amines to interact with 5-HT receptors. Evidence has since demonstrated a clear role for 5-HT receptors in mediating tryptamine-induced contraction of the mesenteric vascular bed (Anwar et al. 2012; Anwar et al. 2013). β-PEA-induced contraction of gastric smooth muscle has also been shown to be mediated via 5-HT receptors (Batista-Lima et al. 2018).

The current study used receptor desensitisation and competitive antagonism to assess whether 5-HT receptors might play a role in mediating β-PEA-induced contraction of aortic rings. The ability of 5-HT to induce vasoconstrictor responses of aortic rings was initially assessed by both cumulative and non-cumulative addition of 5-HT. This was to confirm the presence of functional 5-HT receptors that could be desensitised. Both cumulative and non-cumulative addition of 5-HT induced concentration-dependent contractile responses of rat aortic rings that were potentiated by endothelium removal (Figure 5.4.1). This confirmed the presence of 5-HT receptors in rat aorta, a finding that is consistent with several previous investigations (Forster and Whalley 1982; Villazón et al. 2002; Watts et al. 2012). Most likely, these responses are mediated by 5-HT_{2A} receptors as these receptors predominantly mediate the vasoconstrictor actions of 5-HT in isolated vessels (Watts et al. 2012). As responses were potentiated by endothelium removal, it is likely that 5-HT receptors are present on both smooth muscle and the endothelium. It is likely that endothelial 5-HT receptors mediate the release of endothelium-derived vasodilators, such as nitric oxide (Garcia-Pedraza et al. 2016). As no difference was observed between cumulative and non-cumulative CRC's, cumulative addition of 5-HT was used to assess 5-HT receptor desensitisation.

G-protein coupled receptor (GPCR) desensitisation can be described as the loss of response following the prolonged or repeated administration of an agonist due to uncoupling of a receptor from its signalling cascade (Rajagopal and Shenoy 2018). Desensitisation may be heterologous or homologous in nature. Homologous desensitisation only occurs at receptors to which the agonist binds, whereas heterologous desensitisation is a more generalised effect resulting in a loss of response at multiple GPCRs even in the absence of ligand binding (Kelly et al. 2008). In the current study receptors were considered desensitised following a reduction in the response to a sub maximal dose of 5-HT to ≤ 50% of the initial response. Desensitisation of aortic rings to 5-HT, induced a significant rightward shift of 5-HT CRC's. Unlike 5-HT, no shift was observed for β-PEA CRC's indicating that 5-HT receptors do not mediate trace amine-induced contractile responses.

Although 5-HT receptor desensitisation indicated 5-HT receptors do not mediate β-PEA-induced contraction, further evidence was sought through the use of selective 5-HT antagonists. As 5-HT_{2A} receptors are the main mediator of 5-HT responses in rat aorta (Gilmore and Michael 2011), the non-selective 5-HT_2 antagonist cinanserin was utilised. The concentration of cinanserin used in this study (100 nM), was sufficient to induce a 10-fold shift of 5-HT CRC's (Figure 5.5.5A). Based on the published K_B values of cinanserin (41 nM ,Leysen et al. 1981), the expected shift of the 5-HT CRC at 100 nM cinanserin is approximately 3-fold. This suggests that 5-HT induced contractile responses in the rat aorta are mediated by 5-HT_2 and possible other subsets of 5-HT receptors. Cinanserin failed to

induce any shift of β-PEA generated CRC's (Figure 5.5.5B). Unlike, 5-HT receptor desensitisation, blockade of 5-HT_2 receptors did not reduced the AUC of the β-PEA CRC and therefore indicates that 5-HT_2 receptors do not mediate β-PEA-induced contractile responses in rat aorta.

5.6.4 β-PEA-induced contractile responses are not mediated by dopamine receptors

Dopamine, a well-known TAAR1 agonist, is closely related in terms of structure, synthesis and metabolism to the trace amines (Bunzow et al. 2001; Grandy et al. 2016). The physiological effects of dopamine are mediated by 5 types of dopamine receptor ($D_{1\text{-}5}$) that can be divided into two classes, D_1-class (D_1 and D_5) and D_2-class ($D_{2\text{-}4}$, Missale et al. 1998; Beaulieu et al. 2015). Due to the substantial overlap between dopamine and trace amines, this study investigated trace amine induced vasoconstriction via dopamine receptors.

Dopamine induced concentration-dependent contractile responses of rat aortic rings (Figure 5.5.6). Classically dopamine receptor signalling induces vasodilator responses, although it is well known that at higher concentrations vasoconstriction via α_1-adrenoceptors predominates (Li et al. 2008; Overgaard and Dzavik 2008). In this study dopamine-induced vasodilation was not investigated. However, dopamine-induced vasoconstrictor responses were significantly enhanced in the absence of the endothelium, indicating that there may be an endothelium-derived vasodilator response to dopamine. To establish whether dopamine receptors were responsible for dopamine-induced contractile responses, the D_1 (SCH 39166) and D_2 (haloperidol) receptor antagonists were utilised. Blockade of D_2 receptors by haloperidol induced a significant rightward shift of the dopamine CRC. Similarly, blockade of D_1 receptors by SCH 39166 also significantly shifted the dopamine CRC. These results indicate that dopamine-induced contractile responses are mediated by both D_1 and D_2 receptors. This was unexpected, as within the vasculature both D_1 and D_2 receptors have only been reported to mediate vasodilation (Li et al. 2008; Murphy 2000). Vasoconstrictor responses to dopamine have previously been attributed to dopamine binding to α_1-adrenoceptors (Overgaard and Dzavik 2008). It is possible that the shift in the contractile CRC is a result of non-specific binding to adrenoceptors as haloperidol and SCH 39166 have an affinity for α_1 (K_i = 12 nM, Kroeze et al. 2003) and α_2-adrenoceptors (K_i = 732 nM, Wu et al. 2005), respectively. Based on these K_i values, the expected shift of the dopamine CRC by SCH 39166 (3 µM) antagonising an α_2-adrenoceptor and haloperidol antagonising an α_1-adrenoceptor response (1 µM) is 5.4-fold and 251-fold, respectively. In the current study, SCH 39166 induced an ~6.3-fold shift, indicating possible involvement of α_2-adrenoceptors. Haloperidol induced an ~4-fold shift of the dopamine CRC, therefore this shift cannot be due to antagonism of α_1-adrenoceptors.

Blockade of both D_1 and D_2 receptors did not induce any observable shift of the β-PEA CRC. Furthermore, contractile responses were not reduced in the presence of either haloperidol or SCH 39166. These findings confirm that β-PEA induced vasoconstrictor responses are not mediated by dopamine receptors. Furthermore, as SCH 39166 displays some affinity for α_2-adrenoceptors, this indicates that these also do not mediate β-PEA induced vasoconstrictor responses. Although a role for α_2-adrenoceptors was not directly assessed in this study, their involvement in trace amine-induced vasoconstriction has previously been ruled out (Broadley et al. 2013).

5.7 Conclusion

β-PEA induced contractile responses are not mediated by classical biogenic amine neurotransmitter receptors. It should be considered that the vasoconstrictor actions of β-PEA and other trace amines may be mediated through the release of other endogenous vasoconstrictors or activation of a novel receptor. The TAAR1 selective-antagonist, EPPTB, has low affinity for the rat TAAR1. Without the availability of a rat TAAR1-selective antagonist, the involvement of TAAR1 in trace amine-induced vasoconstrictor responses cannot be definitely determined.

Chapter 6 Investigating receptors involved in β-PEA-induced vasodilation

6.1 Introduction

Chapter 4 demonstrated β-PEA-induced vasodilation is comprised of both endothelium-dependent and -independent mechanisms. The endothelium-dependent mechanism was shown to be reliant on the stimulation of eNOS and subsequent release of nitric oxide. Previous studies of trace amine-induced vasodilation in rat aortic rings demonstrated that endothelium-independent vasodilator responses were not exerted via β-adrenoceptors or receptors for 5-HT, histamine and adenosine (Varma and Chemtob 1993; Varma et al. 1995). These studies predated the discovery of trace amine associated receptors (TAARs, Borowsky et al. 2001; Bunzow et al. 2001). Later work in perfused rat mesenteric vessels identified an endothelium-dependent vasodilator action of the trace amines (Anwar et al. 2012; Anwar et al. 2013). These vasodilator responses were attributed to the action of TAAR1 (Anwar et al. 2013) but were not completely characterised.

The current chapter aims to confirm whether trace amine-induced vasodilation is mediated by TAAR1 or other classical vasodilators.

6.2 Hypothesis

Endothelium-dependent and -independent β-PEA-induced vasodilation is mediated by TAAR1.

6.3 Aims

1. Confirm a role for TAAR1 in mediating β-PEA-induced vasodilation.
2. Assess sensitivity of β-PEA induced vasodilation to antagonism of β_2-adrenoceptors and muscarinic M_3 acetylcholine receptors.

6.4 Materials and methods

6.4.1 Animals

Male Sprague Dawley, rats (350-750g) were obtained and housed as described in Chapter 2. All experiments adhered strictly to the Animals (Scientific Procedures) Act 1986.

6.4.2 Rat Aortic Rings

Tissue viability and the presence or absence of a functional endothelium were confirmed as described in Chapter 2. Aortic rings were pre-constricted with phenylephrine (0.3 μM) prior to constructing cumulative-concentration response curves (CRCs). Cumulative CRCs were obtained for β-PEA, isoprenaline and carbachol. Antagonists or the nitric oxide synthase inhibitor, L-NAME were added to organ baths 15 minutes prior to carrying out CRCs.

6.4.3 Drugs and solutions

All drugs were dissolved in Krebs bicarbonate buffer unless otherwise stated. EPPTB was dissolved in dimethyl sulfoxide (DMSO). To control for DMSO, an equivalent concentration of DMSO was added to organ baths without antagonists. The concentration of DMSO did not exceed 0.25% of the total volume. The composition of Krebs bicarbonate buffer is detailed in Chapter 2.

6.4.4 Data analysis

Vasodilator responses are expressed as a percentage of the contractile response to phenylephrine (0.3 μM) used to pre-constrict tissues. Responses are plotted as the mean response ± the S.E.M where n represents the number of rats in each group. Curve fitting was applied as described in Chapter 2. E_{Max} values are reported as a percentage of the contractile responses to phenylephrine ± S.E.M. Negative logEC_{50} values were obtained for each individual curve. EC_{50} values were calculated from the -logEC_{50} and are reported as the mean value with 95% confidence intervals. Mean EC_{50} values were compared by unpaired/paired, two-tailed Student's t-tests or one-way ANOVA with Dunnett's multiple comparison post-hoc test as appropriate. P-values ≤0.05 were considered statistically significant. AUC was calculated for specified concentration ranges from individual CRCs. The mean AUC was reported as a % of the initial contraction to 0.3 μM phenylephrine by Log[agonist(M)] (%P.Log[M]). AUC's were compared by one-way ANOVA or unpaired, two-tailed Student's t-test as appropriate.

6.5 Results

6.5.1 The murine selective TAAR1 antagonist EPPTB has no effect on β-PEA-induced vasodilator responses

In endothelium-intact aortic rings used for β-PEA experiments in the presence of DMSO, the mean contractile response to 0.3 μM phenylephrine was 1.60 ± 0.07 g (n = 5). This was not different from the response to phenylephrine in endothelium-intact aortic rings used for β-PEA experiments in presence of EPPTB (1.35 ± 0.13 g, n = 7). In endothelium-denuded aortic rings used for β-PEA experiments in the presence of DMSO, the mean contractile response to 0.3 μM phenylephrine was 1.53 ± 0.31 g (n = 4). This was not different from the response to phenylephrine in endothelium-denuded aortic rings used for β-PEA experiments in presence of EPPTB (1.33 ± 0.20 g, n= 6).

DMSO (0.25% vol/vol), used as a vehicle control for EPPTB, had no significant effect on the β-PEA CRC (Figure 6.5.1A, B). The AUC (for the concentration range 1 μM to 1000 μM) of the β-PEA CRCs in the presence of DMSO (251 ± 4 %P.Log[M], endothelium-intact, 276 ± 2 %P.Log[M] ,endothelium denuded) were not different from that obtained in the absence of DMSO (248 ± 4 %P.Log[M], endothelium-intact, 286 ± 1 %P.Log[M], endothelium-denuded). The EC_{50} of β-PEA CRCs in the presence of DMSO were 222 μM (101 – 485 μM, Table 6.5.1) and 142 μM (119 – 171 μM, Table 6.5.1), in endothelium-intact and denuded aortic rings respectively. Neither were found to be different from those obtained in the absence of DMSO (Table 6.5.1). The E_{Max} of β-PEA in the presence of DMSO (35 ± 9% endothelium-intact, 56 ± 19 % endothelium-denuded) was not different from that obtained in the absence of DMSO (47 ± 5 % endothelium-intact, 80 ± 3% endothelium-denuded, Chapter 4).

The TAAR1 selective antagonist, EPPTB (5 μM), had no significant effect on the CRC to β-PEA (Figure 6.5.1C, D). In the presence of EPPTB, the AUC of the β-PEA CRC in endothelium-intact aortic rings (246 ± 4%P.Log[M]) was not different from that obtained in the presence of DMSO (0.25% vol/vol). EPPTB had no significant effect (unpaired, two-tailed, Students T-test) on the EC_{50} (156 μM,139 – 175 μM, Table 6.5.1) or E_{Max} (35 ± 6 %) of β-PEA in endothelium-intact aortic rings. The absence of endothelium had no significant impact on the lack of antagonistic effects of EPPTB (Figure 6.5.1D).

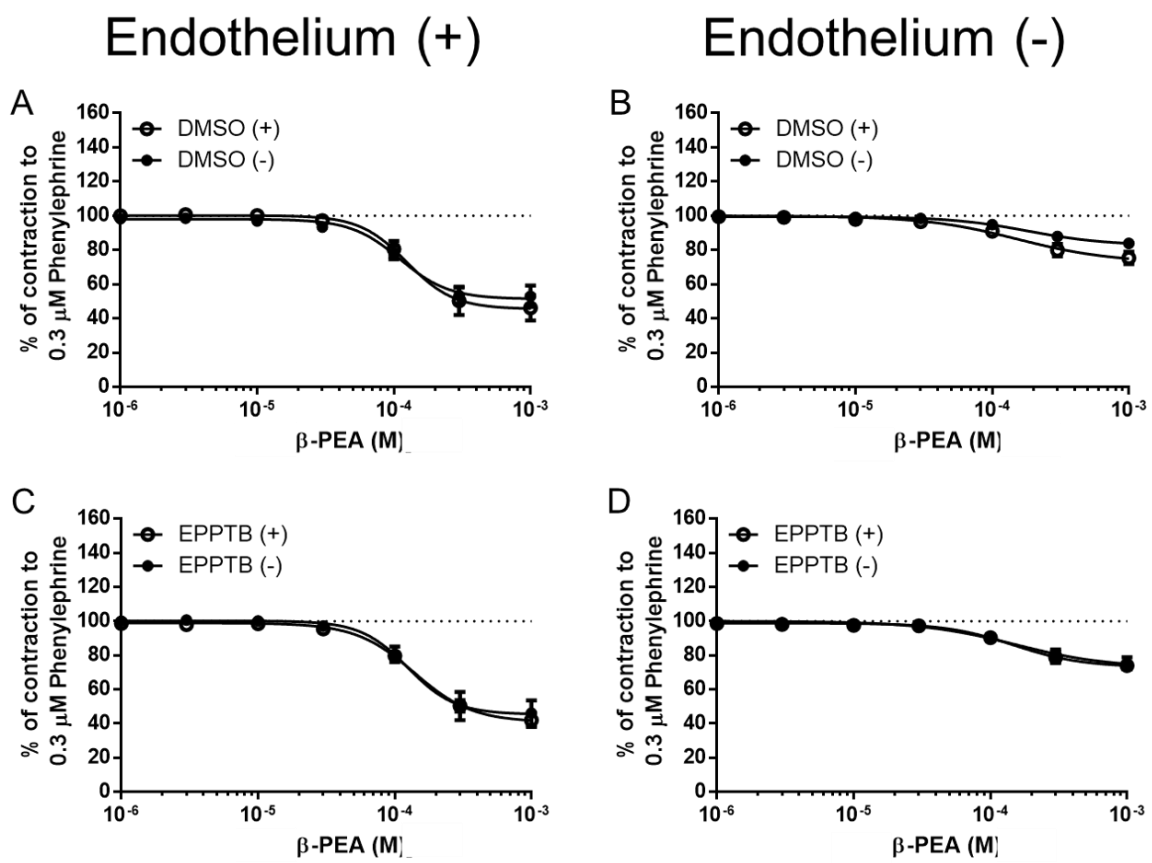

Figure 6.5.1. The effect of EPPTB (5 µM) and DMSO (0.25%) on β-PEA cumulative CRCs in endothelium-intact (A, C) and -denuded (B, D) aortic rings. β-PEA CRCs were obtained in (A) the absence (● n = 17) or presence (○ ○ n = 5) of 0.25% DMSO in endothelium-intact aortic rings. β-PEA CRCs were obtained in (B) the absence (● n = 11) or presence (○ n = 4) of 0.25% DMSO in endothelium-denuded aortic rings. β-PEA CRCs were obtained in the absence (● n = 17) or presence (C, ○ n = 7) of EPPTB in endothelium-intact aortic rings. β-PEA CRCs were obtained in the absence (● n = 11) or presence (C, ○ n = 6) of EPPTB in endothelium-denuded aortic rings. Vasodilator responses are reported as the mean contraction (±SEM) expressed as a percentage of the contractile response to 0.3 µM phenylephrine.

