Relaxin family peptide receptors are a recently de-orphanized group of G-protein coupled receptors with great potential as therapeutic targets for the treatment of anxiety, obesity and diseases involving fibrosis.

Relaxin family peptide receptors – from orphans to therapeutic targets

Emma T. van der Westhuizen[1,6], Michelle L. Halls[1,6], Chrishan S. Samuel[2,3], Ross A.D. Bathgate[2,3], Elaine N. Unemori[4], Steven W. Sutton[5] and Roger J. Summers[1]

[1] Department of Pharmacology, Monash University, Clayton, 3800 Vic, Australia
[2] Howard Florey Institute, Royal Pde, Parkville, 3010 Vic, Australia
[3] Department of Biochemistry & Molecular Biology, University of Melbourne, Royal Pde, Parkville, 3010 Vic, Australia
[4] BAS Medical, San Mateo, CA 94402-2525, USA
[5] Neuroscience, Johnson & Johnson Pharmaceutical Research & Development, LLC, San Diego, CA, USA

The relaxin family peptides have distinct expression profiles and physiological functions. Several of them are the cognate ligands for 4 G-protein-coupled relaxin family peptide receptors (RXFPs; formerly LGR7, LGR8, GPCR135, GPCR142). The relaxin/RXFP1 system has roles in reproductive physiology but is also involved in fibrosis, wound healing and responses to infarction. Relaxin has a potential use in congestive heart failure where fibrosis plays an important role in organ failure. The INSL3/RXFP2 system has biological roles in reproductive biology that may have limited therapeutic potential. However, the recently characterized relaxin-3/RXFP3 system is important in stress/anxiety and body composition. RXFP3 receptor antagonists are potentially novel anti-anxiety and anti-obesity drugs.

EMMA T. VAN DER WESTHUIZEN completed her Bachelor of Science degree with 1st class honours (pharmacology) at the University of Melbourne. In 2004, she received a National Health and Medical Research Council of Australia Dora Lush (biomedical) post-graduate scholarship to conduct her PhD research at Monash University. She has been a finalist for ASCEPT student prizes at two meetings (runner-up in 2005) and in 2007 won the ASCEPT oral prize for the best student oral presentation at the 2007 Molecular Pharmacology of G Protein-Coupled Receptors Symposium. Emma will complete her PhD in 2008, and will continue her studies on G protein-coupled receptor signalling as a post-doctoral fellow in the Institute for Research in Immunology and Cancer, University of Montreal.

MICHELLE L. HALLS completed her Bachelor of Biomedical Science degree with 1st class honours at Monash University in 2002, during which she was awarded the Biomedical Science 2nd Year Prize, the Sir John Monash Award, the Geoff Bentley Prize and the Alan Boura Prize. During her PhD she was supported by an Australian Postgraduate Award and a Postgraduate Excellence Award from the Faculty of Medicine, Nursing and Health Sciences. Michelle was awarded the ASCEPT Oral Prize in 2005 and in 2007, the British Pharmacological Society Oral Prize and the Drug Discovery Biology Initiative Student Poster Prize. Since receiving her PhD, Michelle has received an Australian National Health and Medical Research Council Overseas Biomedical Fellowship and the Mollie Holman Medal for the best PhD from the Faculty in 2007. She will continue her postdoctoral studies at the University of Cambridge in 2008.

Relaxin is a hormone that was discovered in 1926 as a substance influencing the function of the reproductive tract [1]. Subsequently it was discovered to be a peptide with a two chain structure very similar to insulin [2], although relaxin diverged from insulin early in vertebrate evolution to form the separate relaxin peptide family [3,4]. This peptide family is encoded by seven genes in humans, the relaxin genes *RLN1*, *RLN2* and *RLN3* and the insulin-like (INSL) peptide genes *INSL3*, *INSL4*, *INSL5* and *INSL6*. Although these peptides display relatively low primary amino acid sequence homology, phylogenetic analysis indicates that they all evolved from a *RLN3* ancestral gene [4], but subsequently many have been shown to have distinct expression profiles and biological functions. The receptors for relaxin [5], relaxin-3 [6,7], INSL3 [5,8,9] and INSL5 [10] have all been recently 'de-orphanized'. It was originally thought, on the basis of the close

Corresponding author: Summers, R.J. (Roger.Summers@med.monash.edu.au)
[6] These authors contributed equally to the writing of this manuscript.

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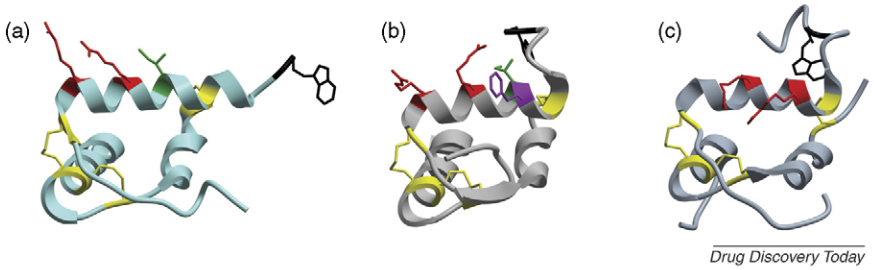

FIGURE 1

Structural comparison of relaxin, relaxin-3 and INSL3. The structure of relaxin **(A)** from X-ray diffraction studies and of relaxin-3 (**B**; PDB code 2FHW) and INSL3 (**C**; PDB code 2H8B) using NMR were modelled using PyMOL software. For all peptides the A-chain is shown in orange and the B-chain in blue; di-sulphide bonds are shown in green; the relaxin receptor binding motif (Arg, Arg, Ile) is shown in red, and the tryptophan essential for INSL3 receptor binding shown in cream.

resemblance between relaxin and insulin that the receptors for relaxin and INSL3 were likely to be related to the known insulin (tyrosine kinase) receptors. This did not, however, sit comfortably with evidence that relaxin caused increases in cAMP in reproductive tissues [11,12] and cell lines [13]. It is now known that, in spite of their structural similarity, relaxin and insulin family peptides have quite distinct signalling mechanisms: the relaxin family peptides activate the G protein-coupled relaxin family peptide

