The apelin receptor APJ: journey from an orphan to a multifaceted regulator of homeostasis

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Abstract

The apelin receptor (APJ; gene symbol *APLNR*) is a member of the G protein-coupled receptor gene family. Neural gene expression patterns of APJ, and its cognate ligand apelin, in the brain implicate the apelinergic system in the regulation of a number of physiological processes. APJ and apelin are highly expressed in the hypothalamo–neurohypophysial system, which regulates fluid homeostasis, in the hypothalamic–pituitary–adrenal axis, which controls the neuroendocrine response to stress, and in the forebrain and lower brainstem regions, which are involved in cardiovascular function. Recently, apelin, synthesised and secreted by adipocytes, has been described as a beneficial adipokine related to obesity, and there is growing awareness of a potential role for apelin and APJ in glucose and energy metabolism. In this review we provide a comprehensive overview of the structure, expression pattern and regulation of apelin and its receptor, as well as the main second messengers and signalling proteins activated by apelin. We also highlight the physiological and pathological roles that support this system as a novel therapeutic target for pharmacological intervention in treating conditions related to altered water balance, stress-induced disorders such as anxiety and depression, and cardiovascular and metabolic disorders.

Key Words
- APJ
- apelin
- G protein-coupled receptor
- homeostasis

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Introduction

G protein-coupled receptors (GPCRs) are activated by a plethora of molecules including neuropeptides, polypeptide hormones and non-peptides such as biogenic amines, lipids, nucleotides and ions. They are classically composed of seven membrane-spanning domains and constitute one of the largest and most diverse gene families in the mammalian genome (Ostrom & Insel 2004). Some novel GPCRs do not have obvious endogenous ligands and are termed orphan receptors, a number of which appear to be constitutively active (Jones *et al.* 2007, Tanaka *et al.* 2007). The cognate ligands for some of these orphan GPCRs have been identified, often based on the cellular and tissue distributions of the orphan GPCRs and occasionally using 'reverse pharmacology', where orphan GPCRs have been used to isolate novel endogenous substances. The human apelin receptor (APJ, gene symbol *APLNR*; O'Dowd *et al.* 1993) is one such GPCR whose endogenous ligand, apelin, has been described (Tatemoto *et al.* 1998). Both APJ and apelin have been implicated as the key mediators of physiological responses to multiple homeostatic perturbations, including cardiovascular control, water balance, hypothalamic–pituitary–adrenal (HPA) axis regulation and metabolic homeostasis. Homeostatic stability is critical in mammalian organisms, and our

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knowledge as to how this vital function is regulated and how this mechanism can go wrong in pathological conditions is still limited.

The apelin receptor, APJ

APJ was first identified as an orphan GPCR, with closest identity to the angiotensin II (Ang II) receptor, type AT_{1a} (O'Dowd *et al*. 1993). In the ensuing years, the receptor was deorphanised when its cognate ligand, apelin, was isolated from bovine stomach extracts (Tatemoto *et al*. 1998). Recently, the apelinergic system has been shown to be critically involved in multiple homeostatic processes.

APJ gene and protein structures

The gene encoding APJ is intronless and is termed *APLNR* in humans and *Aplnr* in the rat and mouse. *APLNR* encodes a 380-amino acid protein and is located on chromosome 11q12 (O'Dowd *et al*. 1993). In the mouse and rat the genes encode 377-amino acid proteins (Devic *et al*. 1999, Hosoya *et al*. 2000, O'Carroll *et al*. 2000) and are present at the chromosomal locations 2E1 and 3q24 respectively. Human APJ shares 92% amino acid sequence homology with mouse APJ (Devic *et al*. 1999) and 90% homology with rat APJ, and there is 96% homology between rat and mouse APJ (O'Carroll *et al*. 2000), indicating a strong evolutionary conservation of the gene. Furthermore, APJ orthologues are present in a number of species including the African clawed frog, rhesus macaque, cow and zebrafish (Devic *et al*. 1996, Margulies *et al*. 2001, Tucker *et al*. 2007, Schilffarth *et al*. 2009) – the latter has ~50% amino acid homology with that of human APJ. The promoter region of the rat APJ gene is TATA less, but contains a potential CAAT box at bp position −1257 relative to the initiating ATG and a number of activator protein 1 and specificity protein 1 (*Sp1*) motifs (O'Carroll *et al*. 2006). There are two transcriptional start sites at −247 and −210 bp (O'Carroll *et al*. 2006). Although the structure/function of the human APJ gene promoter is not known, two transcript variants (one of which encodes the full-length APJ protein and the other which may be non-coding) have been annotated in the National Center for Biotechnology Information (NCBI) database. The cDNA sequence transposed on the gene sequence (NCBI reference sequence NC_000011.9) reveals no introns interrupting the protein-coding sequence, similar to that found in the rat and mouse. There do not appear to be any APJ subtypes in gene databases such as GenBank and Ensembl.

The protein structure of APJ is typical of a GPCR, containing seven hydrophobic transmembrane domains, with consensus sites for phosphorylation by protein kinase A (PKA), palmitoylation and glycosylation (O'Dowd *et al*. 1993). The N-terminal glycosylation of GPCRs has been implicated in receptor expression, stability, correct folding of the nascent protein and ligand binding (Wheatley & Hawtin 1999). Furthermore, the palmitoylation of the C-terminal tail has been reported to play a role in membrane association and, combined with receptor phosphorylation, these fatty acid modifications can influence the internalisation, dimerisation and ligand binding of a GPCR (Huynh *et al*. 2009). Structural studies on APJ have determined that amino acids in both the N-terminal (e.g. Asp^{23} and Glu^{20}) and C-terminal portions of the receptor are required for internalisation (Zhou *et al*. 2003*a*,*c*, Masri *et al*. 2006).

Gene regulation

The regulation of APJ gene expression has not been extensively characterised to date. At the transcriptional level, the region with the highest rat APJ gene promoter activity is found between −966 and −165 bp (O'Carroll *et al*. 2006). Electrophoretic mobility shift, super-shift and competition assays have indicated that the promoter is under complex regulation by *Sp1*, oestrogen receptor, glucocorticoid receptor and CCAAT enhancer-binding protein γ (*C/EBPγ* (*CEBPG*)) transcription factors, with *Sp1* being implicated as a major regulator of rat APJ gene promoter activity (O'Carroll *et al*. 2006).

A number of single-nucleotide polymorphisms (SNPs) have been reported for *APLNR*. A large study of SNPs has found an association of a SNP (rs9943582 (G/A)) in the 5′ flanking region *Sp1*-binding site of *APLNR* with susceptibility to brain infarction (Hata *et al*. 2007), while the 5′UTR G212A variant of *APLNR* has been reported to be associated with slower heart failure progression in idiopathic dilated cardiomyopathy (Sarzani *et al*. 2007). Additionally, two *APLNR* SNPs, rs7119375 (G/A) and rs10501367 (G/A), in the Han Chinese population (Niu *et al*. 2010) and the G212A polymorphism in Italian patients (Falcone *et al*. 2012) may be associated with hypertension, with two further SNPs (rs2282623 (C/T) and rs746886 (C/T)) being reported to be associated with blood pressure (BP) responses to dietary sodium interventions (Zhao *et al*. 2010).

APJ gene is up-regulated in response to acute and repeated stress (O'Carroll *et al*. 2003), changes that are likely to be glucocorticoid dependent. There is also evidence that the endogenous ligand, apelin, regulates the expression of APJ within the gastrointestinal tract

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(Wang *et al*. 2009), while recently the expression of APJ has been shown to be up-regulated in adipose tissues by insulin (Dray *et al*. 2010).

Apelin

APJ remained an orphan receptor until 1998 when Tatemoto *et al*. (1998) identified a 36-amino acid peptide termed apelin, for **AP**J **e**ndogenous **li**ga**n**d.

Apelin gene and protein structures

The gene encoding human apelin, termed *APLN*, is located on chromosome Xq25–26.1 and possesses one intron within its open reading frame of ~6 kb. In the rat and mouse, the genes are termed *Apln* and are located at chromosomal locations Xq35 and XA3.2 respectively. The core promoter regions of these genes have been identified as −207/−1 and −100/+74 bp in rats and humans respectively (Wang *et al*. 2006). Similar to APJ, a CAAT box, but no TATA box, sequence is present in the rat and human promoter regions (Wang *et al*. 2006). Furthermore, rat and human preproapelin cDNAs do not have a classical Kozak consensus sequence (Kozak 1996) surrounding the initiating methionine codon (Lee *et al*. 2000).

Human and bovine *APLN* cDNA sequences encode a 77-amino acid preproprotein (preproapelin) (Tatemoto *et al*. 1998) containing a hydrophobic rich N-terminal region, likely to be a secretory signal sequence (for a review, see Rapoport (2007)). Bovine, human, rat and mouse preproapelin precursors (Habata *et al*. 1999) have 76–95% homology and appear to exist endogenously as a dimeric protein, as a consequence of disulphide bridges formed between cysteine residues (Lee *et al*. 2005).

There are several mature forms of the apelin peptide. As the sequence of the peptide purified by Tatemoto *et al*. (1998) corresponded to the 36 C-terminal amino acids of the preproapelin protein, it was predicted that apelin-36 would constitute a mature form of the peptide. Additionally, as the C-terminal portion of preproapelin also contained lysine (Lys, K) and arginine (Arg, R) residues, and given their potential as sites for proteolytic cleavage, the existence of apelin-17 and apelin-13 peptides was predicted, along with a pyroglutamylated form of apelin-13 ((Pyr^{1})apelin-13) (Fig. 1). These mature forms of apelin lack cysteine residues and are probably only present in monomeric form. The likely secondary structures of apelin-36 and apelin-13 have been determined in aqueous solution, indicating that both possess an unordered structure (Fan *et al*. 2003). The amino acid sequence homology of the mature apelin-36 peptide is more conserved between species than that of preproapelin, with 86–100% homology between bovine, human, rat and mouse amino acid sequences, while the 23 C-terminal amino acids have 100% homology between species (Habata *et al*. 1999), suggesting an important physiological role.