Table 6.5.1 EC_{50} values of β-PEA in the presence or absence of DMSO or EPPTB in endothelium-intact and -denuded aortic rings

Agonist	Endothelium +/-	Antagonist	EC50 (CI95)
β-PEA	+	None	128 µM (7 - 144 µM)
		DMSO (0.25% Vol/Vol)	222 µM (101 – 485 µM)
		EPPTB (5 µM)	156 µM (139 – 175 µM)
	-	None	255 µM (10 – 460 µM)
		DMSO (0.25% Vol/Vol)	142 µM (119 – 171 µM)
		EPPTB (5 µM)	135 µM (108 – 170 µM)

6.5.2 Sensitivity of β-PEA-induced vasodilation to antagonism of muscarinic acetylcholine M_3 receptors

In endothelium-intact aortic rings used for carbachol experiments in the absence of atropine, the mean contractile response to 0.3 μM phenylephrine was 1.60 ± 0.02 g (n = 14). This was not different from the response to phenylephrine in endothelium-intact aortic rings used for carbachol experiments in presence of atropine (1.36 ± 0.25 g, n = 6). The competitive muscarinic acetylcholine receptor antagonist, atropine (100 nM), induced a significant ~50-fold degree of rightward shift of the carbachol CRC in endothelium intact aortic rings (Figure 6.5.2A). The AUC (between 0.01 μM and 100 μM) for the CRC to carbachol was significantly increased (P ≤ 0.001, one-way ANOVA) from 242 ± 5 %P.Log[M] to 369 ± 2 %P.Log[M] in the presence of atropine (Figure 6.5.2). Atropine significantly (P<0.001, unpaired, two-tailed Students T-test) increased the EC_{50} of carbachol from 0.9 μM (0.7 – 1.3 μM, Table 6.5.2) to 37.5 μM (34.1 – 41.2 μM, Table 6.5.2).

In endothelium-intact aortic rings used for β-PEA experiments in the presence of atropine, the mean contractile response to 0.3 μM phenylephrine was 1.23 ± 0.12 g (n = 5). This was not different from the response to phenylephrine in endothelium-intact aortic rings used for β-PEA experiments in absence of atropine (1.47 ± 0.07 g, n = 17). Atropine had no significant effect on the β-PEA CRC in endothelium intact aortic rings (Figure 6.5.2B). In the presence of atropine, AUC (between 1 μM and 1000 μM) of the β-PEA CRC (240 ± 5 %P.Log[M]) was not different from that obtained in the absence of atropine (248 ± 4 %P.Log[M]). Similarly, atropine had no effect on the EC_{50} (121 μM, 95 – 153 μM, Table 6.5.2) or E_{Max} (29 ± 8%) of β-PEA in endothelium intact aortic rings compared with that obtained in its absence (EC_{50} = 128 μM, 7 – 144 μM, E_{Max} = 47 ± 5% Table 6.5.2).

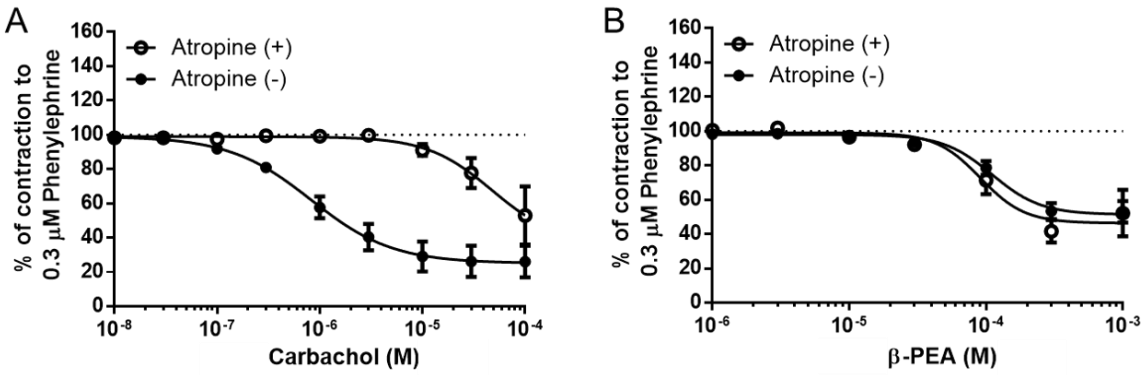

Figure 6.5.2. The effect of atropine (100 nM) on carbachol (A) and β-PEA (B) cumulative CRCs in endothelium intact aortic rings. Carbachol CRCs (A) were obtained in the presence (○ n = 6) or absence (● n = 14) of atropine. β-PEA CRCs (B) were obtained in the presence (○ n = 6) or absence (● n = 17) of atropine. Vasodilator responses are reported as the mean contraction (±SEM) expressed as a percentage of the contractile response to 0.3 μM phenylephrine. Table 6.5.2. EC_{50} values of carbachol and β-PEA in the presence or absence of atropine (100 nM) in endothelium-intact aortic rings

Agonist	Antagonist	EC50 (CI95)
Carbachol	Control	0.9 μM (0.7 – 1.3 μM)
	Atropine (100 nM)	37.5 μM (34.1 – 41.2 μM)
β-PEA	Control	128 μM (7 - 144 μM)
	Atropine (100 nM)	121 μM (95 – 153 μM)

6.5.3 Sensitivity of β-PEA-induced vasodilation to β_2-adrenoceptor antagonism

In endothelium-intact aortic rings used for isoprenaline experiments in the absence of ICI,118,551, the mean contractile response to 0.3 μM phenylephrine was 1.25 ± 0.14 g (n = 6). This was not different from the response to phenylephrine in endothelium-intact aortic rings used for β-PEA experiments in presence of ICI,118,551 (1.21 ± 0.23 g, n = 6). In endothelium-denuded aortic rings used for isoprenaline experiments in the absence of ICI,118,551, the mean contractile response to 0.3 μM phenylephrine was 1.27 ± 0.19 g (n = 6). This was not different from the response to phenylephrine in endothelium-denuded aortic rings used for β-PEA experiments in presence of ICI,118,551 (1.35 ± 0.23 g, n = 6). The β_2-adrenceptor selective antagonist ICI,118,551 (1 μM) induced an ~3-fold and ~2.5-fold degree of shift of the isoprenaline CRC in endothelium-intact and -denuded aortic rings, respectively (Figure 6.5.3A, B). The EC_{50} for the isoprenaline CRC was 3 μM (1 – 7 μM, Table 6.5.3) and 7 μM (5 – 12 μM, Table 6.5.3) in endothelium intact and denuded aortic rings, respectively. ICI,118,551 significantly ($P \leq 0.05$, one-way ANOVA) increased the EC_{50} of isoprenaline in endothelium denuded (16 μM, 13 – 20 μM, Table 6.5.3) but not endothelium intact (9 μM, 4 – 15 μM, Table 6.5.3) aortic rings. No difference (one-way ANOVA) in EC_{50} was found between endothelium-intact or -denuded aortic rings.

In endothelium-intact aortic rings used for β-PEA experiments in the presence of ICI,118,551, the mean contractile response to 0.3 μM phenylephrine was 1.44 ± 0.23 g (n = 6). This was not different from the response to phenylephrine in endothelium-intact aortic rings used for β-PEA experiments in absence of ICI,118,551 (1.47 ± 0.07 g, n = 17). In endothelium-denuded aortic rings used for β-PEA experiments in the presence of ICI,118,551, the mean contractile response to 0.3 μM phenylephrine was 1.52 ± 0.24 g (n = 6). This was not different from the response to phenylephrine in endothelium-denuded aortic rings used for β-PEA experiments in absence of ICI,118,551 (1.52 ± 0.15 g, n = 11). ICI,118,551, had no significant effect on the β-PEA CRC (Figure 6.5.3C, D). ICI,118,551, did not significantly (one-way ANOVA) increase the EC_{50} of β-PEA in endothelium intact (124 μM, 102 – 151 μM, Table 6.5.3) or endothelium denuded (215 μM, 107 – 431 μM, Table 6.5.3) aortic rings, respectively. Similarly, ICI,118,551 did not affect the E_{max} of β-PEA in endothelium-intact (41 ± 9) or endothelium-denuded (68 ± 12 %) aortic rings.

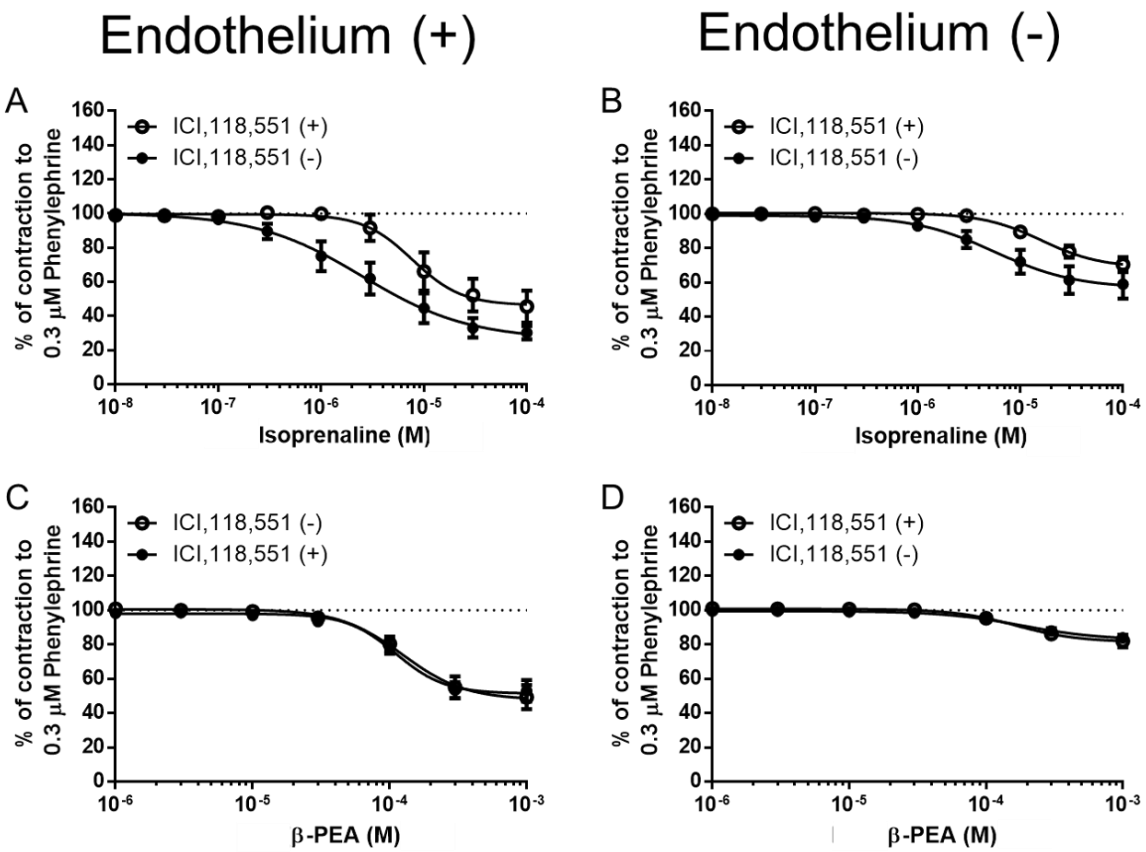

Figure 6.5.3. The effect of ICI,118,551 (1 µM) on isoprenaline (A-B) and β-PEA (C-D) CRCs in endothelium-intact (A, C) and -denuded (B, D) aortic rings. CRCs for isoprenaline were obtained (A) in the presence (○ n = 6) or absence (● n = 6) of ICI,118,551 in endothelium-intact aortic rings. CRCs for isoprenaline were obtained (B) in the presence (○ n = 6) or absence (● n = 6) of ICI,118,551 in endothelium-denuded aortic rings. CRCs for β-PEA were obtained (C) in the presence (○ n = 6) or absence (● n = 17) of ICI,118,551 in endothelium-intact aortic rings. CRCs for β-PEA were obtained (D) in the presence (○ n = 6) or absence (● n = 11) of ICI,118,551 in endothelium-denuded aortic rings. Vasodilator responses are reported as the mean contraction (±SEM) expressed as a percentage of the contractile response to 0.3 µM phenylephrine.

Table 6.5.3. EC_{50} values of isoprenaline and β-PEA in the presence or absence ICI, 118,551 in endothelium-intact and -denuded aortic rings

Agonist	Endothelium +/-	Antagonist	EC50 (CI95)
Isoprenaline	+	Control	3 µM (1 – 7 µM)
		ICI, 118, 551 (1 µM)	9 µM (4 – 15 µM)
	-	Control	7 µM (5 – 12 µM)
		ICI, 118, 551 (1 µM)	16 µM (13 – 20 µM)
β-PEA	+	Control	128 µM (7 - 144 µM)
		ICI, 118, 551 (1 µM)	124 µM (102 – 151 µM)
	-	Control	255 µM (10 – 460 µM)
		ICI, 118, 551 (1 µM)	215 µM (107 – 431 µM)

6.6 Discussion

6.6.1 TAAR1 involvement in β-PEA-induced vasodilation

In Chapter 4 the involvement of TAAR1 in mediating trace amine-induced vasoconstrictor responses was assessed using the only commercially available TAAR1 antagonist, EPPTB (Bradaia et al. 2009). Despite the use of this antagonist, a role for TAAR1 in mediating trace amine-induced vasoconstriction was not definitively determined in rat aortic rings. EPPTB has a low affinity for rat TAAR1 compared with mouse TAAR1 with IC_{50} values of 4539 nM and 28 nM, respectively (Bradaia et al. 2009; Stalder et al. 2011). Furthermore, in Chapter 4 the limit of solubility of EPPTB was 5 μM in organ baths. Although lacking potency at rat TAAR1 receptors, EPPTB remains the only selective antagonist of TAAR1 currently available. At 5 μM, EPPTB had no effect on trace amine induced vasoconstriction. Despite this, it might be possible that TAAR1 is not involved in vasoconstriction but may be involved in vasodilation.