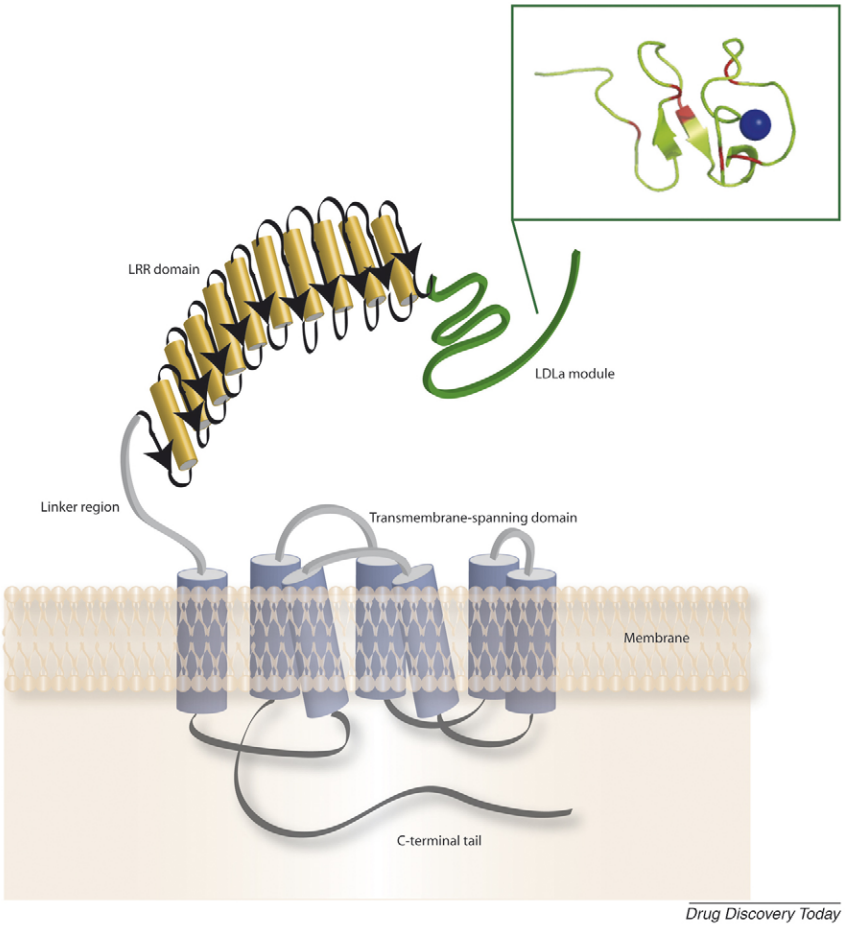

FIGURE 2

Schematic diagram of a type C LGR. RXFP1 and RXFP2 are classified as type C LGRs. These receptors have a large and distinctive ectodomain encompassing an LDLa module at the extreme N-terminus (green line), followed by 10 LRR (LRR domain; coloured orange), a unique hinge region leading into the transmembrane domain (seven transmembrane helices coloured blue), and an intracellular C-terminal tail. Inset – structure of the RXFP1 LDLa module. The NMR solution structure of the RXFP1 LDLa module (PDB code 2JM4) modelled using PyMOL software. The basic chain structure is shown in green, with cysteine residues highlighted in red, and the calcium ion required for correct structure shown in blue. The module runs from N-terminus (left side) to the C-terminal region around the calcium ion.

receptors (RXFP) 1–4, whereas the insulin group activates tyrosine kinases [14]. There is now increasing interest in the RXFP receptors, a group of four receptors comprising the leucine-rich repeat-containing RXFP1 and RXFP2, and the small peptide-like RXFP3 and RXFP4 [15]. The interest stems largely from the increasing realisation that the relaxin family peptides and their receptors have important roles in a wide variety of physiological processes (Figures 1–4).

Relaxin family peptides

The relaxin family peptides are a sub-group of the relaxin-insulin peptide family. All peptides within this family have a uniform two-chain structure, with two inter-chain and one intra-chain disulphide bond. In the human there are seven relaxin family peptides: the human gene 1 (H1-relaxin), human gene 2 (H2-relaxin, commonly referred to as relaxin and equivalent to other species' relaxin-1) and human gene 3 (H3-relaxin), and the insulin/relaxin-like peptides INSL3, INSL4, INSL5 and INSL6. Relaxins (including H1, H2 and H3-relaxin) are distinguished by the relaxin receptor-binding motif Arg^{B13}, Arg^{B17}, Ile^{B20} located within the B chain [16,17].

There is a relatively limited, but ever-expanding, knowledge base for this peptide sub-family. Only a limited number of these peptides currently have defined physiological roles. Although relaxin was initially identified for its influence on parturition in guinea pigs [1], it now has recognized roles in a number of physiological systems including the cardiovascular, renal and reproductive systems, and also in fibrosis and allergic responses [2,18–23]. Relaxin-3 is principally a brain peptide modulating stress and body composition; and INSL3 is responsible for development of the gubernaculum during testis descent and has roles in both male and female germ cell function. Currently, only four of the peptides have identified receptors, although there is a degree of cross-reactivity between peptides and receptors.

The relaxins: relaxin and relaxin-3

In humans, unlike many other species, the levels of circulating relaxin peak during implantation and, thus, the peptide is unlikely to have a major role in childbirth. Relaxin is expressed in a wide variety of human tissues [15] and is considered to be the main circulating relaxin. Relaxin and its receptor are often involved in pathologies that are considered to be age-related, such as fibrosis, wound healing and responses to infarction and are, therefore, likely to represent important pharmacological targets for the treatment of those diseases that may be more prevalent in the elderly [24].

A role for relaxin in protecting several organs from the progression of fibrosis has received strong support from studies with the relaxin knockout mouse that displays a number of changes including: increased lung, renal and ventricular collagen deposition (fibrosis) with age in males [25–28]; increased pulmonary and dermal fibrosis leading to skin thickening in ageing males and females [27,28]; increased lung collagen in a model of allergic airway disease [29]; cardiac hypertrophy and airway fibrosis [30] and increased collagen accumulation in a model of tubulointerstitial fibrosis, a hallmark of progressive renal disease [31]. Thus, the increased degree of fibrosis associated with relaxin knockout mice in all of these experimental models suggests that the receptor for relaxin has therapeutic potential as a target for agents focussed against fibrosis associated with a broad range of diseases.