Although APJ does not bind Ang II (O'Dowd *et al*. 1993), apelin-13 shares a limited homology (four amino acids) with the vasoconstrictive peptide (Lee *et al*. 2000). Moreover, Ang I-converting enzyme 2 (ACE2), which catalyses the C-terminal dipeptide cleavage of Ang I to Ang II, or Ang II to Ang 1–7 (Tipnis *et al*. 2000), also acts on apelin-13 with a high catalytic efficiency, removing the

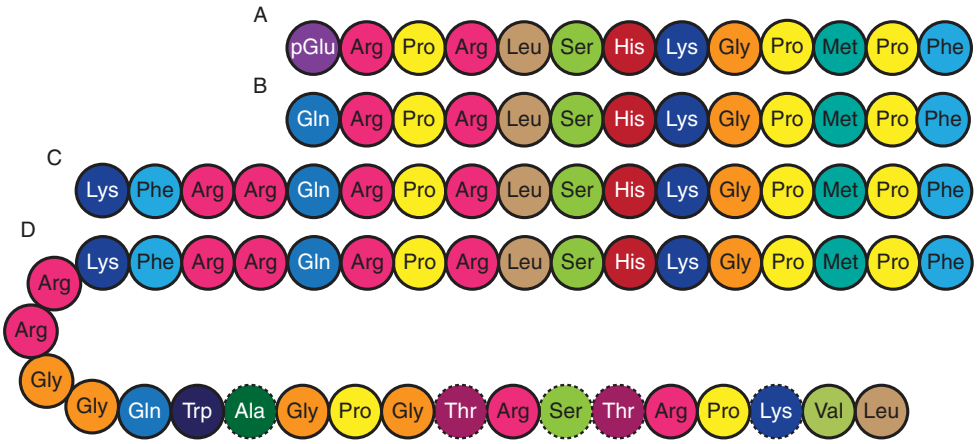

Figure 1
Amino acid sequence of mature rat apelin isoforms. Amino acid sequences of (A) (Pyr^{1})apelin-13, (B) apelin-13, (C) apelin-17 and (D) apelin-36. Black circled residues indicate those identical between human, bovine, rat and mouse.

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C-terminal phenylalanine (Phe, F) residue (Vickers *et al.* 2002). However, this cleavage may not inactivate the peptide, as the apelin isoform K16P, which lacks the terminal Phe, while ineffective at inducing receptor internalisation or regulating blood pressure (BP) (effects associated with the full peptide), still binds to APJ and inhibits forskolin-stimulated cAMP production (El Messari *et al.* 2004).

Gene regulation

The regulation of apelin gene expression is mediated by several effectors, with the involvement of a number of transcription factors. A SNP study has reported a probable role for *Sp1* in the regulation of the expression of *APLN* (Hata *et al.* 2007). Additionally, the cytokine tumour necrosis factor-α (TNFα) has been reported to induce the expression of apelin via phosphatidylinositol 3-kinase (PI3K), c-Jun N-terminal kinase (JNK) and MEK1/2 in adipocytes (Daviaud *et al.* 2006). Furthermore, in studies using lipopolysaccharide (LPS) and cytokines to elicit an immune response in rodents, the expression of apelin mRNA has been reported to be up-regulated, involving the Jak/Stat pathway, while studies using chromatin immunoprecipitation (ChIP) have revealed the binding of Stat3 (Han *et al.* 2008). Mutagenesis, electrophoretic mobility shift assays and ChIP have also been used *in vitro* to determine whether upstream stimulatory factors 1 and 2 (USF1 and USF2) are involved in the expression of apelin in the breast, while *in vitro* ChIP analyses have shown that endogenous USF up-regulates the expression of apelin in the lactating rat breast (Wang *et al.* 2006). Putative hypoxia response elements (HREs) are present in the apelin gene promoter and intron sequence of various species *in silico* (Cox *et al.* 2006), and hypoxia has subsequently been found to up-regulate the expression of apelin in cardiac myocytes (Ronkainen *et al.* 2007), and hypoxia-inducible factor 1α (HIF1α) has been reported to induce the expression of apelin in adipocytes (Glassford *et al.* 2007). Additional studies have determined that hypoxia-inducible apelin up-regulation is mediated by HIF1α at a HRE within the *APLN* intron (conserved in rat and mouse apelin genes) between +813 and +826 bp (Eyries *et al.* 2008), indicating that apelin may play a role in the homeostatic response to low oxygen levels. Insulin has also been shown to increase the expression of apelin in human and mouse adipocytes, via PI3K, protein kinase C (PKC), mitogen-activated and ERK kinase (MAPK) 1 (Boucher *et al.* 2005) and HIF1α (Glassford *et al.* 2007), while aldosterone has been shown to decrease the expression of apelin in 3T3-L1 adipocytes via the p38 MAPK pathway (Jiang *et al.* 2013). Furthermore, studies on white adipocytes have found that the peroxisome proliferator-activated receptor γ co-activator 1α also up-regulates the expression of apelin, possibly indicating that apelin plays a role in energy metabolism (Mazzucotelli *et al.* 2008), while recently in diabetic rats, ghrelin has been reported to reduce apelin mRNA synthesis and release into the lumen (Coskun *et al.* 2013).

APJ distribution

Although it is clear that APJ and apelin mRNAs and proteins are widely distributed in the CNS and peripheral tissues, whether the levels of mRNAs present in most of the regions of the brain and tissues are functionally relevant is not yet known.

Human distribution

Early studies of the expression of APJ mRNA by northern blot and quantitative PCR (qPCR) analyses have revealed strongest signals in the human caudate nucleus, corpus callosum, hippocampus, substantia nigra, subthalamic nucleus, medulla and spinal cord (Matsumoto *et al.* 1996, Edinger *et al.* 1998, Medhurst *et al.* 2003). Recently, the expression of APJ mRNA has also been demonstrated in the human cortex and hippocampus using a sensitive GPCR gene array profiling method – interestingly, APJ transcripts have also been detected in human bone marrow stromal cell lines (Hansen *et al.* 2007). Transcriptomic analysis of multiple brain regions of human donors has revealed a widespread central expression of APJ mRNA with high levels in samples including the hippocampus (e.g. CA4 region), habenular nuclei, paraventricular nucleus (PVN) of the thalamus, supraoptic nucleus (SON) of the hypothalamus and various hindbrain structures (see Allen Brain Atlas: www.brain-map.org). The salient feature of these studies is that APJ has been reported to have a widespread central distribution; although the function of APJ in the majority of brain regions is unknown, foremost among those regions probably important from a functional perspective include the PVN and SON of the hypothalamus.

In the periphery, the expression of human APJ mRNA was originally reported to be strongest in the spleen, with less expression being reported for the small intestine, colonic mucosa and ovary (Edinger *et al.* 1998). A broader qPCR study has also reported strongest expression in the spleen, with high levels also being reported to be present

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in the placenta and weaker levels in the lung, stomach and intestine (Medhurst *et al.* 2003). (Pyr[1])apelin-13-binding sites can be found within the media and intimal layers of muscular arteries and large elastic arteries and veins, while in the lung, apelin-binding sites have a predominantly vascular localisation (Katugampola *et al.* 2001). Furthermore, APJ distribution in cardiovascular tissues, as demonstrated by immunohistochemistry (IHC), indicates APJ to be present in ventricular cardiomyocytes, vascular smooth muscle cells (VSMCs) and intramyocardial endothelial cells (Kleinz *et al.* 2005).

Rat distribution

The expression of APJ mRNA in the rat CNS has been mapped by a variety of techniques including northern blotting, *in situ* hybridisation histochemistry (ISHH), IHC, receptor autoradiography and qPCR. At a detailed anatomical resolution using ISHH, discrete but significant expression of APJ mRNA can be found throughout the rat brain (for comprehensive details, see De Mota *et al.* (2000), Hosoya *et al.* (2000), Lee *et al.* (2000), O'Carroll *et al.* (2000), Medhurst *et al.* (2003) and Xia & Krukoff (2003)), particularly in the PVN and SON where APJ mRNA is present in arginine–vasopressin (VP)-expressing cells (Reaux *et al.* 2001, O'Carroll & Lolait 2003). In many of these regions of the brain it has been confirmed that the APJ gene is translated into immunoreactive protein – interestingly APJ immunoreactivity can be found in both neuronal and glial cell populations (Medhurst *et al.* 2003). (Pyr[1])apelin-13-binding sites can be found in the molecular layer of the cerebellum, the basal surface of the hypothalamic diencephalon and the PVN (Katugampola *et al.* 2001, Hazell *et al.* 2012). In the pituitary, conflicting patterns of APJ mRNA expression have been reported, with labelling of the anterior and intermediate lobes being described in one study (De Mota *et al.* 2000), as opposed to a moderately strong signal in the anterior lobe alone in another investigation (O'Carroll *et al.* 2000). In contrast, APJ immunoreactivity has been found in the nerve terminals of the rat posterior pituitary (Tobin *et al.* 2008).

RT-PCR studies have reported high levels of APJ in the lung and heart, with lower levels being reported for the placenta, thyroid gland, skeletal muscle, costal cartilage, ovary, uterus and adipose tissues (Hosoya *et al.* 2000, Medhurst *et al.* 2003). More detailed ISHH studies have shown prominent cellular labelling of rat APJ mRNA in the parenchyma of the lung, heart, a subpopulation of glomeruli in the kidney and in the thecal cell layer and corpora lutea in the ovary (O'Carroll *et al.* 2000). In addition, (Pyr[1])apelin-13-binding sites are present in the lung and heart and, to a lesser degree, in the kidney cortex (Katugampola *et al.* 2001). Structures showing the strongest expression of APJ and apelin genes in the rat are shown in Fig. 2.