EPPTB had no significant effect on the β-PEA CRCs, compared with vehicle controls (Figure 6.5.1). These results contradict the findings of Batista-Lima et al. (2018), who demonstrated EPPTB was able to block β-PEA-induced relaxation of smooth muscle of rat gastric fundus. However, the previous study used a 10-fold higher concentration of EPPTB than the current investigation. Furthermore, Batista-Lima et al. (2018) examined responses in gastric fundus. It is possible subtle differences may exist between vascular smooth muscle and gastric smooth muscle that could affect the sensitivity of TAAR1 to EPPTB. Alternatively, it is possible that for rat tissues, the concentration (5 μM) used in this study is not sufficient to block rat TAAR1. However, given the published inhibition constant (K_I) of EPPTB for rat TAAR1 is 0.942 μM (Bradaia et al. 2009; Stalder et al. 2011), at a concentration of 5μM should generate an approximately 6.3-fold shift of the CRC. This suggests the concentration of EPPTB used in this study should be sufficient to observe a shift of the β-PEA CRC and therefore TAAR1 does not mediate the vasodilator action of β-PEA.

6.6.2 Endothelium dependent β-PEA induced vasodilation is not mediated by muscarinic acetylcholine M3 receptors

Vasodilation via muscarinic M3 receptors by acetylcholine or other muscarinic agonists such as carbachol is dependent on the presence of an intact and functional endothelium (Jiang et al. 2000). Activation of M3 receptors on the endothelium activates eNOS, release of nitric oxide and subsequent vasodilation (Zhao et al. 2015). As shown in Chapter 4, β-PEA-induced vasodilator responses are comprised of an endothelium-dependent and independent component. It was therefore necessary to establish whether M3 receptors mediate endothelium-dependent β-PEA-induced vasodilation. Carbachol and β-PEA-induced vasodilation was examined in endothelium intact aortic rings in the presence of the non-selective muscarinic antagonist, atropine (1 μM). Atropine induced a significant rightward shift of the carbachol CRC confirming the presence of functional M3 receptors (Figure 6.5.2A). However, atropine had no significant effect on the β-PEA CRC indicating that M3 receptors do not mediate β-PEA-induced stimulation of eNOS and subsequent vasodilation.

6.6.3 β_2-adrenoceptors are not responsible for β-PEA-induced vasodilator responses

In terms of structure, trace amines are closely related to noradrenaline and their vasoconstrictor action can be attributed to their ability to induce noradrenaline release from sympathetic neurones (Broadley 2010; McGeer 2013). Chapter 4 demonstrated that α_1-adrenoceptors are not directly or indirectly activated by β-PEA or the TAAR1 selective agonist RO5256390 to induce a contractile effect. Furthermore Varma and Chemtob (1993) previously demonstrated both tyramine- and β-PEA-induced vasodilation to be resistant to antagonism of β_2-adrenoceptors.

Activation of β_2-adrenoceptors is coupled to cAMP production and subsequent smooth muscle relaxation (Guimaraes and Moura 2001). However, β_2-adrenoceptors are also expressed on vascular endothelium where they initiate vasodilation by stimulation of eNOS (Vanhoutte 2001). In the studies by Varma and Chemtob (1993) and Varma et al. (1995) trace amine induced relaxation was found to be endothelium-independent. However, in Chapter 4 it was demonstrated that traces amines can also induce the release of endothelium-derived nitric oxide resulting in a vasodilator response. To address the involvement of β_2-adrenoceptors in endothelium-dependent β-PEA-induced vasodilation, the sensitivity of β-PEA-induced vasodilation to antagonism by the the selective β_2-adrenoceptor antagonist ICI,118,551, was tested.

The selective β-adrenoceptor agonist isoprenaline induced vasodilator responses in both endothelium-intact and -denuded aortic rings. A significant shift of the isoprenaline CRCs was observed following prior incubation with the β_2-adrenoceptor selective antagonist ICI,118,551, (1μM, Figure 6.5.3A, B) confirming the presence of β_2-adrenoceptors. Prior incubation with ICI,118,551 had no significant effect on β-PEA CRCs. These results are supported by the findings of Varma and Chemtob (1993) who demonstrated tyramine- and β-PEA-induced vasodilation were insensitive to β_2-adrenoceptor blockade (Varma et al. 1995). Collectively, with the study by Varma and Chemtob (1993) it is clear that β-PEA-induced vasodilator responses are not mediated by β_2-adrenoceptors.

6.7 Conclusion

β-PEA-induced vasodilation is not mediated by β2-adrenoceptors or muscarinic M3 acetylcholine receptors. The involvement of TAAR1 in mediating the vasodilator action of β-PEA cannot be definitively determined due to the lack of species specificity of the TAAR1-selective antagonist, EPPTB.

Chapter 7 TAAR1 as an intracellular target

7.1 Introduction

G-protein-coupled receptors (GPCRs) are well established as cell surface receptors that transform an extracellular stimulus into an intracellular signal (Kobilka 2007). An emerging new concept that GPCRs can also signal from intracellular compartments (Jong et al. 2018). represents a new challenge to GPCR research. Most intracellular GCPRs are located at the nuclear membrane (Jong et al. 2018). Although several have been identified at other internal membranes including endosomes (Samaraweera et al. 2001), vesicles and mitochondria (Bénard et al. 2012). Binding sites for GPCR ligands are located inside of these intracellular compartments therefore requiring ligands to cross both the plasma membrane and internal membrane (O'Malley et al. 2003). Although highly permeable ligands may readily cross the plasma and intracellular membranes, less permeable and charged ligands will require active transport mechanisms (Jong et al. 2018).

TAAR1 is a GPCR that has been reported to primarily localise to intracellular compartments (Gainetdinov et al. 2018). To elicit a vascular response, trace amines must therefore gain access the intracellular compartment (Miller et al. 2005; Xie et al. 2008). As trace amines are characterised by their short half-life of less than 30-seconds (Durden and Philips 1980), mechanisms to facilitate their rapid transport to the cytosol must exist. Numerous transporter proteins are reported to display affinity for the trace amines. These transporters fall into two select pathways, uptake-1 (neuronal) and uptake-2 (extraneuronal, Iversen 1973). Uptake-1 transporters, including the dopamine transporter (DAT), noradrenaline transporter (NET) and serotonin transporter (SERT), display a high selectivity and low capacity for their corresponding neurotransmitter (Torres et al. 2003; Rudnick et al. 2014). Although these transporters are generally considered highly specific, at high micromolar to millimolar concentrations, the trace amines are also recognised substrates of these transporters (Raiteri et al. 1977; Danek Burgess and Justice 1999; Liang et al. 2009; Underhill et al. 2019).

Uptake-2 transporters including the Organic Cation Transporters (OCT1-3; Slc22A1-3 Koepsell 2020) and the Plasma Membrane Monoamine Transporter (PMAT; Slc29A4 Wang 2016a), are considered polyspecific and high capacity (Courousse and Gautron 2015). Traditionally they are thought to act as a reserve system for clearance of neurotransmitters following saturation of classical uptake-1 pathways (Courousse and Gautron 2015; Koepsell 2020). The trace amines have been reported as substrates of the uptake-2 transporters at nanomolar concentrations (Engel and Wang 2005; Schomig et al. 2006; Chen et al. 2010; Berry et al. 2016). With the exception of OCT2, all other uptake-2 transporters are reportedly expressed in smooth muscle (Chen et al. 2010; Engel et al. 2004; Wu et al. 2000), making it possible they could mediate transport of trace amines in the vasculature.

Previous investigations of vascular responses to trace amines have demonstrated trace amine-induced vasoconstriction to be resistant to blockade of uptake-1 pathways (Anwar et al. 2013; Broadley et al. 2013). Consequently, the current chapter does not evaluate the influence of uptake-1 transporters on trace amine-induced vascular responses. To date uptake-2 pathways have not been considered as a possible mechanism underlying the vascular responses to trace amines. The current chapter therefore aims establish whether trace amine-induced vascular responses mediated by intracellular TAAR1 and the pre-requisite of trace amine transport across the plasma membrane via uptake-2 pathways. Furthermore, this chapter aims to evaluate the ability of novel tyramine analogues that can cross the membrane independently of uptake-1 or uptake-2 pathways to elicit a vascular response.

7.2 Hypothesis

Trace amine-induced vascular responses are mediated by intracellular TAAR1 and require initial transport across the plasma membrane by uptake-2 transporters.

7.3 Aims

1. Confirm an intracellular site of action for trace amine-induced vascular responses.
2. Confirm plasma membrane transport via uptake-2 pathways is required to elicit vascular responses to the trace amines.
3. Evaluate the vasodilator responses to novel tyramine analogues designed to diffuse across the plasma membrane independently of uptake-2 transporters.

7.4 Materials and methods

7.4.1 Animals

Male Sprague Dawley, rats (350-750g) were obtained and housed as described in Chapter 2. All experiments adhered strictly to the Animals (Scientific Procedures) Act 1986.

7.4.2 Rat aortic rings

Tissue viability and the presence or absence of a functional endothelium were confirmed as described in Chapter 2. Cumulative-concentration response curves (CRC's) were obtained for phenylephrine and β-PEA in aortic rings under baseline tension. Cumulative CRC's were also obtained for β-PEA, tyramine and tyramine analogues in aortic rings pre-constricted with 0.3 μM phenylephrine or 1 μM phenylephrine, in the presence of decynium 22. Where used, the OCT1-3 and PMAT inhibitor, decynium 22 was added to organ baths 15 minutes prior to construction of CRCs.

7.4.3 Drugs and solutions

All drugs were dissolved in Krebs bicarbonate buffer unless otherwise stated. Decynium 22, tyramine and tyramine analogues were dissolved in DMSO (0.05% vol/vol) prior to dilution with Krebs bicarbonate buffer. The source of all drugs used in this chapter and the composition of Krebs bicarbonate buffer are detailed in Chapter 2.

7.4.4 Data analysis

Contractile responses and relaxant responses are expressed as a percentage of the contractile response to high potassium (60mM) Krebs bicarbonate solution and initial contractile response to 0.3 μM or 1.0 μM phenylephrine, respectively, as described in Chapter 2. Responses are plotted as the mean response ± S.E.M where n represents the number of rats in each group. Curve fitting was applied as described in Chapter 2. E_{Max} values were estimated from curve fitting and are reported as a percentage of the contractile responses to 60mM KCl or 0.3-1.0 μM phenylephrine ± S.E.M for contractile or relaxant responses respectively. Negative logEC_{50} values were obtained for each individual curve. EC_{50} values were calculated from the -logEC_{50} and are reported as the mean EC_{50} value with 95% confidence intervals. Mean EC_{50} and E_{max} values were compared by unpaired or paired, two-tailed Student's t-tests or one-way ANOVA supported by Dunnett's multiple comparison post-hoc test as appropriate. P-values ≤0.05 were considered statistically significant. Area under the curve (AUC) for specified concentration ranges was calculated for each individual curve. The mean AUC of contractile CRCs was reported as a % of contraction to 60 mM KCl by Log[agonist(M)] (%.Log[M]). The mean AUC of relaxant CRCs was reported as a % of the initial contraction to phenylephrine by Log[agonist(M)] (%P.Log[M]). AUC's were compared by unpaired, two-tailed Student's t-test or one-way ANOVA as appropriate.

7.5 Results

7.5.1 Sensitivity of phenylephrine- and β-PEA-induced vasoconstriction to blockade of uptake-2 transporters

In endothelium-intact aortic rings used for phenylephrine experiments in the absence of decynium 22, the mean contractile response to 60 mM KCl was 1.63 ± 0.14 g (n = 10). This was not different from the response to 60 mM KCl in endothelium-intact aortic rings used for phenylephrine experiments in presence of decynium 22 (1.66 ± 0.14 g, n = 5). In endothelium-denuded aortic rings used for phenylephrine experiments in the absence of decynium 22, the mean contractile response to 60 mM KCl was 1.28 ± 0.16 g (n = 9). This was not different from the response to 60 mM KCl in endothelium-denuded aortic rings used for phenylephrine experiments in presence of decynium 22 (1.17 ± 0.20 g, n = 5).

The OCT1-3 and PMAT inhibitor decynium 22 (1 μM) induced a significant rightward shift of the phenylephrine CRC (Figure 7.5.1A, B). In the presence of a functional endothelium, decynium 22 induced a 10-fold shift of the phenylephrine CRC. Decynium 22 significantly (P

≤ 0.01, one-way ANOVA) reduced the area under the curve (AUC, between 0.001 µM and 300 µM) of the phenylephrine CRC from 416 ± 5 %.Log[M] to 249 ± 5 %.Log[M] in endothelium-intact aortic rings. The E_{Max} to phenylephrine obtained in the presence of decynium 22 (79 ± 9 % of the contractile response to 60 mM KCl) was significantly lower (P ≤ 0.05, unpaired, two-tailed, Students T-test) than the E_{Max} obtained in the absence of decynium 22 (98 ± 4 %). The EC_{50} of phenylephrine (34 nM, 16 – 70 nM, Table 7.5.1) was significantly increased (P ≤ 0.01, one-way ANOVA) in the presence of decynium 22 (293 nM, 154 – 557 nM, Table 7.5.1).

In the absence of a functional endothelium, decynium 22 induced a 13-fold rightward shift of the phenylephrine CRC (Figure 7.5.1B). Decynium 22 significantly (P ≤ 0.01, one-way ANOVA) reduced the AUC of the CRC to phenylephrine from 439 ± 4 %.Log[M] to 252 ± 4 %.Log[M]. The maximum contractile response to phenylephrine obtained in the presence of decynium 22 (78 ± 8%) was not significantly lower (P = 0.06, unpaired, two-tailed, Students T-test) than the maximum contractile response obtained in the absence of decynium 22 (100 ± 7%). However, the EC_{50} of phenylephrine (15 nM, 8 – 27 nM, Table 7.5.1) was significantly (P ≤ 0.001, one-way ANOVA) increased in the presence of decynium 22 (218 nM, 76 – 625 nM, Table 7.5.1).

In endothelium-intact aortic rings used for β-PEA experiments in the presence of DMSO (0.05% vol/vol) the mean contractile response to 60 mM KCl was 2.10 ± 0.13 g (n = 10). This was not different from the response to 60 mM KCl in endothelium-intact aortic rings used for β-PEA experiments in presence of decynium 22 (1.70 ± 0.13 g, n = 7). In endothelium-denuded aortic rings used for β-PEA experiments in the absence of decynium 22, the mean contractile response to 60 mM KCl was 1.30 ± 0.20 g (n = 9). This was not different from the response to 60 mM KCl in endothelium-denuded aortic rings used for β-PEA in presence of decynium 22 (0.95 ± 0.12 g, n = 7).

Decynium 22 had no significant effect on the mean CRC for vasoconstrictor responses to β-PEA (Figure 7.5.1C, D). In the presence of decynium 22, the AUC (between 1 µM and 1000 µM) of the β-PEA CRC (25 ± 3 %.Log[M]) was not significantly different (one-way ANOVA) from the DMSO (0.05% vol/vol) vehicle control (31 ± 3 %.Log[M]) in endothelium-intact aortic rings. As a complete β-PEA CRC was not generated in the presence of a functional endothelium, neither an EC_{50} nor E_{Max} were calculated.