Although relaxin-3 is the most recently identified relaxin family peptide [32], phylogeny of the relaxin peptide family suggests that it is the ancestral relaxin peptide [3,32]. Relaxin-3 is predominantly localized in the neurones of the nucleus incertus (NI) [32,33]. The role of relaxin-3 is currently defined by the regional distribution of RXFP3 receptors (see below) and data from rat models. In rat forced swim tests, there is increased relaxin-3 mRNA transcription 6 h following the stress [33] suggesting a role for relaxin-3 in stress and anxiety. In addition, injection of relaxin-3 into the paraventricular nucleus increases feeding in satiated rats, suggesting a role for relaxin-3 in appetite regulation [34]. Thus, RXFP3 receptors have significant therapeutic potential as a target for the design of anti-anxiety or anti-obesity agents.

The insulin-like peptides: INSL3 and INSL5

INSL3 was confirmed as the ligand for RXFP2 after the observation that both INSL3 and RXFP2 male knockout animals exhibited cryptorchidism [8,35,36]. INSL3 influences oocyte maturation in females and male germ cell survival [37]. Thus, RXFP2 has therapeutic potential as a target, not only as a treatment for cryptorchidism but also, as a male contraceptive, or to enhance fertility.

INSL5 was identified in a human colon cDNA library [38], but is a non-functional pseudo-gene in dogs and rats [39]. Although INSL5 is expressed in human colon and uterus [3,38], the function of this peptide remains unknown.

Relaxin family peptide receptors (RXFPs)

RXFP1 and RXFP2

RXFP1 (LGR7) and RXFP2 (LGR8) are class C leucine-rich repeat-containing G protein-coupled receptors (LGRs), which share 60% amino acid sequence identity [5]. Both receptors possess the typical seven transmembrane spanning regions of GPCRs, and a unique ectodomain containing 10 leucine rich repeats (LRRs) and an LDL class A (LDLa) module at the extreme N-terminus. The LDLa module distinguishes RXFP1 and RXFP2 from other LGR receptors and indeed all other GPCRs.

RXFP1 receptors from human, rat and mouse all bind relaxin and relaxin-3 peptides with high affinity [40]. This high affinity binding involves the Arg-X-X-X-Arg-X-X-Ile/Val motif in the B chain of the peptides [16,17]. INSL3, INSL5 and INSL6 lack this specific motif and have a very poor affinity for RXFP1. INSL3 is a high affinity agonist at RXFP2 receptors from human, rat and mouse [41] and again, high affinity binding is mediated by specific residues in the B chain of the peptide. Arg-B16 and Trp-B27 are, however, the most important contributors to the binding energy with His-B12, Arg-B20 and Val-B19 all making minor contributions [42]. Relaxin peptides from some species are high affinity RXFP2 agonists, as they contain the critical Arg-B16 and Trp-B27 residues, whereas rodent and rhesus monkey relaxin that lack Trp-B27 have a very poor affinity for RXFP2 [41,43].

Binding and activation of RXFP1 and RXFP2 by relaxin and INSL3 occurs via a three-step mechanism [16,43–45]. Primary ligand binding is between the critical B chain residues of the peptides and the LRRs of the ectodomain [46]. There is a secondary lower affinity binding site in the transmembrane exoloops [43,45], which potentially involves residues in the A chain of the peptide.