Mouse distribution

Negligible levels of APJ expression can be found in the whole brain, cerebellum, hypothalamus, hippocampus and olfactory bulb (Medhurst *et al.* 2003, Regard *et al.* 2008); however, recently, a detailed ISHH characterisation of APJ distribution in the mouse has revealed a very restricted localisation in the CNS, with strong hybridisation specifically in the PVN and SON and also in the anterior pituitary, with marginally lower levels in the posterior pituitary (Pope *et al.* 2012). This suggests a species difference in the central and pituitary distributions of APJ mRNA between mouse and rat. While the significance of this is not known, it may reflect a more extensive role for apelin in mouse pituitary function. Additionally, strong hybridisation can be found in the lung, heart, adrenal cortex, renal medulla, ovary and uterus. These findings confirm the findings of previous qPCR studies, with lower levels also being reported for the thyroid, kidney, spleen, pancreas, skeletal muscle and adipose tissues (Medhurst *et al.* 2003, Regard *et al.* 2008). High levels of APJ-binding sites can be found in the anterior pituitary, while lower levels can be observed in the posterior pituitary, PVN and SON. In the periphery, strong receptor binding can be observed in those tissues that exhibit strong ISHH signals, indicating a good correlation between receptor transcription and translation (Pope *et al.* 2012). Recently, in the mouse embryo (E9.5–10.5) cardiovascular system, APJ mRNA has been shown to predominate in the endothelial layers of arteries and veins and in the endocardial layer of the heart (Kang *et al.* 2013), while APJ protein has been shown to be present in mouse hepatocytes and hepatic tissue (Chu *et al.* 2013).

Apelin distribution

Human distribution

Preproapelin transcripts are present in the human CNS, with highest levels being present in the thalamus and frontal cortex and lower levels in the hypothalamus, midbrain, caudate, hippocampus and basal forebrain (Lee *et al.* 2000). A strong signal can also be observed in the

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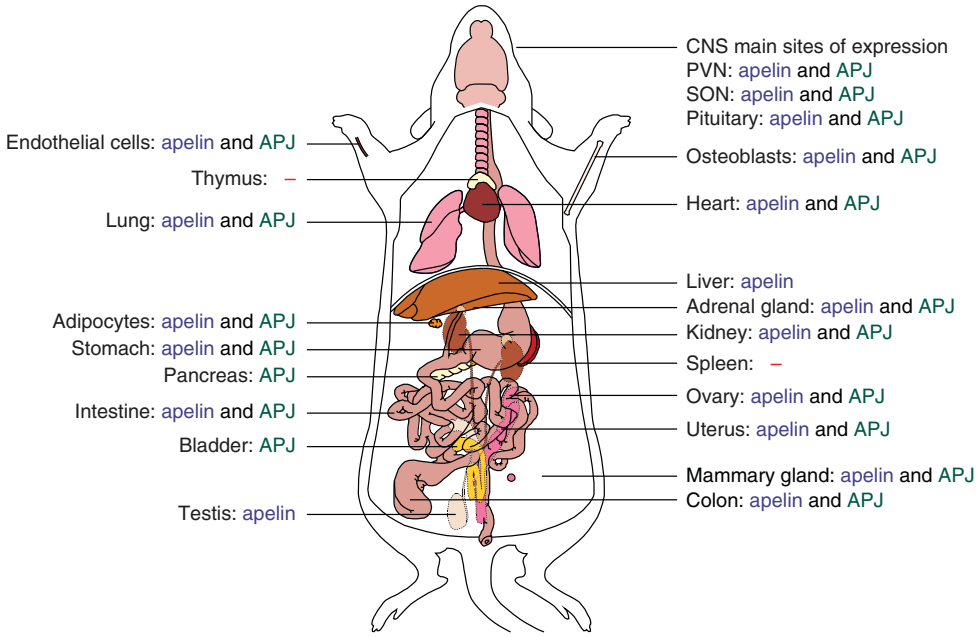

Figure 2

Apelin and APJ gene expression in rat tissues. Gene expression of apelin/APJ in the rat (see text for details). There have been fewer studies demonstrating the expression of apelin and/or APJ protein in the rat (or other species including humans) or determining whether apelin and APJ are localised in different cell populations or co-expressed within a given tissue. Examples of rat tissues where both apelin and/or APJ gene and immunoreactive protein/binding sites have been found (and may be functionally relevant) include the brain, pituitary, lung, heart, gastrointestinal tract, liver and kidney (see text and references Hus-Citharel *et al.* (2008), Wang *et al.* (2009), Zeng *et al.* (2009) and Piairo *et al.* (2011) for details).

spinal cord and pituitary gland, with lower levels being observed in many regions of the brain, including the amygdala, corpus callosum and substantia nigra (Medhurst *et al.* 2003).

In human peripheral tissues, strong levels of apelin expression can be found in the placenta, with lower levels being found in the heart, lung and kidney (Medhurst *et al.* 2003). Immunoreactive apelin is present in the vascular endothelial cells of large conduit vessels, such as the coronary artery and saphenous vein, blood vessels of the kidney and adrenal gland, and vascular and endocardial endothelial cells of the atria and ventricles (Kleinz & Davenport 2004).

Rat distribution

Apelin mRNA has a more abundant and widespread expression than that of APJ mRNA within the rat CNS. High levels of preproapelin transcripts can be found in several regions including the cerebral cortex, claustrum, anterior and posterior cingulate, retrosplenial area and thalamic nuclei (see Lee *et al.* (2000)). Reaux *et al.* (2002) have described an extensive study of apelin protein distribution in the rat brain, using primary antibodies directed against apelin-17. Further studies focusing on the PVN, SON, median eminence and dorsolateral accessory magnocellular nucleus have shown strong labelling for apelin and co-localisation with VP (Brailoiu *et al.* 2002, De Mota *et al.* 2004, Reaux-Le Goazigo *et al.* 2004) and with oxytocin (OT; Brailoiu *et al.* 2002) in the SON and PVN. Apelin is also co-localised with adrenocorticotrophin (ACTH) in corticotrophs and to a lesser extent with growth hormone in somatotropes in the anterior pituitary (Reaux-Le Goazigo *et al.* 2007).

In rat peripheral tissues, apelin levels are high in the mammary gland, ovary, heart and adipose tissues (Habata *et al.* 1999), as well as in the kidney, adrenal gland, intestine, skeletal muscle, vas deferens, testis and uterus (Lee *et al.* 2000, O'Carroll *et al.* 2000, Kawamata *et al.* 2001, Medhurst *et al.* 2003). The expression of apelin mRNA is increased in the mammary gland during pregnancy and lactation, reaching a maximum level around parturition, and the presence of apelin in rat, bovine and human milk has been reported (Habata *et al.* 1999).

There appear to be discrepancies in the distribution of rat preproapelin mRNA and that of apelin immunoreactivity. High levels of preproapelin mRNA have been reported to be present in the hippocampus and cerebral

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cortex where no apelin immunoreactivity is detected, whereas in some cerebral regions, e.g. the thalamus, the bed nucleus of the stria terminalis and the median eminence, the opposite has been observed (Lee *et al.* 2000, Reaux *et al.* 2002). This may be due to the inability of the antibody used in these studies to detect the endogenous apelin isoforms in these regions or because the levels of preproapelin mRNA in these regions are too low to allow detection by ISHH. Additionally, there are other regions, e.g. olfactory regions, piriform and entorhinal cortex and dentate gyrus, where APJ mRNA is expressed, but where there is no apelin immunostaining. This localisation may imply that APJ synthesised in these cell bodies is transported to axon terminals in other regions of the brain where the ligand is expressed and where APJ may be functional. Conversely, APJ mRNA is not expressed in the rat testis where moderate levels of apelin mRNA are present. It is possible that the low levels of (possibly rapidly turning-over) APJ mRNA are below the detection threshold of ISHH or that, assuming that apelin of testicular origin is not redundant, the peptide is binding to a unknown, perhaps related receptor. We cannot exclude the possibility that testicular APJ is also developmentally regulated since the expression of APJ mRNA appears to be higher in infant peripheral tissues than in adult peripheral tissues (Hosoya *et al.* 2000).

Mouse distribution

A single qPCR distribution study has been conducted on the expression of preproapelin in the mouse where the highest signal has been found in the whole brain, with a moderate signal in the heart, kidney and lung, and a low signal in the testis, spleen, ovary and muscle (Medhurst *et al.* 2003).

APJ signalling

APJ binds numerous apelin isoforms and signals through various G proteins to a variety of signalling pathways to culminate in different patterns of activation and desensitisation that may be tissue- and cell type-specific. Recently, APJ has also been reported to heterodimerise with other GPCRs and to signal in the absence of an endogenous ligand.

Endogenous apelin isoforms

The initial synthesis of apelin isoforms included the mature apelin-36, as well as the C-terminal fragments of apelin-17 and apelin-13 and a pyroglutamylated isoform (Pyr^1)apelin-13 (Tatemoto *et al.* 1998). These isoforms were predicted from the potential basic amino acid cleavage sites present in the primary structure of preproapelin (Habata *et al.* 1999). There appear to be three active apelin isoforms in bovine milk and five in bovine colostrum, although the precise isoforms were not identified in the study of Habata *et al.* (1999). Subsequently, a study carried out using gel filtration chromatography revealed the presence of apelin-36 and (Pyr^1)apelin-13 in bovine colostrum (Hosoya *et al.* 2000). Apelin-36 appears to be the most prevalent isoform in the lung, testis and uterus, while apelin-36 and (Pyr^1)apelin-13 predominate in the mammary gland (Kawamata *et al.* 2001), and a single short form of apelin corresponding to (Pyr^1)apelin-13 is present in the hypothalamus (De Mota *et al.* 2004). The latter is also the predominant form in rat whole brain and plasma (De Mota *et al.* 2004) and in human cardiac tissue (Maguire *et al.* 2009), while apelin-17 is present at a lower level in the rat hypothalamus and plasma (De Mota *et al.* 2004). In human plasma, all three isoforms, apelin-13, (Pyr^1)apelin-13 and apelin-17, are present (Reaux *et al.* 2002, Azizi *et al.* 2008). In Chinese hamster ovary (CHO) cells engineered to express the cloned human APJ, apelin-13 binds with high affinity to and associates with APJ more efficiently than apelin-36 and rapidly dissociates from APJ (Hosoya *et al.* 2000). On the other hand, apelin-36 has a higher affinity for APJ in this cell line (K_d=6.3 pM (apelin-36) vs K_d=22.3 pM (apelin-13) (Hosoya *et al.* 2000)) and it is more difficult to dissociate it from the receptor (Kawamata *et al.* 2001).