In endothelium-denuded aortic rings, the AUC of the β-PEA CRC in the DMSO (0.05% vol/vol) vehicle control (89 ± 5 %.Log[M]) was not significantly different from the AUC of the β-PEA CRC obtained in the presence of decynium 22 (66 ± 5 %.Log[M]). In the presence of decynium 22 the E_{max} (117 ± 17%, calculated from the extrapolated β-PEA CRC curve fitting, Figure 7.5.1D) was found to be significantly (P ≤ 0.05) greater than the E_{Max} (76 ± 4%) than that of the DMSO vehicle control. In the absence of endothelium, the EC_{50} of β-PEA in DMSO vehicle controls (64 µM, 2 – 100 µM, Table 7.5.1) was significantly lower (P ≤ 0.001) than in the presence of decynium 22 (636 µM, 276 µM – 1468 µM, Table 7.5.1).

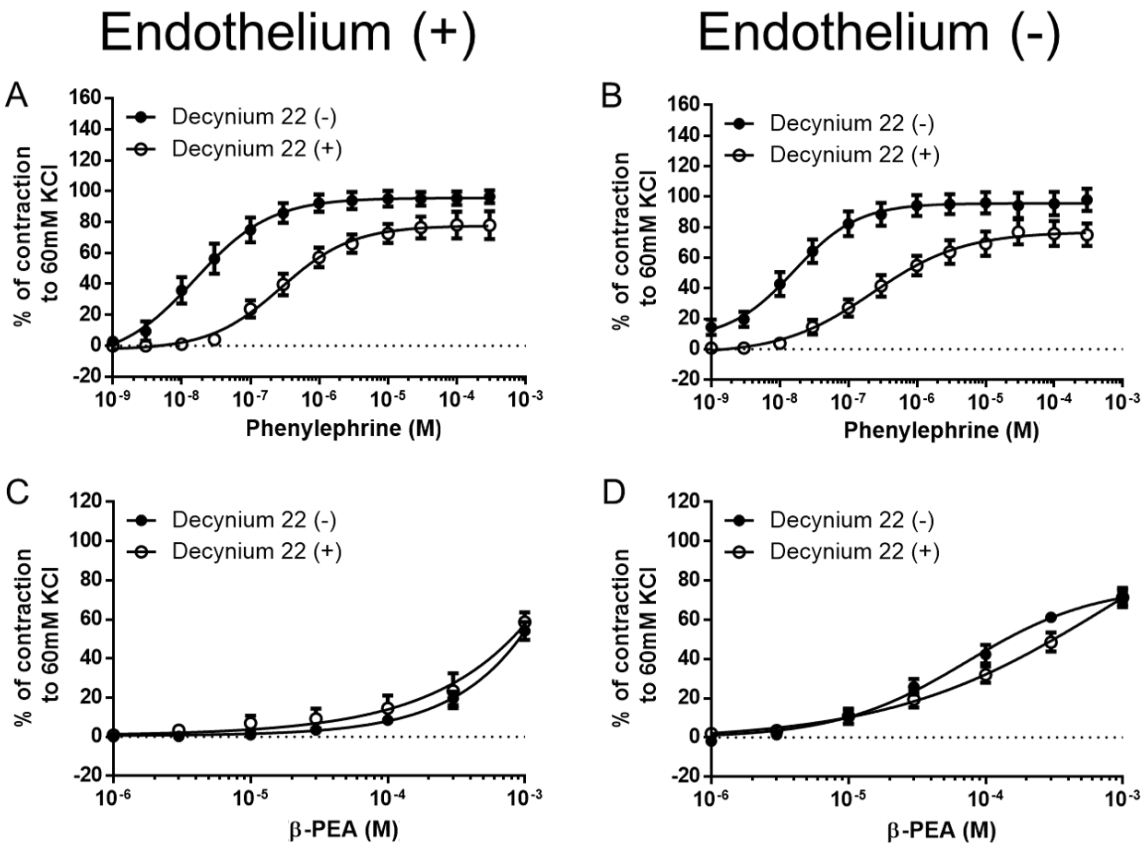

Figure 7.5.1.The effect of decynium 22 (1 μM) on phenylephrine (A-B) and β-PEA (C-D) CRCs in endothelium-intact (A, C) and -denuded (B, D) aortic rings. CRCs for phenylephrine were obtained (A) in the absence (● n = 10) or presence (○ n = 5) of 1 μM decynium 22 in endothelium-intact aortic rings. CRCs for phenylephrine were obtained (B) in the absence (● n = 9) or presence (○ n = 6) of 1 μM decynium 22 in endothelium-denuded aortic ring. CRCs for β-PEA were obtained (C) in the absence (● n = 17) or presence (○ n = 7) of 1 μM decynium 22 in endothelium-intact aortic ring. CRCs for β-PEA were obtained (D) in the absence (● n = 14) or presence (○ n = 7) of 1 μM decynium 22 in endothelium-denuded aortic rings. Contractile responses are reported as the mean percentage (±SEM) of the maximum contractile response to 60mM KCl.

Table 7.5.1. EC_{50} values of contractile agonists in the presence or absence of decynium 22

Agonist	Endothelium +/-	Condition/ Antagonist	EC50 (CI95)
β-PEA	+	0.05% DMSO	ND
		Decynium 22 (1 μM)	ND
	-	0.05% DMSO	64 μM (2 – 100 μM)
		Decynium 22 (1 μM)	636 μM (276 - 1468 μM)
Phenylephrine	+	Control	30 nM (15 – 60 nM)
		Decynium 22 (1 μM)	293 nM (154 – 557vnM)
	-	Control	15 nM (8 – 27 nM)
		Decynium 22 (1 μM)	218 nM (76 - 625 nM)

7.5.2 Sensitivity of β-PEA-induced vasodilation to blockade of uptake-2 transporters

As decynium 22 is a known antagonist of α_1-adrenoceptors (Russ et al. 1996, Figure 7.5.1A, B), the concentration of phenylephrine used to induce a sub-maximal contraction of aortic rings, was increased to 1 µM. In the presence of decynium 22, the mean contractile response to 1 µM phenylephrine was 0.80 ± 0.04 g (n = 5) and 0.86 ± 0.19 g (n = 6) in endothelium-intact and denuded aortic rings respectively. These were found to be significantly different from the mean contractile response to 0.3 µM phenylephrine obtained in the absence of decynium 22 in endothelium-intact (1.47 ± 0.07 g, n = 17, P ≤ 0.001) and -denuded aortic rings (1.52 ± 0.15 g, n = 11, P ≤ 0.05). The baseline tension in endothelium-intact aortic rings pre-constricted with 1 µM phenylephrine was generally lower in the presence of decynium 22 (Figure 7.5.2A). Decynium 22 completely abolished β-PEA-induced vasodilator and restored vasoconstrictor responses in both endothelium-intact and -denuded aortic rings preconstructed with phenylephrine (Figure 7.5.2A, B). Complete CRCs were not obtained in either endothelium-intact or -denuded aortic rings. Therefore, EC_{50} and E_{Max} values were not calculated.

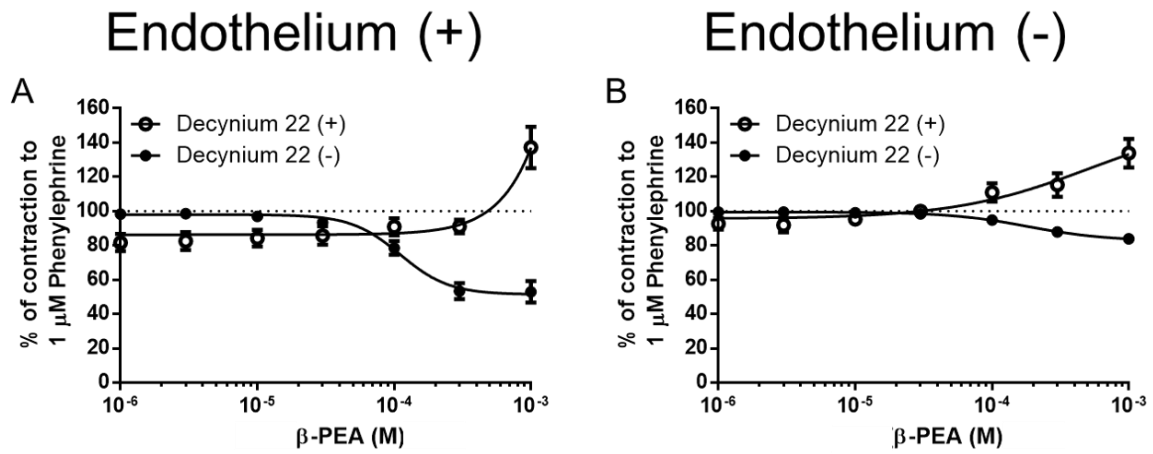

Figure 7.5.2 The effect of decynium 22 (1 µM) on β-PEA-induced vasodilation. CRCs for β-PEA were obtained (A) in the absence (● n = 17) or presence (○ n = 5) of 1 µM decynium 22 in endothelium-intact aortic rings. CRCs for β-PEA were obtained (B) in the absence (● n = 11) or presence (○ n = 6) of 1 µM decynium 22 in endothelium-denuded aortic rings. Vasodilator responses are reported as the mean response (±SEM) expressed as a percentage of the contractile response to 1 µM phenylephrine.

7.5.3 Ability of novel tyramine analogues to induce vasodilation via intracellular TAAR1

To target intracellular TAAR1, novel tyramine analogues with a high lipophilicity, determined by an increase in the calculated -LogD values (CLogD, Figure 7.5.3), were synthesised to promote, monoamine transporter-independent transmembrane diffusion. Tyramine and tyramine analogues (tyramine A1 and tyramine A2) induced concentration-dependent vasodilation of endothelium-denuded aortic rings pre-constricted with 0.3 µM phenylephrine (Figure 7.5.4). In aortic rings used for tyramine experiments the mean contractile response to 0.3 µM phenylephrine was 1.86 ± 0.20 g (n = 3). This was not different from the response to 0.3 µM phenylephrine in aortic rings used for tyramine A1 (1.14 ± 0.12g, n = 3) or tyramine A2 (1.54 ± 0.30 g, n = 3) experiments. The increase in CLogD of tyramine A1 (0.9) did not significantly enhance the vasodilator response compared to tyramine (-1.25). The AUC (between 1 µM and 1000 µM) of tyramine (263 ± 2 %P.Log[M]) and tyramine A1 (252 ± 3 %P.Log[M]) were not found to be significantly different (one-way ANOVA). The EC_{50} of tyramine (290 µM, 276 – 305 µM, Table 7.5.2) was not found to be significantly different (one-way ANOVA) from tyramine A1 (730 µM, 685 µM – 7767 µM, Table 7.5.2). Similarly,

the E_{max} of tyramine (46 ± 2 %) was not significantly different (one-way ANOVA) from tyramine A1 (25 ± 13).

The increased CLogD of tyramine A2 (3.18) was associated with an enhanced vasodilator response compared to tyramine (Figure 7.5.4). The AUC of tyramine A2 (185 ± 9 %P.Log[M]) was significantly ($P \leq 0.01$, one-way ANOVA) lower than that of tyramine (263 ± 2 %P.Log[M]). However, the EC_{50} of tyramine A2 (472 µM, 254 – 875 µM, Table 7.5.2) was not significantly (one-way ANOVA) different from that of tyramine (290 µM, 276 – 305 µM). The E_{max} of Tyramine A2 (-113 ± 32%) was significantly ($P \leq 0.05$) greater than that of tyramine (46 ± 2 %).

To confirm tyramine A2 did not require plasma membrane transporters the effect of decynium 22 (1 µM) on tyramine A2 was assessed (n = 1, Figure 7.5.4). Tyramine A2 continued to induce vasodilator responses in the presence of decynium 22. As only a single repeat was obtained, no statistical analysis was carried out.

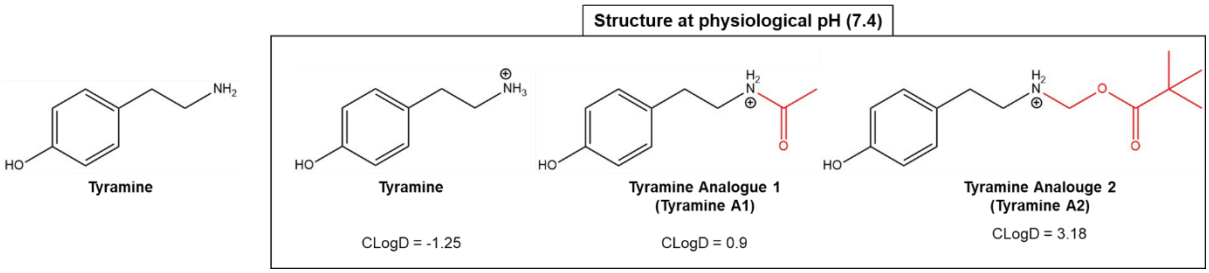

Figure 7.5.3. Structure and calculated LogD (CLogD) values for tyramine and tyramine analogues at physiological pH (7.4).

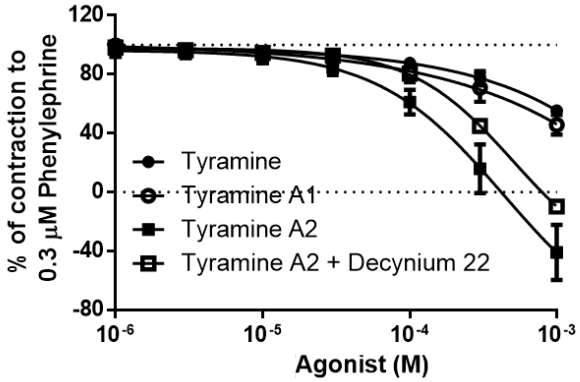

Figure 7.5.4 Tyramine and tyramine analogue-induced vasodilator responses in endothelium-denuded rat aortic rings. Mean cumulative CRCs were obtained for tyramine (● n = 3), tyramine A1 (○ n = 3), tyramine A2 (■ n =3). Also shown is tyramine A2 in the presence of 1 µM decynium 22 (□ n = 1). Vasodilator responses are reported as the mean response (±SEM) expressed as a percentage of the contractile response to 3 µM phenylephrine.

Table 7.5.2. EC_{50} values of tyramine and tyramine analogues

Agonist	Endothelium +/-	EC50 (CI95)
Tyramine	-	290 µM (276 – 305 µM)
Tyramine A1	-	730 µM (685 µM – 7767 mM)
Tyramine A2	-	472 µM (254 – 875 µM)

7.6 Discussion

7.6.1 β-PEA-induced vasoconstriction is mediated by a plasma membrane receptor

TAAR1 is predominately expressed intracellularly and is not readily located at the plasma membrane (Harmeier et al. 2015). Underhill et al. (2019) demonstrated TAAR1 activation first requires trace amines and amphetamines to be transported into the cytoplasm. With the exception of octopamine, trace amines and amphetamines can be transported via DAT in DAT-transfected cell lines (Underhill et al. 2019). However, transport of trace amines may also involve other plasma membrane transporters. Berry et al. (2016) et al previously identified OCT2(SLC22A2) as a high affinity transporter of tyramine in rat brain synaptosomes. The requirement for transport to the cytoplasmic compartment of cells may explain the slow onset in the contractile responses of trace amines reported by Broadley and Richards (2015). Fehler et al. (2010) has previously shown that blockade of DAT, 5-HT and noradrenaline transporters (SERT and NET, respectively) using cocaine does not block β-PEA-induced vasoconstriction. Therefore, this current study did not employ any blockade of these transporters.

To test whether β-PEA-induced vasoconstrictor responses are mediated via an intracellular mechanism, the current study utilised the OCT1-3 and PMAT inhibitor, decynium 22, to block intracellular transport of β-PEA. Decynium 22 induced a significant rightward shift of the α_1-adrenoceptor agonist phenylephrine control (Figure 7.5.1A, B). This is likely because, decynium 22 has previously been reported to act as an α_1-adrenoceptor antagonist (Russ et al. 1996). Furthermore, as decynium 22 marginally reduced the maximum contractile response to phenylephrine, it appears to be acting as a non-competitive antagonist of α_1-adrenoceptors (Schild 1954).