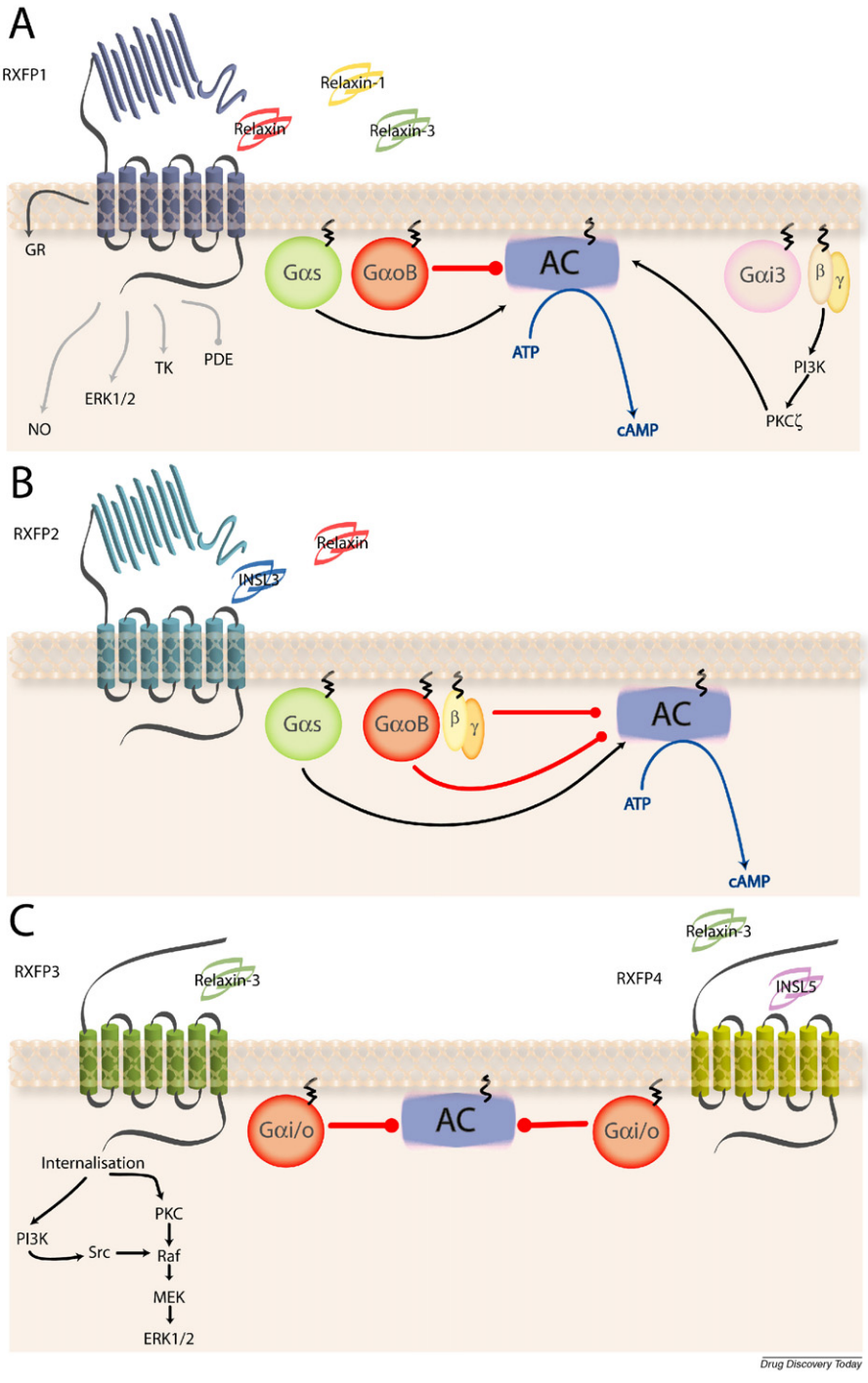

FIGURE 3

Signalling pathways activated by RXFP1 **(A)**. Relaxin, relaxin-1 and relaxin-3 can all bind to RXFP1. Activation of the receptor allows coupling to $G\alpha_s$ and $G\alpha_{oB}$ that activate and inhibit adenylate cyclase respectively, to cause an overall accumulation of cAMP. With time, RXFP1 recruits coupling to $G\alpha_{i3}$ to increase cAMP accumulation further via Gβγ-PI3K-PKCζ. Although the precise mechanisms are as yet undefined, the receptor may also signal via nitric oxide (NO), ERK1/2, tyrosine kinase (TK), phosphodiesterase (PDE) inhibition, or even through direct peptide-induced activation of the glucocorticoid receptor (GR). Signalling pathways activated by RXFP2 **(B)**. INSL3 and relaxin can bind to RXFP2 to activate the receptor and allow coupling to $G\alpha_s$ and $G\alpha_{oB}$ in much the same manner as RXFP1. In HEK293 cells the overall effect is cAMP accumulation but in contrast to RXFP1 there is no delayed coupling of RXFP2 to $G\alpha_{i3}$. Signalling pathways activated by RXFP3 and RXFP4 **(C)**. Relaxin-3 can bind to RXFP3, with relaxin-3 acting as an agonist and inhibiting cAMP production via $G\alpha_{i/o}$. INSL5 acts as an antagonist of the relaxin-3 inhibition of cAMP production. Internalisation or movement into lipid-rich signalling complexes likely occurs since RXFP3 can activate two pathways leading to phosphorylation of ERK1/2 via Raf and MEK: a major pathway via PKC, and a minor pathway via PI3K and Src. Both relaxin-3 and INSL5 can bind to RXFP4, and this receptor inhibits cAMP accumulation via $G\alpha_{i/o}$.