The C-terminal region of the apelin peptide may be responsible for its overall biological activity. N-terminal deletions of apelin-17 reveal that the 12 C-terminal amino acids may be the core requirements for the internalisation and biological potency of APJ (El Messari *et al.* 2004). Apelin-17 induces the internalisation of APJ, which decreases with every N-terminal deletion to apelin-12, while the deletion of the terminal F amino acid results in a peptide that no longer internalises APJ or affects arterial BP. The N-terminal residues within the RPRL motif (residues 2–5) of apelin-13 are critical for functional potency (Medhurst *et al.* 2003), and the C-terminal sequence KGPM (residues 8–11) is important for binding activity and for internalisation (Fan *et al.* 2003). In contrast, the five N-terminal and two C-terminal amino acids of apelin-17 are not required for binding of the peptide to APJ or activation of receptor signalling (e.g. cAMP production) (El Messari *et al.* 2004). Although this

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may indicate a possible dissociation between the conformational states of the receptor responsible for receptor signalling and internalisation, it is also possible that different ligand isoforms may induce differential receptor trafficking and signalling. These studies provide information on the structural importance of key apelin residues critical for efficient binding, activity and internalisation, which have proved significant in the design and synthesis of apelin analogues.

Apelin-13 (F13A): an APJ antagonist

The first structural studies on apelin activity involved the replacement of the C-terminal F residue of (Pyr^1)-apelin-13 with alanine (Ala, A), an analogue termed F13A. Unlike (Pyr^1)apelin-13, F13A is ineffective at inhibiting forskolin-stimulated cAMP accumulation in CHO cells transfected with rat APJ (De Mota *et al.* 2000) and antagonises apelin-13-induced decreases in BP (Lee *et al.* 2005). Additionally, F13A exhibits an approximately eightfold lower potency than (Pyr^1)apelin-13 in intracellular calcium mobilisation; an approximately threefold lower inhibition of cAMP accumulation; and between 2- and 14-fold lower receptor binding efficiency for human APJ expressed in a variety of cell lines (Medhurst *et al.* 2003). However, for human APJ *in vitro*, F13A exhibits binding, calcium mobilisation and internalisation responses comparable to those of (Pyr^1)apelin-13 (Fan *et al.* 2003), while in human cardiac tissue, F13A competes for binding with [^{125}I]-(Pyr^1)apelin-13 in the left ventricle and effectively constricts endothelium-denuded saphenous vein (Pitkin *et al.* 2009). Therefore, although this mutant peptide has been reported to act as an antagonist, it may in fact act as a competitive agonist at APJ.

Cyclic and other apelin analogues

The use of cyclic analogues is a proven method of studying conformational configuration as these analogues restrict the structural backbone of the peptide and confine their secondary structure. In a study carried out to understand the molecular features of apelin required for a signalling response from APJ, three cyclic analogues of apelin-12 (C1, C3 and C4) have proved to be novel APJ agonists, but are less potent than (Pyr^1)apelin-13 in the inhibition of cAMP accumulation and in the phosphorylation of protein kinase B (Akt) and ERK1/2 (Hamada *et al.* 2008), while the use of a bivalent ligand approach incorporating a β-turn within the RPRL region of apelin, which is critical in APJ ligand recognition, has resulted in the identification of a competitive antagonist at APJ (Macaluso *et al.* 2011).

More recently, small-molecule agonists and antagonists of APJ have been identified – the non-peptidic E339-3D6 is a partial agonist of APJ in the inhibition of cAMP production, possesses the ability to act as a vasorelaxant of the rat aorta *ex vivo*, and is a full agonist in terms of APJ internalisation (Iturrioz *et al.* 2010); the CXCR4 small-molecule ALX40-4C (*N*-α-acetyl-nona-D-arginine amide), which inhibits CXCR4 interactions with human immunodeficiency virus (HIV)-1, acts as an antagonist of APJ internalisation (Zhou *et al.* 2003*b*) and ML221, a kojic acid-based small molecule, antagonises apelin-13-mediated activation of APJ in the inhibition of cAMP production (Maloney *et al.* 2012). These agonists and antagonists may provide new tools to explore the function of the apelinergic system and deliver vital information that could lead to potential pharmaceutical therapies targeted at APJ.

Receptor oligomers

Like many other members of the GPCR superfamily, APJ may heterodimerise with other GPCRs to modulate established signal transduction pathways in cultured cells. Using co-immunoprecipitation and fluorescence resonance energy transfer studies, APJ was first reported to dimerise with the Ang II receptor AT_1, which results in an inhibitory effect of apelin on Ang II signalling and on an Ang II-mediated model of atherosclerosis (Chun *et al.* 2008). A further study has established that APJ heterodimerises with the κ-opioid receptor (KOR; Li *et al.* 2012). Treatment with apelin-13 or the KOR ligand dynorphin A induces higher levels of ERK1/2 activation in HEK293 cells stably transfected with APJ and KOR than in HEK293 cells transfected with either APJ or KOR alone. This activation is mediated by increased PKC and decreased PKA activities and results in an increase in cell proliferation (Li *et al.* 2012). The possible heterodimerisation of the native APJ with other co-expressed GPCRs or other signalling proteins *in vivo* may have possible important consequences for understanding APJ function and in the rational design of APJ therapeutics.

Mechanical stretch

APJ is expressed in cardiomyocytes of human and rat hearts (Kleinz *et al.* 2005), and apelin and APJ have been suggested to have roles in cardiac pathophysiology (see below). Apelin and APJ mRNA levels are reduced in neonatal rat ventricular myocytes subjected to mechanical

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stretch, while apelin gene expression has been shown to be reduced in two *in vivo* models of chronic ventricular pressure overload (Szokodi *et al*. 2002). Recently, however, it has been shown that APJ prompts myocardial hypertrophy in response to mechanical stretch by an apelin-independent, β-arrestin-dependent mechanism. This stretch-mediated hypertrophy is diminished by apelin treatment (Scimia *et al*. 2012). Furthermore, the signalling response induced by stretch is pertussis toxin (PTX)-insensitive and G protein-independent, unlike the response observed with apelin. Additionally, stretch diminishes apelin signalling by decreasing G protein activation and increasing β-arrestin recruitment (Scimia *et al*. 2012). Thus, it appears that both apelin and stretch activate APJ to affect cardiac hypertrophy.

G protein-coupling of APJ

APJ was originally hypothesised to couple to $G_{i/o}$ based on initial experiments showing that forskolin-stimulated cAMP production is suppressed by apelin-13 (Tatemoto *et al*. 1998). This coupling hypothesis was strengthened by the inability of (Pyr^1)apelin-13 and apelin-36 to generate Ca^{2+} mobilisation or to release arachidonic acid metabolites into CHO cells stably expressing human APJ (Habata *et al*. 1999). However, both these analogues increase intracellular Ca^{2+} levels in NT2N neurones (Choe *et al*. 2000) and in HEK293 cells stably expressing human APJ (Zhou *et al*. 2003*a*). The coupling of APJ to $G_{i/o}$ has firmly been established by studies demonstrating PTX abrogation of apelin-13- and apelin-36-induced actions in assays measuring extracellular acidification rates (Hosoya *et al*. 2000) and phosphorylation of ERK and p70S6 kinase (Masri *et al*. 2002, 2004). Mouse APJ couples preferentially to $G\alpha_{i1}$ and $G\alpha_{i2}$, but not to $G\alpha_{i3}$, in inhibition of adenylate cyclase and phosphorylation of ERK1/2 (Masri *et al*. 2006). Similarly, human APJ activates ERK1/2 through a $G\alpha_{i2}$-dependent pathway (Bai *et al*. 2008). Moreover, the activation of ERK1/2 by apelin is mediated via PKC in HEK293 cells expressing mouse APJ, indicative of coupling to either G_o or $G_{q/11}$. However, the positive inotropic effect of apelin in rats *in vivo* is only partially abrogated by PTX and by PKC inhibitors. These effects suggest possible PTX-sensitive and -insensitive signalling pathways to be linked to this receptor and indicate that some of the actions of APJ could be mediated by $G_{i/o}$ and/or $G_{q/11}$ coupling (Szokodi *et al*. 2002). Recently, in human umbilical vein endothelial cells (HUVECs), APJ has been shown to activate $G\alpha_{13}$, resulting in the cytoplasmic translocation of class II histone deacetylases HDAC4 and HDAC5 and the activation of the transcription factor *MEF2*, in an apelin-independent manner (Kang *et al*. 2013). Therefore, these studies suggest that apelin signalling may exhibit 'functional selectivity' or 'biased signalling'.

Apelin, signalling through APJ, can trigger numerous intracellular signalling cascades whose final targets are often transcription factors. It is not yet clear through which transcription factors or other cellular effectors many of the actions of apelin are transduced – what is clear is that apelin signals through a diverse set of intermediaries. An overview of the signalling pathways potentially relevant to APJ signalling is shown in Fig. 3.