Blockade of monoamine transport did not block β-PEA induced contractile responses (Figure 7.5.1C, D). The concentration of decynium 22 used in this study has previously been shown to be sufficient to block monoamine transport (Berry et al. 2016). This indicates that β-PEA-induced vasoconstrictor responses are not mediated by an intracellular receptor. Furthermore, as decynium 22 is also an α_1-adrenoceptor antagonist this provides further evidence that α_1-adrenoceptors do not mediate the contractile response to β-PEA. Collectively, the results from the current investigation suggest that vasoconstriction induced by β-PEA is unlikely to be mediated by a receptor located at an intracellular site. These findings provide evidence that β-PEA-induced contractile responses are mediated via a currently unidentified plasma membrane receptor.

Interestingly, the E_{Max} in endothelium-denuded aortic rings was significantly greater in the presence of decynium 22. This is likely explained by sensitivity of the β-PEA vasodilator response to blockade of plasma membrane transport. In the absence of an opposing β-PEA-induced vasodilator response it would be expected that a leftward shift of the β-PEA CRC would be observed. However, this is not the case and it appears that where decynium 22 is present, β-PEA could continue to induce a contractile response at concentrations above those that elicit a maximal contractile response in the absence of decynium 22.

7.6.2 β-PEA-induced vasodilation is mediated by an intracellular receptor

Blockade of plasma membrane transport with decynium 22 (1 μM) completely abolished β-PEA-induced vasodilation in both endothelium-intact and -denuded aortic rings pre-constricted with phenylephrine. Interestingly, β-PEA-induced further contractile responses of pre-constricted aortic rings in the presence of decynium 22. These contractile responses occurred across a similar concentration range to both contractile and vasodilator responses observed in Chapter 4. These surprising results indicate that β-PEA-induced vasodilator responses are mediated by an intracellular receptor whereas vasoconstriction is mediated by

a receptor located on the plasma membrane. This may explain why the E_{Max} of the β-PEA vasoconstrictor CRC is elevated in the presence of decynium 22 as the opposing dilator response is completely abolished.

For a receptor located in an intracellular compartment, it is the intracellular concentration of an agonist that is important in determining receptor binding and activation (Jong et al. 2018). This may explain the high concentrations of β-PEA required to elicit a vasodilator response. Many intracellular GPCRs are activated at the cell surface before being transported to their intracellular site of action to elicit a response (Wang et al. 2018). Although some GPCRs are known to be activated inside intracellular compartments such as the ER, vesicles, endosomes and nuclear membrane (Jong et al. 2018). Intracellular GPCRs are orientated such that the ligand binding domain resides inside intracellular compartments, this necessitates the transport of ligands either by diffusion or active transport across both the plasma membrane and intracellular membrane (O'Malley et al. 2003; Boivin et al. 2008; Barlow et al. 2010). The evidence from this study demonstrates that plasma membrane transport is a pre-requisite to an induced vasodilator response indicating that vasodilation is mediated by an intracellular receptor. Previous research by Underhill et al. (2019) demonstrated transport of trace amines across the plasma membrane to be a pre-requisite for TAAR1 activation. It is likely that intracellular TAAR1 mediates the vasodilator response to β-PEA. It is likely that TAAR1 is orientated such that the ligand binding site also resides inside an intracellular compartment (Underhill et al. 2019). Considering the short half-life of trace amines (Durden and Philips 1980), it is likely that further active transport across the intracellular membrane is required to gain access to the binding site.

Although TAAR1 is a strong candidate for the intracellular receptor mediating the vasodilator effects of β-PEA the only available antagonist of TAAR1, EPPTB (Bradaia et al. 2009), had no effect on β-PEA-induced vasodilation in Chapter 5. However, EPPTB is known to be species-specific, with little affinity for rat TAAR1 (Stalder et al. 2011). Furthermore, it is unknown whether EPPTB is able to cross the plasma membrane. In the absence of an antagonist able to bind to rat TAAR1, it is not possible to confirm whether the intracellular receptor mediating vasodilation in rat aorta is TAAR1. β-PEA-induced vasodilation is insensitive to antagonism of β_2-adrenoceptors, muscarinic acetylcholine M3 receptors (Chapter 5) and receptors for 5-HT, histamine and adenosine (Varma and Chemtob 1993; Varma et al. 1995). The number of potential candidate receptors that could mediate vasodilation has been exhausted. In the rat aorta TAAR1 expression has been confirmed at both the protein and mRNA level (Fehler et al. 2010). Despite the absence of any selective antagonists, TAAR1 remains the most likely candidate receptor that mediates vasodilation as it is a known functionally active intracellular receptor (Gainetdinov et al. 2018; Underhill et al. 2019) and is expressed in rat aorta (Fehler et al. 2010).

7.6.3 Tyramine prodrugs induce vasodilation

The fact that β-PEA-induced vasodilator responses are sensitive to inhibitors of plasma membrane transport strongly suggests that the vasodilator response is mediated at an intracellular compartment, most likely via TAAR1. The concentration of β-PEA required to elicit these responses was in the high micromolar to millimolar range. However, this corresponds to the extracellular concentration. The intracellular concentration required to elicit a response is likely dependent on the affinity of β-PEA for plasma membrane transporters and a kinetic interaction between their transport into the cell and breakdown within the cell (Jong et al. 2018; Koepsell 2020). To investigate the intracellular pharmacology of trace amine-induced vasodilator responses, two novel tyramine analogues (tyramine A1 and tyramine A2, Figure 7.5.3), with a higher lipophilicity than the parent, were synthesised to enhance transporter-independent transmembrane diffusion.

Vasodilator responses to tyramine and tyramine analogues were assessed in endothelium-denuded aortic rings. The vasodilator response associated with the first tyramine analogue, tyramine A1, did not significantly differ from that obtained with tyramine. However, the

second tyramine analogue, tyramine A2, that was more lipophilic than tyramine A1, was associated with an increased vasodilator response. However, the potency of tyramine A2 was not significantly different from that of tyramine or tyramine A1. This may suggest an intracellular contractile response may oppose the vasodilator action of tyramine A2. In cell lines it has previously been shown that, intracellular TAAR1 is coupled to both RhoA and protein kinase A (PKA) (Underhill et al. (2019). In smooth muscle, activation of RhoA increases calcium sensitisation and would therefore promote a vasoconstrictor response (Johns et al. 2000).

The ability of tyramine A2 to induce vasodilation in the presence of blockade of monoamine transport was assessed in a single experiment. Decynium 22 (1 μM) did not abolish tyramine A2-induced vasodilation, indicating that the analogue entered the intracellular environment by transmembrane diffusion and not through the actions of a plasma membrane transporter. Although further experiments will be required to confirm plasma membrane transporter-independent activation of the currently unidentified intracellular receptor and subsequent vasodilator response.

7.7 Conclusion

Vascular responses to the trace amines are mediated via receptors located in distinct cellular compartments. Trace amine-induced vasoconstrictor responses are mediated by a plasma membrane receptor, whereas vasodilator responses are mediated by an intracellular receptor. Trace amine-induced vasodilation is dependent on transport of trace amines across the plasma membrane through transporter proteins. The novel tyramine analogue, tyramine A2, is a strong agonist of the intracellular receptor and is able to cross the plasma membrane independently of plasma membrane transporters.

Chapter 8 Investigating trace amine vascular responses in mesenteric blood vessels

8.1 Introduction

The work in Chapter 4 built on the works of Fehler et al. (2010) and Broadley et al. (2013) to investigate the potential involvement of TAAR1 in mediating trace amine-induced vasoconstrictor responses of rat aorta. Despite the use of a novel TAAR1 agonist, RO5256390, and antagonist, EPPTB, the involvement of TAAR1 in these responses remains unknown. However, in Chapter 4 it was confirmed that the presence of a functional endothelium suppresses the vasoconstrictor response to trace amines through release of nitric oxide. This was investigated further to show trace amines have a secondary role as vasodilators in rat aorta. The vasodilator responses could be partially attenuated by endothelium removal or inhibition of eNOS, indicating that trace amines induce vasodilator responses through stimulation of eNOS and direct interaction with receptors on smooth muscle. These vasodilator responses were demonstrated in Chapter 6 to be insensitive to the muscarinic M_3 acetylcholine receptor antagonist, atropine, the β-adrenoceptor antagonist, ICI,188,551 and the mouse-specific TAAR1 antagonist, EPPTB. In Chapter 7, it was demonstrated that β-PEA-induced vasodilator responses were abolished by the pan-organic cation transporter (OCT1-3) and plasma membrane monoamine transporter (PMAT) inhibitor decynium 22, indicating trace amine-induced vasodilation requires initial transport of trace amines across the plasma membrane. The combined findings of the previous chapters demonstrate that trace amines have a dual functionality mediated by receptors located in separate cellular compartments.

Most research into trace amine induced vascular responses have been carried out in the aorta (Broadley 2010), which is a conductance vessel (O'Brien et al. 1998). In contrast very little research has been carried out in resistance vessels which play an integral role in maintenance of arterial blood pressure (Christensen and Mulvany 1993). Resistance arteries, such as the mesenteric arteries, differ substantially in function from conductance arteries (O'Brien et al. 1998). Large conductance arteries act as both a conduit for blood flow to peripheral tissues and organs and as a cushion to dampen the pressure oscillations resulting from ventricular ejection (Levy et al. 2006). In contrast resistance arteries are considered the major effector of peripheral resistance and blood flow control (Intengan and Schiffrin 2000). The mesenteric vasculature receives approximately 25% of cardiac output and has a large impact on the total peripheral resistance and blood pressure (Christensen and Mulvany 1993).

In the perfused rat mesenteric vascular bed, tryptamine is the only trace amine reported to induce a pressor response (Anwar et al. 2012). However, this contraction was shown to be mediated by 5-HT_{2A} receptors. In the rat aorta, a role for 5-HT_{2A} receptors in mediating β-PEA-induced vasoconstriction was excluded in Chapter 5 as the 5-HT_{2A} antagonist, cinanserin, had no effect on β-PEA-induced vasoconstriction. Interestingly, Anwar et al. (2012) noted that tyramine, β-PEA, tryptamine and 5-HT all induced a vasodilator response when perfusion pressure was raised by phenylephrine infusion (Anwar et al. 2012). This vasodilator response was resistant to 5-HT_7 receptor antagonism, and therefore the authors suggested that the response to be mediated by TAAR1. However, this was not further investigated.

The current chapter aims to extend the work of Anwar et al. (2012) and the findings of Chapter 4 to investigate the vasoconstrictor and vasodilator actions of trace amines in the resistance arteries of the mesentery.

8.2 Hypothesis

β-PEA induces both vasoconstrictor and vasodilator responses in third order mesenteric arteries.

8.3 Aims

1. Confirm β-PEA acts as a vasoconstrictor in isolated mesenteric arteries
2. Investigate β-PEA-induced vasodilator responses in isolated mesenteric arteries

8.4 Materials and Methods

8.4.1 Animals

Male Sprague Dawley, rats (350-750g) were obtained and housed as described in Chapter 2. All experiments adhered strictly to the Animals (Scientific Procedures) Act 1986.

8.4.2 Thin wire myography

Third-order mesenteric arteries were mounted in a Mulvany-Halpern myograph chamber (Models 420A and 620M, Danish Myo Technologies, Denmark) containing pre-warmed (37^{o}C) Krebs bicarbonate solution as described in Chapter 2. Vessels were given a 30-minute equilibration period prior to normalisation of the tension to that generated at 90% of the diameter of the vessel at 100 mmHg (Mulvany and Halpern 1977). After a further 30-minutes tissues were washed prior to testing tissue viability and presence of a functional endothelium as described in Chapter 2. CRCs were obtained for phenylephrine and β-PEA. With carbachol CRCs, the eNOS inhibitor, L-NAME was added to organ baths 15 minutes prior to carrying out CRCs.

8.4.3 Drugs and solutions

All drugs were dissolved in Krebs bicarbonate buffer. The source of all drugs used in this chapter and the composition of Krebs bicarbonate buffer are detailed in Chapter 2.

8.4.4 Data analysis

Contractile responses were expressed as a percentage of the contractile response to high potassium Krebs bicarbonate solution as described in Chapter 2. Vasodilator responses are expressed as a percentage of the initial contractile response to (3 μM) phenylephrine used to pre-constrict tissues. Responses are plotted as the mean response ± the S.E.M where n represents the number of rats. Curve fitting was applied as described in Chapter 2. E_{Max} values are reported as a percentage of the contractile responses to 60mM KCl ± S.E.M. Negative logEC_{50} values were obtained for each individual curve. EC_{50} values were calculated from the -logEC_{50} and are reported as the mean value with 95% confidence intervals. Mean EC_{50} values were compared by unpaired Student's t-test. P-values ≤0.05 were considered statistically significant. Area under the curve (AUC) for specified concentration ranges was calculated for each individual curve. The mean AUC of contractile CRCs was reported as a % of contraction to 60 mM KCl by Log[agonist(M)] (%.Log[M]). The mean AUC of relaxant CRCs was reported as a % of the initial contraction to phenylephrine by Log[agonist(M)] (%P.Log[M]). AUC's were compared by unpaired, two-tailed Student's t-test or one-way ANOVA as appropriate.

8.5 Results

8.5.1 β-PEA-induced vasoconstriction

To confirm blood vessels could contract to a known pharmacological factor, CRC's to phenylephrine were generated in the presence or absence of endothelium. Phenylephrine induced concentration dependent contraction of third order blood vessels independently of the presence of a functional endothelium (

Figure 8.5.1). In the presence of a functional endothelium, the E_{Max} (137 ± 8 %) was not significantly different (unpaired, two-tailed, Students T-test) from the E_{Max} in the absence of endothelium (133 ± 12 %). Similarly, the EC_{50} of phenylephrine in endothelium intact vessels (1.4 µM, 1.0 – 1.8 µM, Table 8.5.1) was not significantly different (unpaired, two-tailed, Students T-test) from the EC_{50} in endothelium denuded vessels (0.8 µM, 0.4 – 1.6 µM, Table 8.5.1).

β-PEA induced vasoconstriction of third-order mesenteric arteries only in the absence of endothelium. In the presence of endothelium, no contractile responses to β-PEA were observed with increasing concentrations of β-PEA (Figure 8.5.2A, C). In the absence of the endothelium a contractile response to β-PEA was only observed at the maximum concentration of β-PEA (1 mM, Figure 8.5.2B, C). The mean contractile response at this concentration of β-PEA was 35 ± 12 % of the contractile response to 60mM KCl. 2/7 endothelium denuded vessels failed to contract even at the highest concentration of β-PEA. As complete CRC's were not obtained for β-PEA neither an EC_{50} nor E_{Max} could not be calculated.

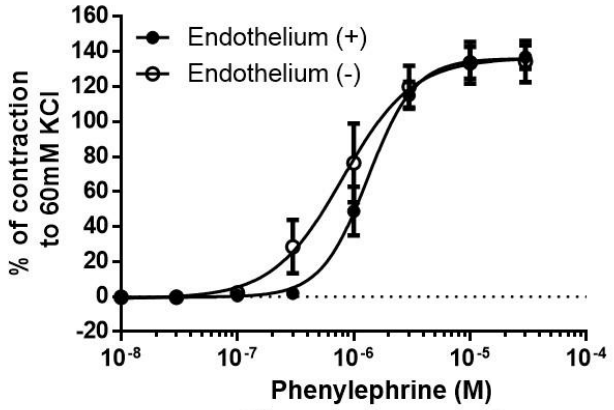

Figure 8.5.1. Mean cumulative CRCs obtained for phenylephrine in the presence (● n = 7) and absence (○ n = 6) of endothelium. Contractile responses are reported as the mean percentage (±SEM) of the maximum contractile response to 60mM KCl. The total area under each CRC were compared by unpaired, two-tailed, Students T-test test.