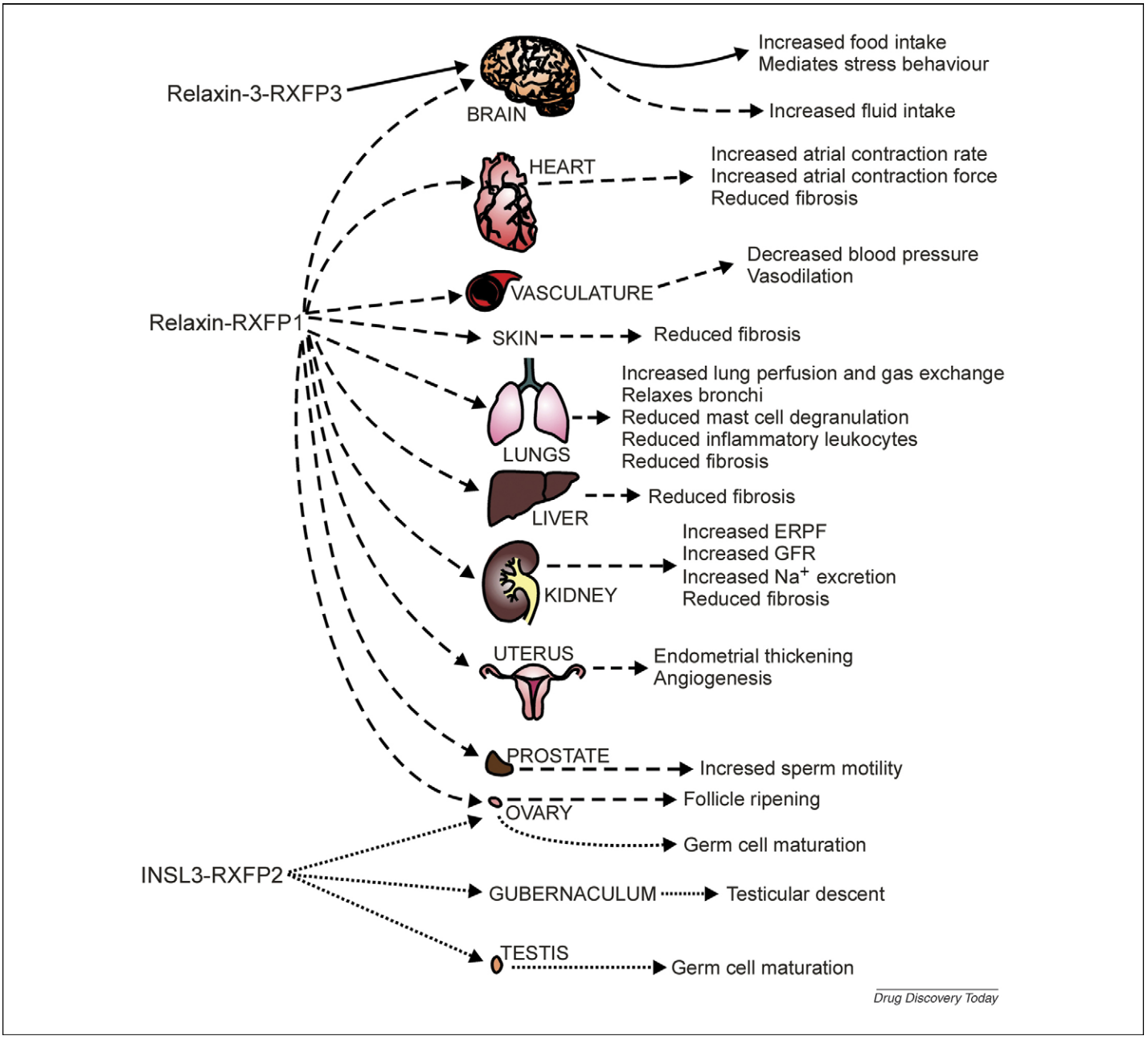

FIGURE 4

Tissue localisation of relaxin family peptides and their receptors with their known functions. Relaxin-3 (H3 relaxin in humans) is predominantly expressed in the brain where it acts on RXFP3 receptors in the brain to increase food intake. mRNA levels of relaxin-3 are increased in stressed rats suggesting a role in behavioural responses to stress. Relaxin (H2 relaxin in humans) is mainly produced by the ovary, where it is secreted into the circulation to act on RXFP1 receptors located at many sites in the body. One of the major general actions of activation of RXFP1 receptors is to decrease fibrosis in many tissues. INSL3 is produced by the Leydig cells of the testis, and is also secreted into the circulation. It acts on RXFP2 receptors in the gubernaculum to facilitate testicular descent into the scrotum. INSL5 and RXFP4 receptor mRNA expression has been determined by RTPCR, and shows high concentrations in kidney, colon and lympohocytes with more modest amounts in brain, prostate, ovary, thyroid and placenta, spleen and pituitary. Receptor protein expression at these sites remains to be confirmed as are the functions of INSL5 acting at RXFP4.

Finally, receptor activation is dependent on the LDLa module, as receptors lacking the LDLa module bind relaxin peptides, but do not generate a cAMP signal [47].

The primary ligand binding sites in RXFP1 and RXFP2 have recently been defined. The high affinity RXFP1 binding site exists at an angle of 45° to five of the parallel pleated sheets of the LRRs, as determined by molecular modelling [16]. Binding to the receptor occurs through generation of a hydrogen-bonding network between the receptor and two residues of the relaxin peptide-binding motif, which is stabilized by a hydrophobic interaction between the third residue of the relaxin peptide binding motif (Ile-B20) and a cluster of residues from neighbouring LRRs in the receptor. Molecular modelling and mutagenesis has also been used to demonstrate the residues in the RXFP2 LRRs that interact

with the key INSL3 B chain residues [46]. RXFP2 Asp-227 is crucial for binding INSL3 Arg-B16, whereas RXFP2 Phe-131 and Gln-133 are involved in INSL3 Trp-B27 binding. Additionally, INSL3 His-B12 probably interacts with RXFP2 Trp-177, INSL3 Val-B19 with RXFP2 Ile-179, and INSL3 Arg-B20 with RXFP2 Asp-181 and Glu-229. Importantly, INSL3 binds to the RXFP2 LRRs in a manner similar to relaxin binding to the RXFP1 LRRs. It is likely that subtle differences in both peptide and receptor structure define ligand-receptor specificities.

The exact position of the secondary binding site in both RXFP1 and RXFP2 is currently unknown. Studies with chimeric RXFP1/RXFP2 receptors, however, suggest that this binding resides in exoloop 2 [43,45]. Importantly, mimicking primary binding alone is not sufficient to activate the receptors. B chain only analogues of INSL3 bind to RXFP2 but act as receptor antagonists [48]. These peptide antagonists are useful research tools and additionally block testis function *in vivo* [48]. Both primary and secondary binding are necessary for receptor activation and it is likely that compounds that bind to the secondary binding site alone will also be receptor antagonists.

The mode by which the LDLa module directs ligand-activated cAMP signalling is unknown. It is likely, however, that it involves specific side chain interactions. An RXFP1 receptor mutant, with the LDLa module substituted by the LDLa module from the second ligand binding domain of the LDL receptor LB2, binds relaxin normally but does not cause cAMP accumulation [49]. Additionally, mutation of specific residues predicted from the NMR structure of the RXFP1 LDLa module causes perturbation of cAMP signalling [49]. The LDLa module may act as a tethered ligand of the RXFP1-relaxin receptor complex and mimicking this interaction may produce a receptor agonist. Although isolated LDLa modules do not rescue the function of RXFP1 receptors lacking the LDLa module, they do act as RXFP1 antagonists [47]. Therefore, ligand activation of RXFP1 and RXFP2 is a complex process and production of small molecule agonists may require novel approaches that target the transmembrane G-protein interaction directly or produce conformational changes in receptor structure to cause or enhance signalling by allosteric mechanisms.

RXFP3 and RXFP4

RXFP3 (GPCR135; SALPR) and RXFP4 (GPCR142; GPR100) are activated by relaxin-3 and INSL5, respectively. They are G protein-coupled receptors of ~400 amino acid residues that lack the LRR and LDLa module present in RXFP1 and RXFP2. Structurally, these receptors are homologous with small peptide receptors that respond to somatostatin or angiotensin II. RXFP3 and RXFP4 are both encoded by a single exon, and have distinct expression profiles. RXFP3 is most strongly expressed in the brain [50], whereas RXFP4 shows a widespread distribution, with mRNA detected in the colon, thyroid, salivary gland, prostate, placenta, thymus, testis, brain and kidney [6]. The amino acid sequence of RXFP3 is highly conserved between many different species, however, RXFP4 is only present in some species and, like the cognate ligand INSL5, is a pseudogene in rats and dogs [3]. While RXFP3 has important central roles, particularly in modulating behavioural responses to stress [33] and in appetite regulation [34,51], the role of RXFP4 remains to be identified. RXFP3 binds exclusively to relaxin-3 and the B-chain monomer of relaxin-3 [7], whereas RXFP4 binds relaxin-3, the relaxin-3 B-chain and INSL5 [6,52]. The receptor regions and specific residues involved in ligand binding, however, remain to be identified for these receptors.

RXFP signalling

RXFP1 and RXFP2 couple to stimulatory and inhibitory G-proteins

Constitutively active mutants of RXFP1 and RXFP2 (Asp 637 Tyr) cause cAMP accumulation [5,14]. Recent studies of cAMP accumulation have revealed distinct patterns of G-protein coupling and cAMP signalling for RXFP1 and RXFP2 [53]. Both receptors initially couple to $G\alpha_s$ to increase and $G\alpha_{oB}$ to modulate negatively cAMP production [53]. Only RXFP1, however, can recruit coupling to $G\alpha_{i3}$ with time, and activates a $G\beta\gamma$-PI3K-PKCζ pathway to cause a further surge in cAMP accumulation [53–55].