APJ signalling to ERK1/2

Both apelin-13 and apelin-36 activate the phosphorylation of ERK1/2 in CHO cells stably expressing mouse APJ (Masri *et al*. 2006). The activation of ERK1/2 is time- and dose-dependent and is mediated via a PTX-sensitive G_i-protein, yet independent of the βγ-complex, in a Ras-independent and a PKC- and MEK-dependent pathway (Masri *et al*. 2002). Similar studies using exogenously transfected human APJ in HEK293 cells have described the activation of ERK1/2 via $G\alpha_{i2}$ by apelin, with no activation of p38 MAPK (Bai *et al*. 2008). Studies on hippocampal cultures expressing APJ, and on mouse hearts, have also shown that apelin mediates the activation of ERK1/2 (O'Donnell *et al*. 2007, Simpkin *et al*. 2007). However, apelin does not activate ERK1/2 in human osteoblasts and dose dependently decreases the phosphorylation of ERK1/2 in mouse cortical neurones, cells that endogenously express APJ (Xie *et al*. 2006, Zeng *et al*. 2010).

The stimulation of some GPCRs by agonists leads to the activation of metalloprotease isoenzymes, members of the a disintegrin and metalloproteinase family of peptidase proteins, to produce ligands that activate the epidermal growth factor receptor (EGFR) and subsequently ERK1/2. The GPCR and EGF transactivation pathways are cell specific and depend on various parameters such as the G protein type, receptor type and cellular network (Liebmann 2011). In VSMCs, the activation of ERK1/2 by Ang II via AT_I is partially dependent on the transactivation of EGFR (Eguchi *et al*. 1998); however, this pathway is not activated by (Pyr^1)apelin-13 in HEK293 cells expressing rat or mouse APJ, suggesting that APJ does not activate ERK1/2 via the transactivation of EGFR in these cells (A-M O'Carroll, S Tilve & GR Pope, 2010, unpublished observations).

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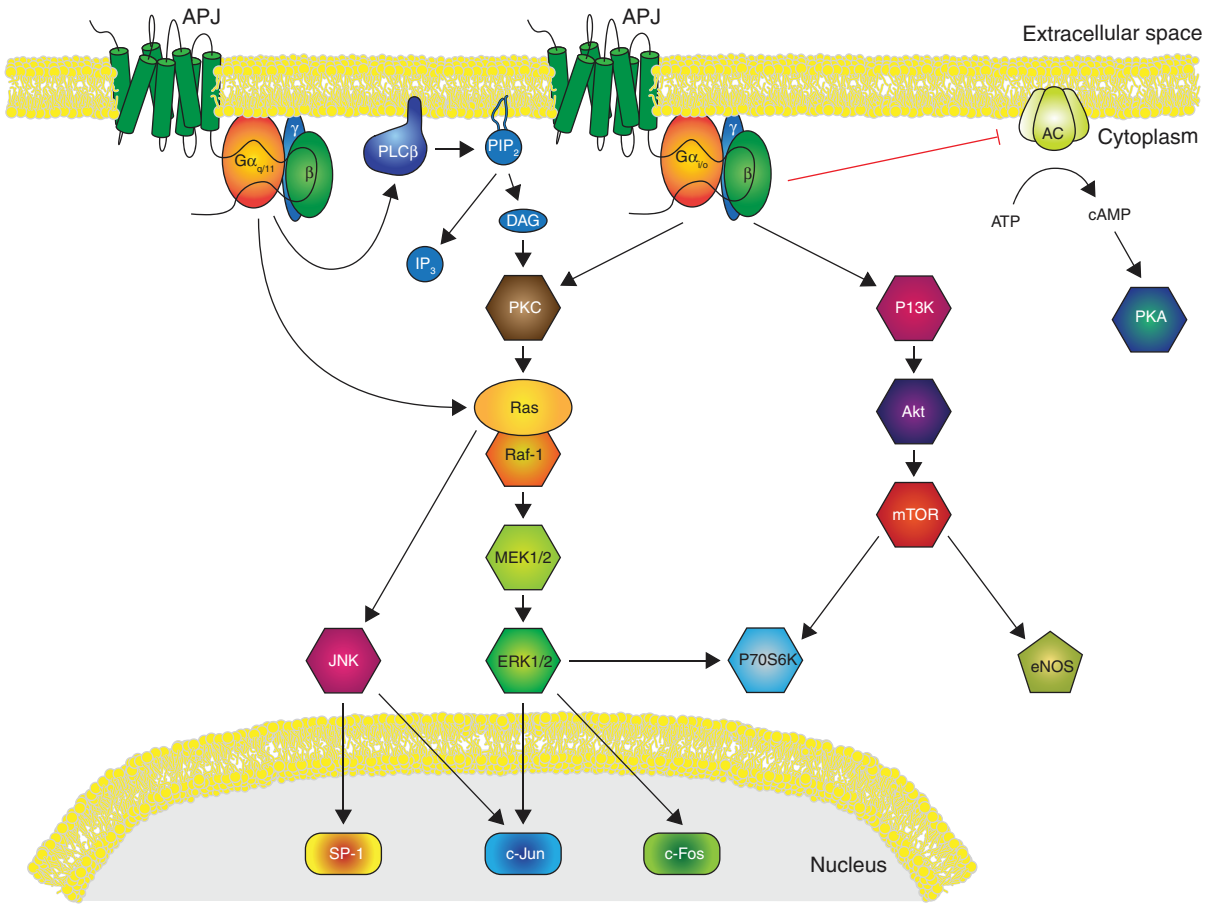

Figure 3
Overview of APJ signalling pathways. Schematic diagram of APJ signalling pathways. Coupling to $G_{q/11}$ stimulates PLC-β signalling, including the hydrolysis of phosphatidylinositol 4,5-biphosphate (PIP_2) to IP_3 and diacyl glycerol (DAG). DAG subsequently activates PKC, which is an activator of the small G-protein, Ras. Ras then either activates a cascade leading to the activation of JNK, and the transcription factors *SP1* and *c-Jun* or the MAPK cascade of Raf-1, MAPK-/ERK kinase (MEK1/2) and ERK1/2. ERK1/2 have a variety of substrates including numerous transcription factors (e.g. *c-Jun* and *c-fos*) and other kinases (e.g. p70S6K). $G_{q/11}$ also signals independently of PKC, but still via Ras and the MAPK cascade. $G_{i/o}$ stimulates the MAPK cascade via PKC, and it can also activate phosphoinositide 3-kinase (PI3K) with the subsequent activation of Akt and mammalian target of rapamycin (mTOR), leading to the activation of both p70S6K and endothelial nitric oxide synthase (eNOS). Furthermore, $G_{i/o}$ signalling inhibits adenylate cyclase (AC) activity. In contrast, G_s activates AC, increasing cAMP synthesis from ATP, leading to the activation of protein kinase A (PKA). Thin black arrows indicate activation pathways and the red blunted arrow indicates inhibition.

Apelin and the PI3K/Akt pathway

The phosphorylation, and thus activation, of Akt has been shown to be a downstream effector of apelin signalling; this was first shown to occur via a PTX-sensitive G-protein and PKC (Masri *et al.* 2004). Apelin-13- and apelin-36-mediated Akt phosphorylation in CHO cells expressing mouse APJ takes place via coupling to $G\alpha_{i1}$ or $G\alpha_{i2}$. Apelin also activates Akt in HUVECs (Masri *et al.* 2006) and in osteoblasts, where it has proliferative and anti-apoptotic effects (Xie *et al.* 2006, 2007, Tang *et al.* 2007). Additionally, apelin activates Akt in rat hippocampal neuronal cultures, suggesting a neuroprotective role (O'Donnell *et al.* 2007), while in mouse cortical neurones, the neuroprotective action of apelin is blocked by the PI3K inhibitor wortmannin, implicating the PI3K/Akt pathway in this process (Zeng *et al.* 2010). APJ signalling via PI3K/Akt is also involved in proliferation and anti-apoptotic actions in rat and human VSMCs respectively (Cui *et al.* 2010, Liu *et al.* 2010). Studies carried out *in vivo* have shown the involvement of apelin in cardioprotection via Akt-mediated signalling (Simpkin *et al.* 2007); however, the protective effect of apelin in ischaemia/reperfusion may be independent of Akt signalling (Kleinz & Baxter 2008).

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Apelin-induced activation of p70S6K

Apelin induces the dual phosphorylation of the S6 ribosomal protein kinase (p70S6K) in HUVECs, where apelin promotes cell proliferation via PTX-sensitive, ERK1/2-, mammalian target of rapamycin (mTOR)-, and Akt-dependent intracellular cascades (Masri *et al.* 2004). In pluripotent embryonic stem cells, apelin induces the phosphorylation (but not full activation) of p70S6K, via the upstream activation of ERK1/2 (D'Aniello *et al.* 2009). Although in one study of hypoxia apelin has been shown to induce the phosphorylation of p70S6K via mTOR in mouse embryonic endothelial cells (Eyries *et al.* 2008), the protective action of apelin in the rat ischaemic heart does not appear to be mediated by p70S6K (Kleinz & Baxter 2008).

APJ signalling via nitric oxide synthase

APJ signalling via nitric oxide synthase (NOS) was first reported in anaesthetised rats, where the hypotensive action of apelin is abrogated by the NOS inhibitor L-N^G-nitroarginine methyl ester (L-NAME; Tatemoto *et al.* 2001). Similar findings have been observed in mice, where apelin-induced phosphorylation of endothelial NOS (eNOS, NOS3) was observed in isolated mouse endothelial cells (Ishida *et al.* 2004). In the isolated rat aorta, apelin stimulates the transport of L-arginine and enhances the activity of eNOS to stimulate the production of nitric oxide (Jia *et al.* 2007), while post-infarct treatment of rats with (Pyr^1)apelin-13 significantly increases serum nitric oxide levels (Azizi *et al.* 2013). Apelin has also been implicated in signalling via NOS in the aortic ring of diabetic mice and in the control of glucose metabolism in mice, as validated by studies carried out with a NOS inhibitor and eNOS knockout (KO) mice (Zhong *et al.* 2007, Duparc *et al.* 2011). However, the cardioprotective role of apelin in mice does not appear to be mediated via eNOS activity (Simpkin *et al.* 2007). Studies in humans have shown that the relaxation of splanchnic arteries by apelin-13 is mediated in part by nitric oxide (Salcedo *et al.* 2007) and that, *in vivo*, apelin induces the vasodilation of peripheral resistance vessels via a nitric oxide mechanism (Japp *et al.* 2008).