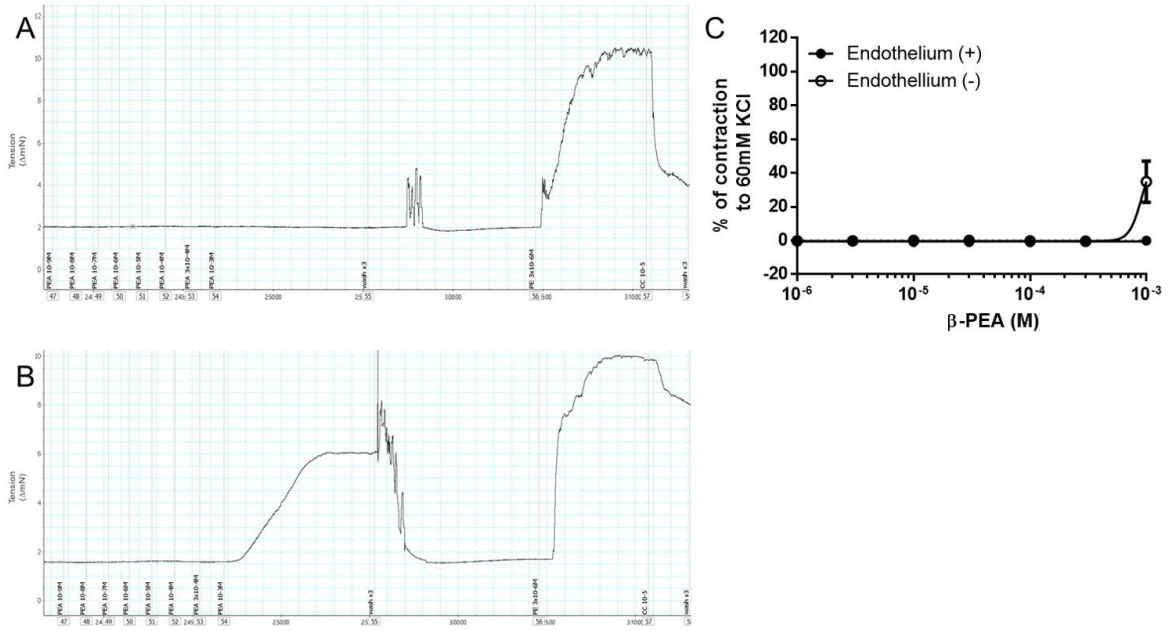

Figure 8.5.2. β-PEA-induced vasoconstrictor responses in rat 3rd order mesenteric arteries. Representative trace of β-PEA induced contraction in the presence (A) or absence (B) of endothelium. Cumulative CRC's for β-PEA were obtained (C) in the presence (● n = 6) and absence (○ n = 7) of endothelium. Contractile responses are reported as the mean percentage (±SEM) of the maximum contractile response to 60mM KCl.

Table 8.5.1 EC_{50} values of vasoconstrictors in the presence or absence of inhibitors or antagonists in endothelium intact and denuded aortic rings.

Agonist	Endothelium +/-	Condition/Antagonist	EC50 (CI95)
Phenylephrine	+	Control	1.4 μM (1.0 – 1.8 μM)
	-	Control	0.8 μM (0.4 - 1.6 μM)
β-PEA	+	Control	ND
	-	Control	ND

8.5.2 β-PEA-induced vasodilation

To assess β-PEA induced vasodilator responses in the presence and absence of endothelium, it was first necessary to assess the success of endothelium removal against a known endothelium-dependent vasodilator (carbachol). In the presence of endothelium, carbachol induced concentration-dependent vasodilator responses of vessels pre-constricted with 3 μM phenylephrine (Figure 8.5.3A). The EC_{50} and E_{max} of carbachol were 214 nM (134 – 342 nM, Table 8.5.2) and 10± 2 % of the initial contraction to phenylephrine, respectively. In the absence of endothelium, vasodilator responses to carbachol were significantly reduced (Figure 8.5.3A). The AUC (between 0.01 μM and 10 μM) of the carbachol CRC in endothelium-denuded vessels (226 ± 2 %.Log[M]) was significantly ($P \leq 0.001$, one-way ANOVA) greater than the AUC of the carbachol CRC in endothelium-intact vessels (99 ± 7 %.Log[M]). In the absence of endothelium, the mean vasodilator response at the maximum concentration of carbachol was 79 % (71 – 86 %). This was found to be statistically significant ($P \leq 0.001$) from the baseline tension (100% of the initial contractile response to phenylephrine). As a complete CRC was not obtained in the absence of endothelium, neither an EC_{50} nor E_{Max} were obtained.

Inhibition of eNOS using L-NAME (100 μM) significantly reduced carbachol induced vasodilator responses (Figure 8.5.3B). In the presence of L-NAME the AUC of the carbachol

CRC in endothelium-intact vessels (196 ± 2 %.Log[M]) was significantly (P ≤ 0.001, one-way ANOVA) greater than in the absence of L-NAME. In the presence of L-NAME, the mean vasodilator response at the maximum concentration of carbachol was 66 % (37 – 96 %). This was not statistically significant (P = 0.055) from the baseline tension (100% of the initial contractile response to phenylephrine). As a complete CRC was not obtained in the absence of endothelium, neither an EC_{50} nor E_{Max} were obtained. To confirm complete removal of endothelium, the effect of L-NAME in endothelium denuded vessels was also assessed. In the absence of endothelium, L-NAME did not further reduce vasodilator responses to carbachol (Figure 8.5.3C). The AUC in the presence of L-NAME in endothelium-denuded vessels (218 ± 1 %.Log[M]) was not significantly (one-way ANOVA) different from the absence of L-NAME.

β-PEA induced concentration-dependent vasodilation of pre-constricted vessels (Figure 8.5.4). The AUC (between 0.01 µM and 1 mM) of the CRC to β-PEA in endothelium-intact vessels was 235 ± 6 %.Log[M]. In the presence of a functional endothelium, β-PEA had an EC_{50} of 29 µM (10 – 68 µM, Table 8.5.2) and an E_{Max} of -7 ± 7 % of the contraction to phenylephrine. In some cases (n = 3), following addition of the maximum concentration (1 mM) of β-PEA, a vasoconstrictor response was observed (Figure 8.5.4A). These increased tension within the range of 16 – 59 % of the contractile response to phenylephrine.

Endothelium removal had no significant effect on the CRC to β-PEA (Figure 8.5.4C The AUC of the β-PEA CRC was 260 ± 5 %.Log[M]. This was not found to be significantly different (unpaired, two-tailed, Students T-test) from the presence of endothelium. The EC_{50} (50 µM, 26 – 89 µM, Table 8.5.2) and E_{Max} (6 ± 13 %) in endothelium denuded vessels, were not significantly different (unpaired, two-tailed, Students T-test) from endothelium intact vessels. Like endothelium intact vessels, following addition of the maximum concentration (1 mM) of β-PEA, a vasoconstrictor response was observed in some cases (n = 3, Figure 8.5.4B). These increased tension within the range of -26 – 73 % of the contractile response to phenylephrine.

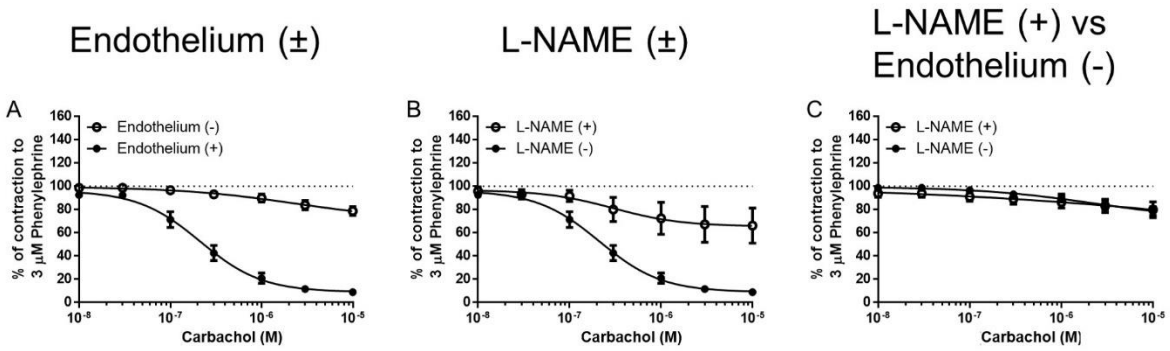

Figure 8.5.3. effect of endothelium removal or inhibition of eNOS with L-NAME (100 µM) Carbachol induced vasodilator responses in rat 3rd order mesenteric arteries. Mean cumulative CRC's were obtained for carbachol (A) in the presence (● n = 7) and absence (○ n = 6) of endothelium. Cumulative CRC's were obtained for carbachol (B) in endothelium-intact third-order vessels in the absence (● n = 7) or presence (○n = 5) of L-NAME. Cumulative CRC's were obtained for carbachol (C) in endothelium -denuded third-order vessels in the absence (● n = 6) or presence (○n = 5) of L-NAME. Vasodilator responses are reported as the mean response (±SEM) expressed as a percentage of the contractile response to 3 µM phenylephrine.

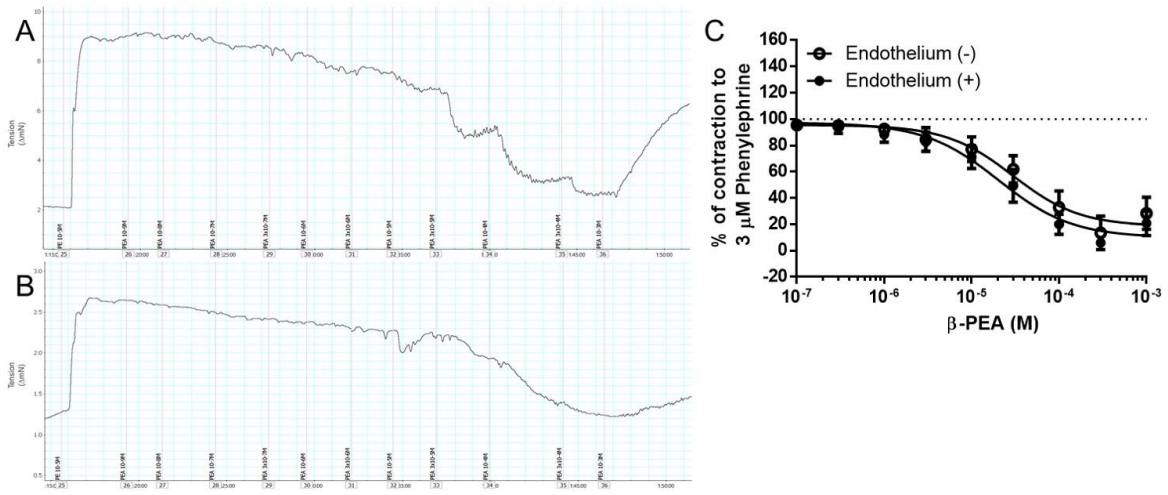

Figure 8.5.4. β-PEA-induced vasodilator responses in rat 3rd order mesenteric arteries. Representative trace of β-PEA induced vasodilation in the presence (A) or absence (B) of endothelium. Mean cumulative CRCs were obtained for β-PEA (C) in the presence (● n = 6) and absence (○ n = 7) of endothelium. Vasodilator responses are reported as the mean response (±SEM) expressed as a percentage of the contractile response to 3 µM phenylephrine.

Table 8.5.2. EC_{50} values of vasodilators in the presence or absence of inhibitors or antagonists in endothelium intact and denuded aortic rings

Agonist	Endothelium +/-	Condition/Antagonist	EC50 (CI95)
Carbachol	+	Control	214 nM (134 – 342 nM)
		L-NAME	ND
	-	Control	ND
		L-NAME	ND
β-PEA	+	Control	29 µM (10 – 68 µM)
	-	Control	50 µM (26 – 89 µM)

8.6 Discussion

8.6.1 β-PEA is a poor vasoconstrictor of mesenteric arteries

In the presence of a functional endothelium, β-PEA did not induce vasoconstrictor responses even at the maximum concentration used (1 mM). The concentrations of β-PEA used in this investigation were more than sufficient to induce vasoconstrictor responses in rat aortic rings, as reported in Chapter 4. Anwar et al. (2012) previously reported a lack of contractile activity to bolus doses of β-PEA in the perfused rat mesenteric vascular bed. However, the maximum concentration of β-PEA used in their study (100 nM) was likely far below the threshold required to elicit a vasoconstrictor response. In the absence of endothelium, β-PEA induced a vasoconstrictor response at the maximum concentration of β-PEA (1mM). However, the ability of β-PEA to induce a vasoconstrictor response at this concentration was also variable. β-PEA failed to elicit a contraction in a small number (2/7) of vessels. The poor contractile response to β-PEA in mesenteric vessels may be due to low cell surface expression of receptors in mesenteric vessels. Alternatively, as β-PEA also induced strong vasodilator responses in mesenteric arteries, the vasodilator response may predominate in the mesenteric vessels. Vasodilator responses to β-PEA may need to be blocked to unmask the contractile response.

As concentration dependent contractions were not generated to β-PEA, concentration dependent contractile responses were assessed using the α_1-adrenoceptor agonist phenylephrine. This was to rule out the possibility that third order arteries could not respond to increasing concentrations of a vasoconstrictor. Unlike β-PEA, third-order mesenteric arteries contracted in a concentration dependent manner to phenylephrine. Phenylephrine induced contractile responses were found to be independent of the presence of a functional endothelium. These results demonstrate that third-order mesenteric arteries are capable of responding to an increasing concentration of a known vasoconstrictor.

8.6.2 β-PEA is primarily a vasodilator in mesenteric arteries

In Chapter 4, β-PEA was shown to induce vasodilation of pre-constricted rat aortic rings. The current chapter extended the investigation of β-PEA-induced vasodilator responses to isolated third-order mesenteric arteries. In the rat aorta, β-PEA-induced vasodilator responses were partially attenuated by endothelium removal. Therefore, to fully investigate β-PEA in isolated mesenteric vessels, it was first necessary to establish the effects of endothelium removal against the known vasodilator, carbachol.

In the presence of a functional endothelium, carbachol induced large vasodilator responses of pre-constricted arteries (Figure 8.5.3). Following endothelium removal, vasodilator responses to carbachol were almost completely abolished, with weak but statistically significant dilator responses only at high concentrations of carbachol. To establish whether the remaining dilator responses were due to residual endothelium, nitric oxide release was inhibited using the eNOS inhibitor, L-NAME. L-NAME reduced dilator responses in endothelium intact vessels to a similar degree as endothelium removal. However, in the absence of endothelium, no difference was observed in the presence or absence of L-NAME. It is possible that any residual response in endothelium intact tissues is down to release of endothelium-derived hyperpolarisation factor (EDHF) or prostacyclin (PGI_2, Jiang et al. 2000; Durand and Gutterman 2013). The impacts of PGI_2 and EDHF were not further investigated. However, M1 and M3 muscarinic acetylcholine receptors have been reported to induce vasodilation of rat mesenteric arteries that is independent of the presence of a functional endothelium (Tangsucharit et al. 2016). This may explain the residual vasodilator response to carbachol in endothelium-denuded vessels and endothelium-intact vessels in the presence of L-NAME.