Evidence for activation of these pathways occurs in cells endogenously expressing the receptors: activation of the delayed $G\alpha_{i3}$ pathway and relaxin-stimulated translocation of PKCζ occurs in THP-1, MCF-7, pregnant human myometrial and mouse mesangial cells, all of which endogenously express RXFP1 [54]. Increases in cAMP accumulation are important for relaxin-mediated myometrial inhibition in the rat, and decidualisation of human endometrial stromal cells [56]. In a rat model of myocardial infarction, pertussis toxin (PTX; Gi/Go inhibitor) inhibited relaxin-induced cAMP accumulation and chronotropic and inotropic responses in atria [57], suggesting a functional and potential therapeutic relevance for these signalling pathways *in vivo*. With regard to the RXFP2 receptor, gubernacular cells (which endogenously express RXFP2) increase cAMP accumulation in response to INSL3 [9], whereas in testicular germ cells and oocytes (both of which endogenously express RXFP2) a decrease in cAMP accumulation occurs in a PTX-sensitive manner [37].

RXFP1 also activates a number of other therapeutically relevant pathways. A number of cells that endogenously express RXFP1 show rapid activation of ERK1/2, including human endometrial stromal cells, THP-1 cells and primary cultures of human coronary artery and pulmonary artery smooth muscle cells [58]. A recent study has linked increases in ERK1/2 phosphorylation in response to relaxin, with a protective decrease in apoptosis in cardiac myocytes following apoptosis-inducing treatment with hydrogen peroxide [59]. Relaxin also increases nitric oxide in both an acute and chronic manner [60], and a number of studies have linked this to therapeutically relevant outcomes [61–70]. Relaxin reduces infarct size in a rat model of stroke, in a nitric oxide-dependent manner [71]. Relaxin-induced nitric oxide production was also found to decrease the recruitment of leukocytes to areas of inflammation.

RXFP3 and RXFP4 couple to inhibitory G-proteins

RXFP3 and RXFP4 receptors both couple to inhibitory G-proteins and inhibit forskolin-stimulated cAMP production [6,7]. A functional role for inhibition of cAMP production in regions of the brain that express RXFP3 has not been shown. RXFP3 is, however, also strongly coupled to ERK1/2 activation in recombinant cell systems and a neuronal cell line [72]. Activation of ERK1/2 is important in mediating obesity and stress signals in the brain,

suggesting a physiologically-relevant mechanism for the effects of relaxin-3 detailed above [73,74]. ERK signalling is also important in other areas of the brain that express RXFP3 receptors, such as the amygdala where it has a role in long-term potentiation and in the formation of long term memories in rats [75]. ERK1/2 activation also has a role in the amygdala and visual cortex in mediating signals for pain perception [76] and light stimulation. The consequences of cAMP inhibition in the regions that express RXFP4 receptors, has also been little investigated, although, decreases in cAMP may be associated with decreased secretion of calcitonin from C cells of the thyroid [77].

Tissue distribution and function of relaxin family peptides and their receptors

Reproductive system

For many years, attention rested on relaxin as a reproductive hormone that acted on the pubic symphysis, cervix, uterus, nipples and mammary glands with an emphasis that varied with species. In many species, including humans, there is growth and an increase in elasticity of pubic joint cartilage during pregnancy [22] but, while it has been demonstrated that relaxins can promote ripening of the cervix, clinical trials of topically applied peptide failed, possibly owing to poor pharmacokinetic properties of the preparation. Relaxin inhibits uterine contractility in rats, mice and pigs, but not in sheep, cows or humans [15,22]. Relaxin is important in the development of the mammary nipple and mammary gland and binding sites are present in the mammary glands of a number of species, including humans [78,79]. In humans and other primates there is increasing evidence that relaxin prepares the endometrium for implantation. Relaxin is associated with endometrial angiogenesis, thickening and bleeding [80,81] and plasma levels of relaxin are highest in the first trimester, at the time corresponding to embryo implantation. The role of relaxin, however, is probably facilitatory, rather than mandatory, for implantation, since this still can occur in humans and other primates that have had ovaries removed [82]. Relaxin is also produced in the male reproductive tract, is present in semen, and increases sperm motility and penetration into oocytes [83]. As well as having a potential function in males, it is also tempting to speculate that relaxin in semen targets the female reproductive tract to prepare the endometrium for implantation.

Studies of the INSL3/RXFP2 system are less extensive, but in humans, RXFP2 mRNA is found in the uterus and testis [5]. RXFP2 epitopes are found in spermatocytes, spermatids and Leydig cells from the human testis, and in the epididymal epithelium [84]. INSL3 acts on RXFP2 receptors located on the gubernaculum [9] to facilitate testis descent reflected by the finding that both *INSL3* and *RXFP2* male knockout mice display bilateral cryptorchidism [8,35,36]. Although both RXFP3 and RXFP4 mRNA are found in human testis [6,7] and RXFP4 is found in placenta and prostate their functions in these tissues are unknown.

Brain

It is now clear that relaxin family peptides influence many physiological systems in addition to the reproductive system. RXFP1 receptor distribution in the brain includes the circumventricular organs (subfornical organ – SFO; and organum vasculosum of the lamina terminalis – OVLT) and the neurosecretory magnocellular hypothalamic nuclei (i.e. paraventricular and supraoptic nuclei) [85,86] where they control plasma osmolality [87,88]. RXFP1 mRNA has been demonstrated in human brain using RT-PCR [5]; RXFP2 mRNA is expressed in the rat brain [89]; RXFP3 is predominantly expressed in the brain, whereas RXFP4 is expressed in various tissues, including the brain [6]. RXFP3 mRNA is present in human brain and in rat brain, where it is found in areas such as the supraoptic and paraventricular nucleus [6,7]. Recently, a chimeric peptide, relaxin-3 B-chain/INSL5 A-chain, was shown to be a selective, high affinity ligand for RXFP3 and RXFP4. In the rat [22], relaxin-3/INSL5 labels RXFP3 receptors in the cerebral cortex, olfactory bulb and superior colliculus [50].