Involvement of reactive oxygen species in APJ signalling

Increased cellular levels of reactive oxygen species (ROS), a byproduct of the mitochondrial aerobic respiratory chain or generated via the NAPDH oxidase complex, are widely known as the cause of oxidative stress that is associated with cell death (apoptosis and/or necrosis) and some common human cardiovascular (e.g. atherosclerosis and ischaemia/reperfusion injury) and neurodegenerative (e.g. Parkinson's and Alzheimer's) diseases (Valko *et al.* 2007). Intracellular ROS are also implicated in the normal signalling of many GPCRs, whereby molecular species such as superoxide (O_2^-) and hydrogen peroxide generated following the activation of GPCRs target a number of signalling cascades including protein tyrosine kinases, PKC, Ca^{2+} channels, MAP kinases and immediate early genes such as *Egr1* (e.g. see Bae *et al.* (2011) for a review). APJ signalling alters intracellular ROS, stimulating myocardial catalase generation and inhibiting hydrogen peroxide generation, to regulate cardiomyocyte hypertrophy (Foussal *et al.* 2010), while long-term post-infarct treatment with (Pyr^1)apelin-13 reduces ROS injury (Azizi *et al.* 2013), thus exhibiting cardioprotective activity. Additionally, apelin prevents neuronal apoptosis in mouse cortical neurones by the reduction of ROS generation and activation of Akt (Zeng *et al.* 2010), while the chronotropic effect of apelin-13 in the rostral ventrolateral medulla (RVLM) appears to be mediated by NAPDH oxidase-derived superoxide production (Yao *et al.* 2011).

Biological actions of the apelinergic system

Although progress has been made in recent years in clarifying the physiological significance of apelin/APJ, much remains to be discovered about the expression of the apelinergic system and precisely how it affects numerous physiological functions. Since the discovery of the apelin ligand, both apelin and APJ have been implicated as key regulators of central and peripheral responses to multiple homeostatic perturbations. These include playing pivotal roles in the regulation of cardiovascular function, angiogenesis, fluid homeostasis and energy metabolism and acting as neuroendocrine modulators of the HPA axis responses to stress. It is becoming apparent that the apelinergic system may play a pathophysiological role within many of these regulatory systems.

The central mRNA expression of preproapelin in regions of the hippocampus, hypothalamus, thalamus and midbrain shares a distribution pattern, as shown by ISHH, similar to that of angiotensinogen (Ang II precursor) (Lee *et al.* 2000). Ang II is part of the rennin–angiotensin system (RAS), which controls extracellular fluid volume and arterial vasoconstriction, thereby regulating mean arterial blood pressure (MABP). The central actions of the RAS include the regulation of drinking behaviour, salt

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appetite and VP secretion (Marc & Llorens-Cortes 2011). Importantly, the RAS plays a critical role in the pathogenesis of heart failure (Kim & Iwao 2000). Interestingly, apelin exerts many physiological effects that appear to oppose those exerted by Ang II (Tatemoto *et al*. 2001, Chen *et al*. 2003, Cheng *et al*. 2003, O'Carroll *et al*. 2003, De Mota *et al*. 2004, Ishida *et al*. 2004, Lee *et al*. 2006). More recently, apelin has been shown to block many Ang II-initiated processes, perhaps partly by dimerisation between APJ and AT_1 (Chun *et al*. 2008).

Cardiovascular roles of apelin/APJ

It is clear that apelin has both peripheral and central cardiovascular effects. However, experiments carried out in animal models have yielded conflicting results about the role of peripheral apelin in the regulation of vascular tone, with both pressor and depressor responses being described (Lee *et al*. 2000, 2005, Ishida *et al*. 2004). In anaesthetised intact rats, the overall effect of peripherally administered apelin is the reduction of MABP (Lee *et al*. 2000, Reaux *et al*. 2001, Tatemoto *et al*. 2001, Cheng *et al*. 2003). This hypotensive action is blocked by the NOS inhibitor L-NAME, indicating a nitric oxide-mediated pathway (Tatemoto *et al*. 2001). In conscious rats, the effect is even less clear, with both increases and decreases in MABP being reported (Cheng *et al*. 2003, Kagiyama *et al*. 2005). Discrepancies among these reports may reflect the conscious state of the animal or the different apelin isoforms used in these studies; it is unknown which specific apelin peptide may be responsible for the (patho)-physiological roles of apelin. Further evidence that APJ plays a role in the regulation of BP comes from a study on mice with a global deletion of APJ, where a transient decrease in systolic BP observed in conscious wild-type (WT) mice following i.p. injection of (Pyr^1)apelin-13 is abolished in APJ KO mice (Ishida *et al*. 2004). However, while peripheral apelin is a vasodilator in the human saphenous vein, in vessels denuded of endothelium, apelin acts as a vasoconstrictor (Katugampola *et al*. 2001, Maguire *et al*. 2009). Therefore, peripheral apelin may act as an antihypertensive factor (Lee *et al*. 2000, 2005, Ishida *et al*. 2004), and sensitivity to the peripheral administration of apelin might be altered in hypertensive disease (Lee *et al*. 2005).

Central administration of (Pyr^1)apelin-13 – the predominant apelin isoform in the cardiovascular system – increases MABP (Seyedabadi *et al*. 2002, Kagiyama *et al*. 2005). While i.c.v. injections of (Pyr^1)apelin-13 have no effect on MABP or heart rate (HR) in anaesthetised rats (Reaux *et al*. 2001), i.c.v. injections increase both MABP and HR in conscious rats (Mitra *et al*. 2006). Central administration of (Pyr^1)apelin-13 also increases c-*fos* (*Fos*) expression in the PVN, suggesting that the pressor effect of apelin may originate from the PVN. In addition, microinjection of apelin-13 into the nucleus tractus solitarius and RVLM of rats increases arterial BP (Seyedabadi *et al*. 2002). Apelin expression is also increased in the RVLM of spontaneously hypertensive rats (SHRs) compared with that in normotensive Wistar Kyoto (WKY) rats (Zhang *et al*. 2009), and microinjection of an apelin-neutralising antibody into the RVLM of SHRs lowers BP. Overexpression of the rat apelin gene in the RVLM of WKY rats, using an adeno-associated virus type 2–apelin viral vector, elevates BP and results in cardiac hypertrophy, while microinjection of apelin-13 into the RVLM of WKY rats increases BP and HR (Zhang *et al*. 2009). More recently, however, it has been shown that microinjection of apelin-13 into the subfornical organ, which detects circulating signalling molecules, decreases BP and HR (Dai *et al*. 2013).

Additionally, the apelinergic system has an important role in cardiac function. In the isolated rat heart, infusion of apelin-16 induces a potent dose-dependent positive inotropic effect, with an EC_{50} of 40–125 pM in humans and ~33 pM in rats (Szokodi *et al*. 2002, Maguire *et al*. 2009), an effect also observed in the failing heart (Berry *et al*. 2004). In mice, administration of apelin increases myocardial contraction while reducing cardiac preload and afterload, without causing hypertrophy (Ashley *et al*. 2005). Furthermore, apelin increases the shortening of sarcomeres in cardiomyocytes (Farkasfalvi *et al*. 2007), an effect that is impaired in isolated ventricular myocytes from apelin and APJ KO mice (Charo *et al*. 2009). Apelin KO mice have an impaired response to cardiac pressure overload, thus suggesting a role for apelin/APJ in the sustainability and amplification of the cardiac response to stress (Kuba *et al*. 2007). There is also evidence for a role in essential hypertension (EHT) as circulating levels of apelin-12 are decreased in patients with EHT (Sonmez *et al*. 2010). Functionally, the apelinergic system plays a role in the Cripto signalling pathway (which stimulates signalling by the transforming growth factor Nodal or growth/differentiation factors 1 and 3, via activin type IB and type IIB receptors) in mammalian cardiac myogenesis (D'Aniello *et al*. 2009).

Cardiovascular development defects have been reported in APJ KO mice, where a loss of homozygous mutants has been described (Charo *et al*. 2009, Roberts *et al*. 2009, Kang *et al*. 2013), but not in apelin KO mice

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(Charo *et al.* 2009), indicating possible ligand-independent effects of the receptor. This effect may perhaps be explained by the recent report that APJ signals independently of apelin in response to cardiac mechanical stretch (Scimia *et al.* 2012). APJ KO embryos at E10.5, when lethality begins, have poorly developed vasculature of the yolk sac, delayed formation of the atrioventricular cushion and unusually formed cardinal veins and dorsal aorta (Kang *et al.* 2013). APJ KOs that survive do not reveal any apparent morphological differences (Roberts *et al.* 2009, Kang *et al.* 2013); however, they have decreased vascular smooth muscle layer recruitment and myocardial defects including thinning of the myocardium, enlarged right ventricles and ventricular septal defects (Kang *et al.* 2013), suggesting an involvement of apelin/APJ signalling in cardiovascular development.