β-PEA-induced vasodilator responses were observed across a similar concertation range in both endothelium-intact and -denuded arteries pre-constricted with phenylephrine (Figure 8.5.4). Unlike the rat aorta, endothelium removal did not attenuate vasodilator responses to

β-PEA. This indicates that, in the mesenteric arteries, vasodilator responses to β-PEA are independent of the presence of a functional endothelium. However, this is in contrast to the findings of Anwar et al. (2012), who demonstrated β-PEA-induced vasodilation to be susceptible to L-NAME in the perfused mesenteric vascular bed. These differences between the current investigation and that of Anwar et al. (2012) may be explained by subtle differences in the endothelium throughout the mesentery. The current investigation used third-order vessels which at the level of the endothelium may lack receptors that can respond to β-PEA, whereas the endothelium of larger vessels encompassing the entire vascular bed may contain a larger pool of receptors that respond to β-PEA. β-PEA was a more potent vasodilator in third-order mesenteric vessels than rat aortic rings as it was able to reduce the tension of pre-constricted vessels to baseline levels. This was not achieved in aortic rings, even at the highest concentration of β-PEA (1 mM). This and the lack of contractile responses, indicate that in the mesenteric vasculature the predominate response to β-PEA is vasodilation.

An interesting observation in isolated mesenteric arteries, was an unexpected contractile response at the maximum concentration of β-PEA. This occurred in approximately 50% of vessels and was independent of the presence of a functional endothelium (Figure 8.5.4). In vessels that had not been subjected to pre-constriction with phenylephrine, β-PEA was established to be a poor vasoconstrictor. In the presence of endothelium, β-PEA did not induce any contractile responses and a contractile response was only observed at the maximum concentration (1 mM) in endothelium denuded vessels. It is possible that the vasodilator response to β-PEA may have become desensitised, therefore unmasking the contractile response to β-PEA. However, further research will be required to determine whether this contractile effect is due to β-PEA or a return to the tension induced by phenylephrine.

8.7 Conclusion

Clear differences exist between trace amine-induced responses in resistance, third-order mesenteric arteries, and conductance arteries such as the aorta. In the mesentery, β-PEA predominately induces a vasodilator response. This vasodilator response is independent of the presence of a functional endothelium and reduced the tension to baseline in pre-constricted tissues.

Chapter 9 General Discussion

9.1 Summary

This thesis sought to gain a deeper understanding of the pharmacology of trace amine-induced vascular responses. Previous work has largely focused on identifying the receptors involved in the vasoconstrictor actions of the trace amines, concluding that TAAR1 was responsible (Herbert et al. 2008; Fehler et al. 2010; Broadley et al. 2013; Broadley and Richards 2015). Although discovered in 1993 (Varma and Chemtob 1993; Varma et al. 1995), the vasodilator actions of the trace amines have been overlooked in favour of their vasoconstrictor actions. Vasodilation was not again reported until 2012 (Anwar et al. 2012), where TAAR1 was suggested to mediate this response. Despite the use of TAAR1 selective reagents the current study was unable to conclusively confirm a role for TAAR1 in either vasoconstriction or vasodilation.

The current work has identified that trace amine-induced vascular responses in rat aorta are mediated by receptors located in separate cellular domains. Vasoconstriction appears to predominantly be mediated by receptors localised to the cell surface. In contrast, vasodilation is mediated by intracellular receptors, requiring trace amines to be transported via uptake-2 pathways to elicit a vasodilator response. The study also characterised variation in the vasoconstrictor response to trace amines. It was observed that the vasoconstrictor response is unpredictable and unstable in immature rats, becoming predictable and sustainable after rats attain sexual maturity. This effect was conserved across different rat strains. However, the vasoconstrictor actions of the β-PEA were found to be less potent in aortic rings from female animals.

9.2 Mechanism of trace amine-induced vasoconstriction

Historically the vasoconstrictor effects of the trace amines have been attributed to the sympathomimetic release of noradrenaline from sympathetic neurones (Broadley 2010). Considering that structurally the trace amines are closely related to classical neurotransmitters (Lindemann and Hoener 2005) such as 5-HT, noradrenaline and dopamine, it is not surprising that evidence indicates the trace amines can also exert their effects independently of noradrenaline release. For example, tryptamine is reported to induce a vasoconstrictor effect via activation of 5-HT receptors (Anwar et al. 2012; Anwar et al. 2013; Stollak and Furchgott 1983). In addition, β-PEA has also been reported to induce contraction of rat gastric fundus via 5-HT receptors (Batista-Lima et al. 2018). However, in isolated blood vessels the vasoconstrictor effects of trace amines have previously been demonstrated to be resistant to blockade of $\alpha_{1/2}$-, β_2-adrenoceptors, monoamine oxidase inhibitors and inhibitors of uptake-1 transporters (Broadley et al. 2013; Fehler et al. 2010). Consequently, the vasoconstrictor responses to trace amines have been attributed to TAAR1 (Broadley et al. 2013; Fehler et al. 2010).

Expression of TAAR1 has previously been confirmed at both the protein and mRNA level in rat aorta (Fehler et al. 2010). Previous studies about the involvement of TAAR1 in trace amine-induced vasoconstriction have been reliant on the comparison of the potency order of vasoconstrictor responses with the published potency orders in cell lines (Broadley et al. 2013; Bunzow et al. 2001; Fehler et al. 2010). Although a valid method, the potency order of trace amine-induced vasoconstriction does not recapitulate that of cell lines (Broadley et al. 2013). The major limitation of these previous studies is the lack of TAAR1 selective reagents, leaving the involvement of TAAR1 without definitive proof.

In Chapter 5 a systematic approach utilising several antagonists of classical neurotransmitters and selective TAAR1 reagents was used to identify the receptor or receptors involved in trace amine-induced vasoconstrictor responses. This approach has confirmed that vasoconstrictor responses to β-PEA are not mediated by α_1-adrenoceptors, 5-HT receptors or D_1 and D_2 dopamine receptors. Chapter 5 also details the first use of the

TAAR1-selective agonist, RO5256390, and selective antagonist, EPPTB, in isolated rat blood vessels. Despite the use of selective reagents, this study was unable to confirm a role for TAAR1 in mediating the vasoconstrictor actions of trace amines. Although RO5256390 was able to induce a vasoconstrictor response, EPPTB had no effect on either RO5256390- or β-PEA-induced contractile responses. As detailed in Study Limitations, EPPTB has very low affinity for rat TAAR1 and displays poor solubility. Based on the published affinity constant (K_I) of EPPTB for rat TAAR1 (0.942 μM Bradaia et al. 2009), the expected shift at a concentration of 5 μM is 6.3-fold. However, this degree of shift may be difficult to observe, suggesting a higher concentration of EPPTB would be required. The limit of solubility for EPPTB in this study was 5 μM in organ baths and therefore EPPTB does not appear to be a suitable tool for the study of TAAR1 responses in rats. Without the development of more soluble TAAR1 antagonists a role for TAAR1 in mediating trace amine-induced vasoconstriction cannot be definitively confirmed.

9.3 Mechanism of trace amine-induced vasodilation

Vasodilator responses to the trace amines were first reported in rat aorta by Varma and Chemtob (1993). This initial report found vasodilation to be independent of the presence of a functional endothelium. A later study seeking to clarify the receptors involved, found the vasodilator response was not mediated by β-adrenoceptors or receptors for 5-HT, histamine and adenosine (Varma et al. 1995). At a time prior to the discovery of trace amine associated receptors (TAARs, Borowsky et al. 2001; Bunzow et al. 2001) the authors suggested the role of novel tyramine receptors. Later work in the perfused rat mesenteric vascular bed reported an endothelium-dependent vasodilator response to numerous trace amines (Anwar et al. 2012). As the response was resistant to blockade of 5-HT_7 receptors, the authors attributed the response to TAAR1, and it was not further investigated (Anwar et al. 2012; Anwar et al. 2013). Although both Varma and Chemtob (1993) and Anwar et al. (2012) described clear trace amine-induced vasodilator responses, there was some discrepancy about the involvement of endothelium-derived mediators. This difference may be a result of the different vascular models utilised in each study. Large conductance arteries such as the aorta, act as a conduit for blood flow and dampen pressure oscillations that originate from ventricular ejection, having only a minor impact on blood pressure (Levy et al. 2006). In contrast resistance vessels such as the mesenteric arteries are the major effectors of both peripheral resistance and blood flow control (Intengan and Schiffrin 2000). It is possible that for the mesenteric arteries to carry out their role as a major effector of blood pressure, trace amine-induced vasodilation also requires endothelium-derived mediators.

Chapter 4 clarified that in rat aorta, β-PEA-induced vasodilation has both an endothelium-dependent and -independent mechanism. At the level of the endothelium, β-PEA-induced vasodilation was found to be entirely dependent on the release of nitric oxide. Chapter 6 then determined β-PEA-induced vasodilator responses were not mediated by β_2-adrenoceptors or muscarinic M_3 acetylcholine receptors. The involvement of TAAR1 was not confirmed despite the use of the selective TAAR1-antagonist, EPPTB. However as later described in Study Limitations, this may be due to the low affinity of EPPTB for the rat TAAR1.

9.4 Intracellular vs extracellular responses to trace amines

Since its discovery in 2001, TAAR1 has proven difficult to characterise pharmacologically, due to the high concentrations of trace amines required to elicit a vascular response which raises questions about the selectivity of TAAR1 agonists (Broadley 2010; Fehler et al. 2010). Numerous studies have demonstrated TAAR1 is predominantly localised within intracellular compartments (Bunzow et al. 2001; Miller et al. 2005; Xie et al. 2008). For intracellular receptors, it is the intracellular concentration of an agonist that is important in determining receptor binding and activation (Jong et al. 2018), which may explain the high concentrations required to induce a vascular response. Furthermore, in order to elicit a vascular response, it is necessary for trace amines to gain access to the intracellular compartment. Considering

that trace amines characteristically have half-lives of less than 30 seconds (Durden and Philips 1980), mechanisms to facilitate their rapid transport to the intracellular environment must exist. Neuronal and extraneuronal (uptake-1 and uptake-2 respectively, Iversen 1973) transporters have affinity for the trace amines.

Uptake-1 transporters have a high selectivity and low capacity for their corresponding neurotransmitters (Torres et al. 2003). Although tuned towards classical neurotransmitters, (dopamine – DAT, serotonin – SERT, noradrenaline – NET), at high micromolar concentrations trace amines are also substrates of these transporters (Raiteri et al. 1977; Danek Burgess and Justice 1999; Liang et al. 2009; Underhill et al. 2019). In contrast the uptake-2 transporters, such as the organic cation transporters (OCT1-3, Slc22A1-3, Koepsell 2020) and the Plasma Membrane Monoamine Transporter (PMAT, Slc29A4, Wang 2016a), are considered poly-specific and high capacity (Courousse and Gautron 2015). The trace amines are substrates of uptake-2 transporters at nanomolar concentrations (Berry et al. 2016; Chen et al. 2010; Engel and Wang 2005; Schomig et al. 2006). Excluding OCT2, all other uptake-2 transporters are known to be expressed in smooth muscle (Chen et al. 2010; Engel et al. 2004; Wu et al. 2000), making them strong candidates for rapid uptake of trace amines in the vasculature.

Chapter 7 explored the possibility that uptake-2 transporters are a pre-requisite for trace amines to elicit a vascular response through an intracellular receptor. Blockade of uptake-2 pathways using the pan-OCT1-3 and PMAT inhibitor, decynium 22 (1 µM), failed to abolish vasoconstrictor responses to β-PEA. In contrast, the same concentration of decynium 22 was able to completely abolish β-PEA-induced vasodilation, resulting in further β-PEA-induced contraction. These results provide evidence that β-PEA-induced vasoconstriction and vasodilation are mediated by receptors located in distinctly separate cellular compartments. As β-PEA-induced vasodilation was abolished by blockade of uptake-2 transport, the vasodilation is clearly mediated by an intracellular receptor. Furthermore, blockade of uptake-2 pathways restored β-PEA-induced contractile responses in pre-constricted tissues. As β-PEA-induced contraction was not sensitive to uptake-2 blockade, trace amine-induced contraction is reliant on a receptor or receptors localised to the cell surface. Further evidence that trace amine-induced vasoconstriction does not rely on plasma membrane transport was previously provided by Fehler et al. (2010) who showed that the vasoconstrictor action of trace amines, including β-PEA, are not sensitive to blockade of uptake-1 pathways. However, Underhill et al. (2019), demonstrated that intracellular TAAR1 is coupled to both RhoA and protein kinase A (PKA). Activation of RhoA would promote a vasoconstrictor response through increased calcium sensitisation (Johns et al. 2000). It is therefore possible that trace amine-induced vasoconstrictor responses may be mediated by receptors located at both the plasma membrane and at an intracellular site.

Further evidence for the involvement of an intracellular receptor in mediating the vascular effects of trace amines was provided using novel tyramine analogues. By increasing the lipophilicity of tyramine, plasma membrane transporter-independent transmembrane diffusion is increased (Waring 2010). The increase in lipophilicity of tyramine analogue A2 (tyramine A2) was associated with a significant enhancement of the vasodilator response. However, the increase in lipophilicity was not associated with a leftward shift of the CRC which would be expected following an increase in the intracellular concentration of agonist. This suggests that tyramine A2 may have a higher efficacy for TAAR1 as evidenced by the increased maximum vasodilator response. However, as no shift in the CRC was observed this could indicate the existence of an opposing vasoconstrictor response which may be unmasked in aortic rings which have not been pre-constricted. It is likely that TAAR1 may be coupled to multiple downstream signalling pathways such as RhoA and PKA (Underhill et al. 2019). Activation of PKA signalling would lead to a vasodilator response in smooth muscle, whereas RhoA signalling would promote a contractile response (Johns et al. 2000). Although no vasoconstrictor action was observed for tyramine A2, as tyramine A2 is structurally similar to the parent compound it is likely that it can still induce a vasoconstrictor response

via receptors located at the cell surface. Interestingly, although only a single experiment was carried out, the presence of decynium 22 (1 μM), did not abolish tyramine A2-induced vasodilation. This suggests, tyramine A2 was able to cross the plasma membrane independently of plasma membrane transporters to elicit a response at an intracellular receptor.

9.5 Predominant vascular effects of trace amines in different vascular beds

Previous investigation of trace amine responses in the perfused rat mesenteric vascular bed have demonstrated tryptamine to be the only trace amine capable of causing a vasoconstrictor response (Anwar et al. 2012), although this was demonstrated to be mediated by 5-HT_{2A} receptors (Anwar et al. 2013). It was noted that tyramine, β-PEA, tryptamine and 5-HT could also induce a vasodilator response when perfusion pressure was raised by phenylephrine infusion (Anwar et al. 2012). The vasodilator response was resistant to blockade of 5-HT_7 receptors but sensitive to inhibition of eNOS using L-NAME (Anwar et al. 2013).

The findings in the mesenteric vasculature, an example of a resistance bed (Christensen and Mulvany 1993), are distinctly different from those of the rat aorta, a conductance vessel (O'Brien et al. 1998). Chapter 4 and previous studies have demonstrated trace amines, including β-PEA, tyramine, octopamine and tryptamine induce contraction of rat aortic rings (Fehler et al. 2010; Broadley et al. 2013). To date the receptor or receptors responsible for the vasoconstrictor response remain unknown. Previous studies and Chapter 4 have shown trace amines have a second function as a vasodilator in the aorta (Varma and Chemtob 1993; Varma et al. 1995). Although, the receptors responsible have not been identified, data presented in Chapter 4 provides evidence that the vasodilator response is comprised of both an endothelium-dependent and -independent component. This contrasts with the findings in the mesentery where trace amine-induced vasodilation was shown to be entirely dependent on endothelium-derived nitric oxide (Anwar et al. 2012; Anwar et al. 2013). It was hypothesised that the predominant vascular response, vasoconstriction or vasodilation may vary between vascular beds.