Cardiovascular system

Relaxin has important roles in the increased plasma volume, cardiac output and heart rate associated with pregnancy, and also decreases blood pressure and vascular resistance. In pregnant rats, glomerular filtration rate and effective renal plasma flow increase, while vascular resistance decreases in parallel with increases in plasma levels of relaxin [90]. In both female and male rats, administration of relaxin increases renal plasma flow and glomerular filtration rate and also causes the classical reduction in plasma osmolality associated with pregnancy [91]. The mechanism suggested to explain the actions of relaxin on the kidney involves the induction of vascular gelatinase that cleaves big endothelin (ET) to yield ET_{1-32} that acts on the ET_B receptor to increase production of the vasodilator NO [23].

Vasodilation in response to relaxin is common in arterioles, capillaries and venules in reproductive tissues, heart, liver and caecum. The mechanisms suggested for the vasodilator actions of relaxin are activation of NOSIII via cAMP, induction of NOSII [60] and modification of the extracellular matrix of the vessel walls [23]. While the relevance of these cardiovascular actions in humans is yet to be fully determined, the experimental findings reported to date suggest that relaxin may take part in the cardiovascular adjustments of pregnant women, directed to increasing cardiac output, upregulation of organ and decidual perfusion and optimisation of body fluid homeostasis [20,21].

While the heart is clearly a target organ for relaxin in rodents and contains binding sites for relaxin, RXFP1 RNA, and clear positive inotropic and chronotropic actions, corresponding evidence in humans is far from convincing. Relaxin has no positive inotropic effect in human atria (Summers & Castro, unpublished observations) and radiolabelled relaxin does not bind to human atria (Tan and Summers, unpublished observations). mRNA for RXFP2 has been demonstrated in human [5] and rat [92] kidney as has RXFP4 [6] but there are no known functional correlates for these expression patterns to date.

Connective tissue homeostasis

Relaxin has established physiological roles in inhibition of collagen biosynthesis and promotion of collagen breakdown in reproductive tissues, but it is also becoming clear that it has similar effects in non-reproductive tissues, leading to the sugges-

tion that relaxin has potential as an anti-fibrotic agent. Relaxin acts directly on TGF-β-stimulated human dermal fibroblasts [93], lung fibroblasts [94] and cardiac fibroblasts [95] to promote a decrease in type I and type III collagen synthesis, together with an increase in matrix metalloproteinase (MMP) expression and activation. It has been used successfully to modify the extracellular matrix in the dermis [96], lung [94], liver and kidney [97]. Interestingly, in all of these experimental paradigms, relaxin only affects aberrant collagen deposition, induced by pro-fibrotic stimuli, surgery or chemical means, but has little effect on normal collagen levels.

Insights from knockout animals

Studies with the relaxin knockout mouse demonstrate that the peptide is an endogenous mediator of collagen turnover in non-reproductive tissues. Increased interstitial collagen is found in heart [25], lung [27], kidney [26] and skin [28]. The effect is particularly prevalent in older male mice and associated with abnormal function of these organs. In the heart, there is atrial hypertrophy and impaired left ventricular (LV) diastolic filling and venous return, associated with increased LV collagen content and ventricular chamber stiffness [25]. In the lung, there is increased weight (total mass) and collagen and structural changes associated with altered peak expiratory flow and lung recoil [27]. Increased kidney collagen in male relaxin knockout mice is associated with focal interstitial fibrosis and a more general diffuse increase in glomerulosclerosis, from 6-months of age onwards [26], leading to impaired renal function with increased serum creatinine and urinary protein [26]. Collagen levels also increased in the dermis of relaxin-deficient animals [28,98]. Treatment of relaxin knockout mice with recombinant relaxin in the early and developed stages of fibrosis causes a reversal of collagen deposition in the lung [27], heart [95] and kidney [26], consistent with an anti-fibrotic action for this peptide.

With regard to reproduction, RXFP1 knockout female mice show normal fertility and litter size [99], but a small proportion are incapable of delivering their litters, with approximately 15% of the pups found dead soon after birth and all pups dead within 24–48 h. The phenotype is similar to relaxin knockout female mice [100], further supporting the hypothesis that relaxin facilitates birth by promoting growth of and softening the cervix [22]. Those pups that survive delivery die later because relaxin is also required for nipple development [101], and pups are unable to grasp the small nipples of mice lacking either relaxin or RXFP1 receptors [99,100]. In relaxin [102] and RXFP1 knockout males [99] fertility is markedly diminished with disrupted spermatogenesis associated with an increased rate of apoptosis of meiotic spermatocytes [99]. The testis and epididymidis weights are lower than in wild-type controls. In tissues other than reproductive tissues, recent studies in the RXFP1 knockout mouse have demonstrated an increase in interstitial collagen in the lung [103], while other organs have yet to be examined.

In the RXFP2 or INSL3 knockout male mouse, the testes fail to descend into the scrotum, the mouse is sterile and in both cases the effect is attributable to failure of the gubernacular ligament to develop [8,35,36]. INSL3 acts directly on RXFP2 in the gubernaculum [9] and this has a dramatic effect in transgenic female mice overexpressing INSL3 during embryonic development, where the ovaries descend [104]. While RXFP2 knockout female mice are fertile [8], approximately 20% of INSL3-deficient female mice are infertile [36].

The phenotypes of neither RXFP3 nor RXFP4 knockout mice have been reported. It remains to be shown whether deletion or overexpression of relaxin-3, RXFP3 and/or RXFP4 will show a significant phenotype.

Possible therapeutic applications for drugs that target RXFP1

Update on clinical trials using relaxin

Since the 1980s, recombinant human relaxin has been developed by three companies, Genentech, Connetics and BAS Medical. During that time, over 500 human subjects have been treated with relaxin for indications including cervical ripening, scleroderma, fibromyalgia and orthodontics. Although the scleroderma trial showed a decreased modified Rodnan Skin Score (phase II), increased predicted creatine clearance (phase II and phase III) and a moderate but not clinically meaningful reduction in diastolic blood pressure (phase II and phase III) [105], these past trials have failed to show a definitive clinical use for relaxin, but were invaluable in demonstrating specific biological activities of this hormone in humans. The following sections provide a brief update on the status of trials currently being conducted or recently completed.