Apelin appears to have a role to play in the pathophysiology of the cardiovascular system – it has been implicated in vascular diseases, heart failure, and ischaemia and subsequent reperfusion. In vascular diseases, the expression of apelin is up-regulated in the atherosclerosis of human coronary artery (Pitkin *et al.* 2010). Yet its role is undetermined, as conflicting evidence has been found in KO studies, indicating both antagonistic and inducing roles for apelin in atherosclerotic formation (Hashimoto *et al.* 2007, Chun *et al.* 2008). During heart failure, plasma apelin levels rise in the early stages of disease and stabilise or lower as the condition develops (Chen *et al.* 2003, Chong *et al.* 2006, Miettinen *et al.* 2007). However, APJ mRNA is decreased in the weakened and enlarged heart of humans with idiopathic dilated cardiomyopathy (Foldes *et al.* 2003). Apelin may have a cardioprotective role in hypoxia and ischaemia, where the cardiac levels of apelin and APJ respectively are increased (Atluri *et al.* 2007, Ronkainen *et al.* 2007, Sheikh *et al.* 2008, Zeng *et al.* 2009). Apelin may also play a protective a role in ischaemia/reperfusion injury (Simpkin *et al.* 2007, Zeng *et al.* 2009), although the method of signalling appears to be independent of the characteristic myocardial kinase cascade, termed the reperfusion injury salvage kinase pathway (Kleinz & Baxter 2008). Post-infarct treatment with (Pyr^1)apelin-13 reduces infarct size and increases HR, with a long-term antioxidant cardioprotective action (Azizi *et al.* 2013).

Role of apelin/APJ in angiogenesis

Apelin is an angiogenic factor (Kasai *et al.* 2004, Kalin *et al.* 2007) and mitogen of endothelial cells (Masri *et al.* 2004). Significantly, apelin is required for the normal development of frog heart (Cox *et al.* 2006, Inui *et al.* 2006) and formation of murine blood vessels (Kidoya & Takakura 2012). Additionally, the development of the retinal vasculature is stunted in apelin KO mice (Kasai *et al.* 2008), and apelin is necessary for hypoxia-induced retinal angiogenesis (Kasai *et al.* 2010), and is also involved in non-neovascular remodelling of the retina (McKenzie *et al.* 2012).

The apelinergic system has been implicated in tumour neoangiogenesis. In brain tumours, the expression of apelin and APJ is up-regulated in microvascular proliferations (Kalin *et al.* 2007), while tumour cell lines overexpressing apelin show increased growth (Sorli *et al.* 2007). The pathophysiological effects of apelin in angiogenesis have also been reported for the liver, where the apelinergic system is a factor in portosystemic collaterisation and splanchnic neovascularisation in portal hypotensive rats (Tiani *et al.* 2009) as well as in neovascularisation during liver cirrhosis (Principe *et al.* 2008). However, apelin may have therapeutic effects in ischaemia recovery due to vessel regeneration and endothelial proliferation (Eyries *et al.* 2008) and blood vessel diameter regulation (Kidoya *et al.* 2010). These findings indicate that apelin is a crucial factor for angiogenesis and that there may be therapeutic potential in both the disruption of its signalling (e.g. tumours) and the stimulation of APJ expression (e.g. ischaemia recovery).

Role of apelin/APJ in fluid homeostasis

The detection of APJ mRNA expression in areas of the brain critical for the control of fluid homeostasis led to the hypothesis that apelin may play a role in the regulation of body fluid balance. VP, along with OT, is synthesised primarily in the neurones of the mPVN and SON, which project to the posterior pituitary and release the peptides into the systemic circulation. The predominant endocrine function of VP from this source is to increase water permeability in the renal collecting duct cells, thereby allowing the retention of water.

The regulatory actions of apelin on thirst and drinking behaviour have been reported. In water-replete animals, a significant increase in water intake is observed following i.p. (Kawamata *et al.* 2001) or i.c.v. (Taheri *et al.* 2002) injection of apelin, whereas in other studies apelin has been reported to reduce water intake post i.c.v. injection (Clarke *et al.* 2009) or to have no effect (Reaux *et al.* 2001, Mitra *et al.* 2006). Additionally, in water-deprived rats, an inhibitory effect (Reaux *et al.* 2001) or lack of any effect

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(Mitra *et al*. 2006) of apelin on drinking behaviour is observed, while in apelin KO mice, the dehydration-induced drinking response is comparable to that observed in WT mice (Kuba *et al*. 2007). The expression of apelin and APJ mRNAs, and labelling of apelin-immunoreactive magnocellular cells, are increased by dehydration (O'Carroll & Lolait 2003, Reaux-Le Goazigo *et al*. 2004), while the labelling of VP-immunoreactive cells decreases, implying the differential regulation of these peptides in response to dehydration (Reaux-Le Goazigo *et al*. 2004). Recently, however, abnormal fluid homeostasis has been demonstrated in APJ KO mice, manifested by a decrease in drinking behaviour and an inability to concentrate urine to levels observed in controls during water deprivation (Roberts *et al*. 2009), suggesting an antidiuretic effect of apelin *in vivo*. However, in lactating rats, apelin induces diuresis and has direct effects on renal vasculature (Hus-Citharel *et al*. 2008). APJ is also necessary in dehydration-induced signalling in the subfornical organ, implicating the apelinergic pathway in responses to hyperosmotic stimuli (Roberts *et al*. 2010).

In the hypothalamo–neurohypophysial system, the physiological effects of apelin appear to be mediated by VP, perhaps by a direct action on APJ-containing vasopressinergic neurones. There is evidence that apelin regulates the actions of VP through the modulation of VP neurone activity and VP secretion (Reaux *et al*. 2001, Taheri *et al*. 2002, De Mota *et al*. 2004, Reaux-Le Goazigo *et al*. 2004, Tobin *et al*. 2008); however, contradictions exist which remain to be resolved. In virgin female rats, direct administration of apelin into magnocellular SON neurones via microdialysis activates VP cell bodies (Tobin *et al*. 2008), while in *in vitro* release studies on SON explants, apelin has been shown to inhibit somatodendritic VP release (Tobin *et al*. 2008). These data imply the differential regulation of axonal and dendritic VP release by apelin. In lactating rats on the other hand, the inhibition of vasopressinergic neurone activity by i.c.v. injected apelin, and an inverse relationship between plasma apelin and VP concentrations, can be observed (De Mota *et al*. 2004). These disparate effects of apelin on VP neurones may be dependent on the physiological conditions of the animals, as lactation is associated with phenotypic changes in the PVN and SON, which include elevations in VP levels (Poulain *et al*. 1977, Burbach *et al*. 2001). Additionally, in humans, increased or decreased plasma apelin concentrations have been found under water-loading conditions or under raised osmolality, respectively – effects that are in contrast to the effects of VP (Azizi *et al*. 2008). Moreover, apelin stimulates the release of both VP and corticotrophin-releasing hormone (CRH) from rat hypothalamic explants *in vitro* (Taheri *et al*. 2002) and stimulates the secretion of VP in ruminants (Charles *et al*. 2006, Sato *et al*. 2012). Thus, data to date indicate a major physiologically active role for APJ in the central mechanisms of water intake and fluid retention; however, the nature of these responses is not clear-cut.

Metabolic actions of apelin/APJ

A number of studies have pointed out an emerging involvement of apelin in energy metabolism and a role for adipocyte-derived apelin in the (patho)-physiology of obesity has been reported. Both apelin and APJ mRNAs are present in mouse, human and rat adipose tissue (Boucher *et al*. 2005, Kleinz *et al*. 2005, Dray *et al*. 2010), and their levels increase in adipose tissue and plasma with obesity. This highlights APJ as an intriguing therapeutic target for metabolic disorders. However, the expression of plasma apelin is increased only in obese humans and in mouse models of obesity associated with hyperinsulinaemia (Boucher *et al*. 2005, Castan-Laurell *et al*. 2008), indicating that obesity or high-fat feeding may not be the main cause for the rise in the expression of apelin, and implying a close relationship between apelin and insulin both *in vivo* and *in vitro*. Insulin directly acts on adipocytes *in vitro* to stimulate the production of apelin (Sorhede Winzell *et al*. 2005), and the expression of apelin mRNA is down-regulated in the adipocytes of mice treated with the β-cell toxin streptozotocin, which leads to a fall in plasma insulin levels (Boucher *et al*. 2005, Wei *et al*. 2005). In mice, nutritional status influences apelin levels *in vivo* – fasting inhibits plasma levels, which are then restored by re-feeding (Boucher *et al*. 2005, Dray *et al*. 2010) – thus strengthening the implication that insulin regulates apelin gene expression and secretion. Additionally, apelin, perhaps through APJ expressed in pancreatic islet β-cells, regulates the secretion of insulin – apelin inhibits glucose-stimulated insulin secretion *in vivo* in mice and in isolated islets of Langerhans *in vitro* (Sorhede Winzell *et al*. 2005). Interestingly, in a recent study, apelin has been shown to alleviate diabetes-induced reduction of pancreatic islet mass and to improve the insulin content of pancreatic islets in type 1 diabetic mice (Chen *et al*. 2011).

Apelin may have a positive effect in the metabolic syndrome (a combination of risk factors that when occurring together increase the risk of coronary artery disease, stroke and type 2 diabetes (T2D)). Apelin KO mice have reduced insulin sensitivity, are glucose intolerant

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and are hyperinsulinaemic (Yue *et al.* 2010). The peripheral administration of apelin reduces peak plasma glucose concentrations by increasing glucose uptake in skeletal muscle and adipose tissue (Dray *et al.* 2008) and improves insulin sensitivity in both apelin KO (Yue *et al.* 2010) and obese high-fat diet fed (Attane *et al.* 2012) mice, with the insulin-sensitising effects continuing for up to 4 weeks, with no tolerance to the actions of apelin. Apelin increases glucose uptake, both *in vitro* (Zhu *et al.* 2011) and *in vivo*, through both insulin-dependent and -independent pathways (Dray *et al.* 2008). Apelin may also decrease body adiposity, independently of altered food intake, by increasing energy expenditure through the activation of mitochondrial uncoupling proteins 1 and 3 (Higuchi *et al.* 2007). Clinical studies have shown a promising therapeutic value for apelin, as apelin displays beneficial glucose-lowering effects in human adipose tissue (Castan-Laurell *et al.* 2008) and plasma apelin levels correlate with glucose (Soriguer *et al.* 2009) and HbA1c (Dray *et al.* 2010) levels. Apelin is linked to the pathogenesis of T2D – plasma apelin concentrations are increased in insulin-resistant patients (Li *et al.* 2006), in type T2D patients (Cavallo *et al.* 2012) and in morbidly obese T2D individuals (Soriguer *et al.* 2009), perhaps indicating a compensatory role of apelin in the reduction of insulin resistance. However, conversely, plasma apelin levels are reduced in newly diagnosed T2D patients (Erdem *et al.* 2008) and increased in T2D patients and obese non-diabetic individuals (Boucher *et al.* 2005, Dray *et al.* 2010). The increased expression of apelin in plasma and adipose tissue of obese individuals can, however, be reversed by a hypocaloric diet (Castan-Laurell *et al.* 2008). As a result of such studies, similarities between the function of apelin and that of insulin, and a link between this adipokine and glucose homeostasis, have been hypothesised.