The data reported in Chapter 8 confirms that β-PEA is a very poor vasoconstrictor of isolated third-order mesenteric arteries. Unlike the rat aorta where endothelium removal significantly enhanced vasoconstrictor responses, in the mesenteric vessels, β-PEA only caused a contractile response at millimolar concentrations in endothelium-denuded vessels. By contrast β-PEA induced vasodilator responses of pre-constricted mesenteric vessels at concentrations as low as 1 μM. In contrast to rat aortic rings, trace amine-induced vasodilation of third-order mesenteric vessels was shown to be almost completely independent of the presence of a functional endothelium. This contrasts with the findings of Anwar et al. (2012), although may be explained by differences between a single isolated blood vessel and the entire vascular bed.

9.6 Potential alternative receptors involved in vascular responses to trace amines

As described above and in detail in Chapter 7, trace amine-induced vasoconstrictor and vasodilator responses are mediated at two cellular locations. Vasoconstriction is mediated at the cell surface whereas vasodilation appears to be mediated by an intracellular receptor. These responses may be mediated by distinct subsets of the same receptor (Underhill et al. 2019) or by separate receptors. As numerous studies have reported TAAR1 localisation to be predominantly intracellular (Bunzow et al. 2001; Miller et al. 2005; Xie et al. 2008), it is possible that the vasoconstrictor actions of the trace amines are mediated by an alternative receptor at the cell surface.

Possible candidate receptors reside within the TAAR family, of which only TAAR1-4 are able to respond to the trace amines (Ferrero et al. 2012). Aside from TAAR1, TAAR4 is the only TAAR with a known affinity for any of the classical trace amines, and is able to respond to both β-PEA and tryptamine (Borowsky et al. 2001). However, tyramine and octopamine are able to induce a vasoconstrictor response in rat aortic rings, neither of which are agonists of TAAR4, which is likely to eliminate TAAR4 as a candidate receptor (Chapter 3, Borowsky et al. 2001; Khwanchuea et al. 2008; Fehler et al. 2010;Broadley et al. 2013). Furthermore, like TAAR1, TAAR4 is poorly expressed at the cell surface (Borowsky et al. 2001). This provides further evidence against TAAR4 as a possible candidate as this study has shown that the vasoconstrictor response is likely to be mediated by a cell surface receptor. Trace amines have been reported to cause contraction of isolated human blood vessels (Maguire et al. 2002). As TAAR4 is a pseudogene in humans (Borowsky et al. 2001) it is impossible for TAAR4 to mediate these responses. Other candidates include TAAR2 and TAAR3, although very little is known about these receptors as almost all TAAR research has focused on TAAR1 since many psychotropic drugs have an affinity for this receptor (Gainetdinov et al. 2018).

As reported in Chapter 7, the vasodilator effects of trace amines are mediated by an intracellular receptor. Although the selective TAAR1 antagonist, EPPTB, had no effect on β-PEA-induced vasodilator responses, it does not have sufficient affinity for rat TAAR1 compared to its limit of solubility for a lack of effect to be taken as evidence that vasodilation is not mediated by TAAR1 (Study Limitations, Stalder et al. 2011). As TAAR1 is predominately an intracellular receptor (Bunzow et al. 2001; Miller et al. 2005; Xie et al. 2008), it remains a strong candidate for trace amine-induced vasodilator responses. In neurones, TAAR1 has been shown to be a functional target for the trace amines and amphetamines (Underhill et al. 2019). Within neurones, TAAR1 is coupled to RhoA and protein kinase A (PKA) that is dependent on the subcellular compartment (Underhill et al. 2019). This provides evidence that as an intracellular receptor, TAAR1 is functional and may mediate trace amine-induced vasodilator responses.

9.7 Physiological roles

When the trace amine, tyramine, is administered systemically, the cardiovascular effects include vascular constriction and an increase in cardiac rate and contractile force (Ghose 1984). The diet is the major source of trace amines in the plasma (VanDenBerg et al. 2003). Foods rich in trace amines include, cheese, chocolate and wine (Branchek and Blackburn 2003). Normally, ingestion of foods rich in trace amines has no effect on the cardiovascular system as trace amines are rapidly catabolised by monoamine oxidase enzymes in the intestines and liver before reaching the systemic circulation (VanDenBerg et al. 2003). To induce an 30 mmHg increase in systolic blood pressure requires approximately 500 mg of orally administered tyramine under fasting conditions (Bieck and Antonin 1988). Trace amine concentrations in a typical meal are generally low (≤5 mg, VanDenBerg et al. 2003). However certain foods such as aged cheeses contain significantly higher levels of trace amines (up to 1000 mg/Kg tyramine, Gillman 2018). When eaten in sufficient amounts, such foods can provide enough tyramine to elicit an increase in systolic blood pressure (VanDenBerg et al. 2003).

This study has demonstrated β-PEA, induces vasodilator responses of third order mesenteric arteries at concentrations in excess of 1 μM (Chapter 7). Furthermore, previous work by Anwar et al. (2012), demonstrated a vasodilator effect of trace amines in the perfused rat mesenteric vascular bed at concentrations as low as 10 nM. Following a meal, the concentration of circulating trace amines most likely reaches the level required to induce a vasodilator response in the mesenteric vasculature. A vasodilator effect in the mesenteric vasculature would increase blood flow to the gastrointestinal tract, aiding in digestion and absorption (Broadley et al. 2009; Broadley 2010). Therefore physiologically, the trace amines may act to aid in the digestion and absorption of nutrients following a meal.

Throughout the current study and in all previous studies of vascular trace amine responses (Varma and Chemtob 1993; Varma et al. 1995; Herbert et al. 2008; Khwanchuea et al. 2008; Broadley et al. 2009; Fehler et al. 2010; Herbert et al. 2011; Anwar et al. 2012; Anwar et al. 2013; Broadley et al. 2013; Broadley and Broadley 2017), high micromolar to millimolar concentrations of trace amine have been required to elicit either a vasoconstrictor or vasodilator response. The current study (Chapter 7) demonstrated dependence on plasma membrane transport, via uptake 2 transporters, to elicit a vasodilator response at an intracellular receptor. A response generated by an intracellular receptor is dependent on the intracellular concentration of agonist (Jong et al. 2018). Chapter 7 demonstrated β-PEA to be a more efficacious vasodilator in mesenteric arteries than in the aorta. It is likely that the greater magnitude of the vasodilator response in mesenteric vessels is mediated by both receptor and transporter functionality. Although plasma membrane transport was not assessed in the present study, it is plausible that the same transporters mediate uptake of trace amines in both the mesenteric and aortic vasculature. Furthermore, there may be greater expression of plasma membrane transporter and TAAR1 in mesenteric arteries which would account for the greater vasodilator response. A greater expression of transporters and TAAR1 in the mesenteric vessels would facilitate the vasodilation of the mesenteric arteries following a meal thereby aiding the digestion and absorption.

A recent study by Rafehi et al. (2019) found strong interindividual variation in the blood pressure response to orally administered tyramine. Interestingly the authors found that increases in blood pressure were generally short lived, although it was also observed that the effects of tyramine on blood pressure often varied with respect to both intensity and time of onset (Rafehi et al. 2019). The study by Rafehi et al. (2019) utilised both male and female volunteers between 18 and 49 years of age that were genotyped for numerous MAO-A, CYP2D6, and OCT1 polymorphisms. No statistically significant differences were found between gender and age for the metabolism of tyramine, however the variation of tyramine blood pressure responses were not examined for age or gender (Rafehi et al. 2019). Variability in onset of contraction may be an effect of the age of volunteers, although the age and gender were not investigated in the onset of these responses. Highly variable onset in the contraction of rat aorta to β-PEA, tyramine and RO5256390 with age and sexual maturity was reported in Chapter 3. It is possible that this effect is conserved in humans and may explain the variation in onset of the pressor response in the study by Rafehi et al. (2019).

9.8 Clinical Relevance

It is well established that excessive levels of circulating trace amines and amphetamines are linked with detrimental cardiovascular events (Broadley 2010). Ingestion of dietary trace amines, particularly from cheese and chocolate, have long been linked with onset of migraine headache (Hanington and Harper 1968). Many studies support dietary trace amines as a migraine trigger (Kohlenberg 1982; Vaughan 1994; Borkum 2016). However, several studies suggest no evidence of any association between dietary intake and migraine onset, suggesting other constituents such as flavonoids to be the true cause (Jansen et al. 2003; Borkum 2016). Intracranial and extracranial arteries have been suggested to play a nociceptive role in migraine (Asghar et al. 2011; Shevel 2011). Vasodilation of the intracranial arteries has been reported during induced and spontaneous migraine onset (Asghar et al. 2011; Amin et al. 2013). Considering trace amines act as vasodilators in both rat aorta and mesenteric vasculature, it is possible that elevated plasma trace amines induce the vasodilator component of migraine headaches. Although to date, the effects of trace amines in the intercranial and extracranial vessels is unknown.

Increased plasma levels of tyramine have been reported in hypertensives (Andrew et al. 1993). Tyramine infusion induces a significantly greater increase in systolic blood pressure in hypertensive individuals compared with normotensive (Colombo et al. 1989). It is possible that trace amines play an important physiological role in blood pressure regulation in hypertensive individuals. As trace amines have been demonstrated to act as vasodilators,

they may play an important role in reducing blood pressure. Alternatively, as tyramine pressor responses are larger in hypertensives, it has been suggested hypertensives may be more susceptible to the vasoconstrictor action of the trace amines (Broadley 2010). It is likely that in individuals suffering from endothelial dysfunction, that the vasodilator response to trace amines is abrogated due to the lack of endothelium-derived vasodilators (Rajendran et al. 2013). Alternatively, in the hypertensive individual, receptors mediating the vasoconstrictor actions of trace amines could be upregulated whilst receptors or uptake-2 transporters required for vasodilation are downregulated.

Therapeutic and illicit use of amphetamine and amphetamine-like drugs are commonly linked with adverse cardiovascular events. (-)-S-cathinone, pseudoephedrine and phenylpropanolamine have strong links with acute myocardial infarction (AMI) and acute myocardial ischaemia (Oosterbaan and Burns 2000; Pederson et al. 2001; Al-Motarreb et al. 2002; Manini et al. 2005). (-)-S-cathinone and 3, 4-methylenedioxymethamphetmine (MDMA) have both been shown to induce vasoconstriction of coronary arteries (Al-Motarreb and Broadley 2003; Baker et al. 2007; Herbert et al. 2008; Broadley et al. 2009). Considering the structural and functional overlap with the trace amines (Bunzow et al. 2001; Lindemann and Hoener 2005), amphetamine-like drug-induced vasoconstriction is likely mediated by the same receptors that mediate trace amine-induced vasoconstriction. However, it is unknown whether amphetamine-like substances share the vasodilator properties of the trace amines. Furthermore, it is unknown if trace amines can induce a vasodilator response in the coronary vessels.

9.9 Study Limitations

Previous studies of vascular trace amine responses have been impeded by the lack of selective TAAR1 reagents (Baker and Broadley 2003; Baker et al. 2007; Fehler et al. 2010; Anwar et al. 2012; Anwar et al. 2013; Broadley et al. 2013). As a result, studies have relied on comparison of the potency order of trace amine-induced vascular responses with the published potency orders of trace amines in TAAR1 expressing cell lines (Bunzow et al. 2001). Although a viable method, there are some inconsistencies between the potency order in cell lines and that of trace amine-induced vascular responses (Fehler et al. 2010; Broadley et al. 2013), highlighting the need for TAAR1 selective compounds.

Considerable efforts have led to the development of several synthetic TAAR1-selective reagents. Hoffman-La Roche synthesised the first TAAR1 agonist RO5166017 through structural modification of the α_2-adrenoceptor agonist S18616 (Revel et al. 2011). Since then, a number of full agonists, such as RO5256390, and partial agonists including RO5203648 have been developed (Revel et al. 2012; Revel et al. 2013). Although a wide variety of TAAR1 agonists have been identified, compounds displaying TAAR1-antagonism have remained elusive. Despite a sizeable effort, to date, only a single TAAR1 antagonist, N-(3-Ethoxy-phenyl)-4-pyrrolidin-1-yl-3-trifluoromethyl-benzamide (EPPTB), has been identified (Bradaia et al. 2009).

The current study utilised both the TAAR1-selective agonist, RO5256390, and selective antagonist, EPPTB, to confirm a role for TAAR1 in mediating trace amine-induced vascular responses. Despite being considered a TAAR1-selective agonist, at concentrations in excess of 3 µM RO5256390 also has affinity for alternative receptors including 5-$HT_{2A/B}$, opiod, imdazoline and muscarinic M_3 acetylcholine receptors (Revel et al. 2013). The highest concentration of RO5256390 used in the current investigation was 30 µM which could indicate RO5256390-induced responses may in part involve alternative receptors. Although any involvement of α_1-adrenoceptor involvement was excluded in Chapter 5, the involvement of alternative receptors was not evaluated. The involvement of alternative receptors, in particular, 5-HT_2 receptors, in RO5256390-induced contractile responses remains to be evaluated.

The TAAR1-selective antagonist, EPPTB, has a high affinity for mouse TAAR1 with an IC_{50} of 27.5 nM (Bradaia et al. 2009). In contrast, the affinity of EPPTB for rat and human TAAR1 is very poor with IC_{50}'s of 4539 nM and 7487 nM, respectively (Bradaia et al. 2009). In addition to its poor affinity for rat TAAR1, the use of EPPTB is somewhat limited by its poor solubility (Stalder et al. 2011). The current investigation found 5 µM to be the limit of solubility. However, other studies have successfully utilised concentrations of 50-100 µM (Batista-Lima et al. 2018; Koh et al. 2019). However, at concentrations in excess of 10 µM, EPPTB also displays affinity for classical aminergic receptors and transporters, in particular 5-HT_{1B}, D_1-like, 5-$HT_{2A,}$ 5-HT_{5A}, α_2-adrenoceptors and the dopamine and noradrenaline transporters (Bradaia et al. 2009). Despite receptor selectivity issues, low affinity for rat TAAR1 and poor solubility, EPPTB remains the only TAAR1 antagonist discovered to date (Bradaia et al. 2009; Stalder et al. 2011; Gainetdinov et al. 2018). Consequently, it was decided to utilise EPPTB at the limit of solubility (5 µM) which is below the levels reported for non-specific binding (Bradaia et al. 2009). In Chapter 5 and Chapter 6, EPPTB was shown to have no significant effect on trace amine-induced vascular responses. Considering the poor affinity for rat TAAR1 and low solubility, EPPTB is not a suitable tool to assess TAAR1 responses in the rat.

The lack of TAAR1 specific reagents is a major barrier to investigations of trace amine-induced vascular responses. Without the development of a rat and human specific TAAR1 antagonist, the involvement of TAAR1 in the vascular responses to trace amines will remain inconclusive. Future studies of trace amine-induced vascular responses should consider the use of TAAR1 knockout and overexpressing rats (Harmeier et al. 2015). The use of TAAR1 knock out and overexpressing animals may provide conclusive evidence for TAAR1 involvement in trace amine-induced vascular responses.

9.10 Conclusion

Although the receptors mediating vascular responses to the trace amines remain unknown, it is now clear that trace amine-induced vascular responses are dependent on the subcellular localisation of receptors. Receptors localised to the cell surface of smooth muscle mediated a vasoconstrictor response, whereas receptors located in the intracellular compartment of smooth muscle and endothelial cells mediate a vasodilator response. Which response, vasoconstriction or vasodilation, predominates is dependent on the vascular bed. Vascular responses to the trace amines are conserved across gender and strain of rat. However, trace amine-induced contractile responses become more stable following the period of sexual maturity.

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