Apelin/APJ and the neuroendocrine response to stress

As has been noted previously, APJ is localised in the hypothalamic pPVN and the anterior pituitary gland, key areas involved in the stress response. Apelin mRNA is also present in these areas, co-localising with VP in the mPVN, SON and pituitary. Additionally, apelin immunostaining of cell bodies and fibres is highest in the hypothalamus, with large numbers of apelin-positive cell bodies present in the PVN and SON. The presence of APJ and apelin in VP- and CRH-containing hypothalamic nuclei, which are pivotal to the HPA axis responses to stress, suggests a role for apelin/APJ in neuroadenohypophysial hormone release.

A role for apelin in the regulation of the HPA axis responses to stress is supported by studies showing that central administration of (Pyr^1)apelin-13 increases the expression of *c-fos*, an indicator of neuronal activity, in the PVN (Kagiyama *et al.* 2005). Furthermore, administration of apelin-13 stimulates the release of CRH and VP from hypothalamic extracts *in vitro* (Taheri *et al.* 2002), effects consistent with stimulation of the stress axis. APJ mRNA levels increase in the PVN in response to acute and chronic stress and following adrenalectomy (O'Carroll *et al.* 2003), implying negative regulation of the expression of APJ mRNA by glucocorticoids. Additionally, dexamethasone, a glucocorticoid agonist, decreases apelin mRNA levels in 3T3-L1 mouse adipocytes (Wei *et al.* 2005).

Apelin may potentially stimulate the secretion of ACTH either directly at the level of the pituitary corticotroph or via an indirect action on the hypothalamus involving the release of both VP and CRH. Consistent with the expression of apelin and APJ in anterior pituitary corticotrophs, administration of apelin-17 directly increases the release of ACTH, while also augmenting K^+-stimulated ACTH release, in an *ex vivo* perfusion system of anterior pituitary glands, suggesting possible autocrine or paracrine functions for apelin in this tissue (Reaux-Le Goazigo *et al.* 2007). Central administration of (Pyr^1)apelin-13 in rats also increases plasma ACTH and CORT levels while decreasing prolactin, luteinising hormone and follicle-stimulating hormone levels (Taheri *et al.* 2002). However, increases in plasma ACTH and CORT levels observed after i.c.v. administration of (Pyr^1)apelin-13 in mice are reduced to control levels by pre-treatment with the CRH receptor antagonist α-helical $CRH_{9–41}$ (Jaszberenyi *et al.* 2004, Newson *et al.* 2009), while (Pyr^1)apelin-13-mediated increases in plasma ACTH levels are abolished in VP V1b receptor KO mice (Newson *et al.* 2009), indicating that apelin also modulates the release of ACTH via an indirect action on the hypothalamus involving both CRH- and VP-dependent mechanisms. Recently, using APJ KO mice, APJ has been shown to play a regulatory role in the modulation of the HPA axis responses to some acute stressors including LPS challenge (an immune stressor), insulin-induced hypoglycaemia (a metabolic stressor) and forced swim (a physical/psychological stressor) (Newson *et al.* 2013). These studies suggest that other peptides cannot compensate for the loss of APJ to directly, or indirectly, induce the release of ACTH in response to stress. Thus, the integration of neurobehavioural responses to stress may be more complicated

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than previously envisioned, with apelin/APJ exerting a pivotal neuroregulatory role.

Other functions of the apelinergic system

Apelin was first isolated from stomach extracts, and studies on the actions of apelin in the gastrointestinal system have found functional, and possible cell survival, roles (Wang *et al*. 2004, 2009, Susaki *et al*. 2005, Han *et al*. 2007). In the gastrointestinal system, apelin/APJ may be regulators of hormone (Wang *et al*. 2004) and gastric acid (Ohno *et al*. 2012) secretion. Apelin/APJ may also have a direct effect on vascular smooth muscle, including vasoconstriction, which may affect renal glomerular hemodynamic function in the rat kidney (Hus-Citharel *et al*. 2008). Some studies have also proposed an immunological role for apelin as it reduces the production of cytokines in mouse spleen cells (Habata *et al*. 1999, Horiuchi *et al*. 2003, Leeper *et al*. 2009), suggesting that apelin may modulate neonatal immune responses through rodent and bovine colostrum and milk. APJ is also a co-receptor of HIV entry into target cells (Choe *et al*. 1998, Edinger *et al*. 1998, Zhang *et al*. 1998), an action that is blocked by apelin (Zou *et al*. 2000). APJ may contribute to HIV-1 infection and pathogenesis in CNS-based cells as viral envelope proteins can mediate fusion with APJ-positive, cluster of differentiation 4 (CD4)-negative cells, provided that CD4 is added in trans (Puffer *et al*. 2000), and HIV can infect APJ-expressing cells despite their CD4 status (Zhou *et al*. 2003*a*). Other possible roles for apelin and APJ in the rodent CNS include antinociception (Xu *et al*. 2009, Lv *et al*. 2012*b*), enhancement of depressive behaviour (Lv *et al*. 2012*a*), and facilitation of passive avoidance learning (Telegdy *et al*. 2013). Apelin may also have a role in neuroprotection, as apelin pre-treatment protects hippocampal neurones against N-methyl-D-aspartate (NMDA) receptor-mediated excitotoxic injury (O'Donnell *et al*. 2007), possibly via the phosphorylation of Akt and ERK1/2 (Zhou *et al*. 2000, Cheng *et al*. 2012), and prevents apoptosis in cultured mouse cortical neurones (Zeng *et al*. 2010).

Furthermore, apelin and APJ are expressed in osteoblasts where they may induce cell proliferation and promote survival (Xie *et al*. 2006, 2007, Tang *et al*. 2007, Wattanachanya *et al*. 2013); however, an increase in bone mass can be observed in apelin KO mice (Wattanachanya *et al*. 2013). Recently, apelin has been reported to have a potential role in the pathophysiology of osteoarthritis (OA), as apelin is present in synovial fluid, and OA patients have elevated plasma apelin concentrations (Hu *et al*. 2011). Blood plasma levels of apelin are reduced in patients with polycystic ovary syndrome (Chang *et al*. 2011, Olszanecka-Glinianowicz *et al*. 2012), consistent with the role played by apelin/APJ in metabolic disturbances such as insulin resistance.

Future directions

At present, there is a paucity of APJ-selective ligands with which to explore the physiological roles of APJ, and pharmacological studies on whole animals are also confounded by the presence of the target receptor in multiple tissues. Therefore, there is a major requirement for selective pharmacological tools to assess the possibility of non-ligand-mediated effects, such as the modulation of other GPCR signalling pathways by receptor dimerisation, as has been reported recently in the case of APJ and the KOR (Li *et al*. 2012). The demonstration of hetero-APJ dimerisation/oligomerisation *in vivo* may be facilitated by the development of fluorescent APJ ligands, as has been shown recently for the OT receptor (Albizu *et al*. 2010), and/or 'bivalent' ligands, allowing the simultaneous binding of APJ and its partner (Shonberg *et al*. 2011). Additionally, further in-depth studies into apelin fragments are required to establish whether different intracellular signalling responses result from 'functional selectivity' or 'biased signalling'. Future studies utilising RNA interference knockdown of expression, or conditional KO animals (up/down-regulating the expression of APJ in a time- and tissue-dependent manner), would circumvent the known limitations of KO models as well as potential co-morbid complications arising from the peripheral consequences of the absence of APJ. The use of KO animals and the potential of emerging pharmacological agents will no doubt prove useful in studies investigating the role of the apelinergic system in the variety of functions in which it has been implicated.

Stress can impair growth and development while contributing to behavioural, endocrine, metabolic, cardiovascular, autoimmune and allergic disorders. From current knowledge, the possibility that apelin/APJ alters this balance during development cannot be excluded. It has been shown that APJ plays a role in the HPA axis responses to various acute stressors (Newson *et al*. 2013), and previous studies have implicated APJ in the hypothalamic response to repeated stress (O'Carroll *et al*. 2003). Acute and chronic stress has pathological outcomes in individuals displaying genetic vulnerabilities. Acute stress triggers immunological reactions, alterations in BP, gastrointestinal symptoms, and neurological and

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psychological responses, while chronic stress causes a variety of disorders, including physical, behavioural and neuropsychiatric manifestations and cardiac, vascular and metabolic diseases. Notably, the apelinergic system is implicated in a number of these conditions – such as cardiovascular and metabolic disorders – whether the involvement of APJ in the stress response contributes to pathological outcomes is yet to be clarified.

Elevated levels of apelin have been detected in many pathological states or disease processes, such as heart disease, atherosclerosis, tumour angiogenesis and diabetes (Chen *et al.* 2003, Sorli *et al.* 2007, Pitkin *et al.* 2009, Dray *et al.* 2010). However, in many systems, apelin has been shown to have positive effects, for example in the cardiovascular system, where it has a cardioprotective effect (Simpkin *et al.* 2007, Smith *et al.* 2007, Kleinz & Baxter 2008). This has led to speculation that apelin and APJ could be future targets for therapeutic strategies (for comprehensive reviews, see Lee *et al.* (2006) and Sorli *et al.* (2006)). For this potential to be realised, a greater understanding of the regulation of APJ expression in both physiological and pathophysiological states is required.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review.

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