Cervical ripening

Evidence for the ability of relaxin to ripen the cervix in several mammalian species, including the pig and the rat, is overwhelming. The ability of relaxin to cause similar changes in women was initially tested by Genentech in the early 1990s in three multinational Phase II clinical trials. These trials employed topical application of relaxin (intra-vaginal) in women with post-date pregnancies [106]. These studies failed to show that relaxin ripened the cervix, but pharmacokinetic data indicated that a contributing factor may have been the failure of penetration of relaxin into the cervical tissue.

More recently, BAS Medical tested relaxin for its ability to ripen the cervix following 24 h intravenous administration. Using this route, there was no doubt that relaxin was systemically available, a fact confirmed by periodic sampling of plasma from the women in the trial. Despite the clear bioavailability of relaxin, cervical changes measured by the Bishop score were not different between the relaxin- and placebo-treated groups. Small decreases in blood pressure and improvements in markers of renal function, however, were observed and were consistent with effects of relaxin observed in previously conducted trials [107].

Congestive heart failure

Congestive heart failure (CHF) is a condition in which the heart fails to pump blood adequately to the organs of the body. Frequently, this inability is associated with sympathetic nervous system overactivity, decreased cardiac output and systemic vasoconstriction. Part of the biology of relaxin during human pregnancy involves a decrease in systemic vascular resistance, which falls by about 30% during the first trimester of pregnancy [108], when relaxin levels are at their peak [109]. Previous clinical trials also suggested that exogenously administered relaxin is associated with systemic vasodilation.

Chronic administration of relaxin in the scleroderma trials was accompanied by consistent, small decreases in systolic blood pressure of a magnitude similar to that seen during pregnancy (5 mmHg) [107]. In a retrospective analysis, it was also observed that a subset of hypertensive subjects (systolic blood pressure ≥140 mm Hg at baseline) responded to relaxin with larger decreases (15–20 mmHg) in systolic blood pressure over the 6-month dosing period.

Declining renal function in patients with CHF is highly correlated with mortality, suggesting that a therapeutic agent that can improve or preserve renal function could have a very large impact on this patient population. One of the most consistently observed findings in the scleroderma trials was that relaxin improved predicted creatinine clearance [107]. These data are consistent with preclinical studies also demonstrating that relaxin improves renal function [91], and with the view that relaxin contributes to the natural improvement in renal function of pregnant women, leading to the suggestion that exogenously administered relaxin may improve renal function in patients with CHF.

A small, pilot clinical trial of the effects of short-term relaxin infusion on haemodynamics and renal function in subjects with stable CHF was recently conducted by Drs Thomas Dschietzig and Christoph Richter at the Charité Hospital in Berlin, Germany. Although the study was not placebo-controlled, improvements during dosing in hemodynamic parameters, including increased cardiac output and decreased systemic vascular resistance and pulmonary capillary wedge pressure, were demonstrated [110]. Markers of renal function, including serum creatinine, BUN, and uric acid, showed decreases in all subjects during relaxin administration, consistent with improved renal function. These results suggest that relaxin infusion may be able to help alleviate the vasoconstriction and renal dysfunction in patients with HF. These data were used to support initiation of a 330-patient, randomized placebo-controlled trial of relaxin treatment in patients with acute HF in approximately 50 sites internationally. The endpoints in this study are improvements in symptoms of HF, including breathing difficulty, and in renal function. The study is expected to be completed late in 2008.

Pre-eclampsia

Pre-eclampsia is a disorder that occurs only in pregnant women and is believed to be the result of profound systemic vasoconstriction, leading to hypertension, and dysfunction of organs, including the kidney. Renal dysfunction in women with pre-eclampsia is reflected by elevated serum uric acid and proteinuria. Recently, studies have suggested that the vasoconstriction associated with this disorder may be owing to the inhibition of vasoactive/angiogenic factors, such as vascular endothelial growth factor (VEGF), by soluble receptors (s-flt1) that competitively inhibit binding of the factors to their cell-associated receptors [111]. Ironically, these inhibitory factors may be elaborated by the placenta in response to poor blood flow that results from impaired trophoblast invasion.

Preclinical evidence that relaxin plays a role in the development and maintenance of the endometrial vasculature dates back to the 1950s when investigators observed remarkable histological changes in the endometrium following administration of a relatively crude preparation of relaxin to monkeys [112]. In previous clinical trials of relaxin, frequent reports of menorrhagia and metrorrhagia by women during infusion of relaxin also suggested that increased blood flow to the human endometrium occurs following exogenous relaxin administration; an effect potentially mediated via its inhibitory actions on platelet aggregation [113,114] For this reason, and because relaxin specifically upregulates VEGF in endometrial cells [115] and improves renal function via increased blood flow [91], the peptide is currently being tested in the US in a small trial of women with severe pre-eclampsia. Renal function, uterine and umbilical blood flow, as well as clinical signs and symptoms in mother and baby, are being monitored in this study. It is suggested that if relaxin is able to increase blood flow to the endometrium/placenta, it may be able to break the cycle of ischaemia at that site, and result in a decrease in the elaboration of anti-angiogenic/vasodilatory factors by the placenta.

Possible therapeutic applications for drugs that target RXFP3

Since RXFP3 has only recently been shown to be the relaxin-3 receptor, RXFP3 modulators have yet to be tested in humans. The therapeutic potential of RXFP3 agonists/antagonists can currently only be presumed, based on anatomical, physiological and pharmacological experiments performed in lower species. Studies published so far indicate potential therapeutic uses of RXFP3 modulators to treat stress/anxiety and metabolic disease.

Parallel evolution of relaxin/insulin superfamily ligands with their cognate receptors [3] combined with sequence conservation of both ligands and receptors in various species suggests that these ligand/receptor systems modulate basic functions. A phylogenetic analysis of the relaxins and their co-evolved receptors further suggests the RXFP3/relaxin-3 and RXFP4/INSL5 ligands/receptors are the most ancient ligand/receptor pairs.

Because of the nature of afferent and efferent projections of the nucleus incertus, the predominant source of relaxin-3 in the rat, it has been proposed that this nucleus is central to a midline behaviour control network of the brainstem [50,116]. The nucleus incertus, also known as the nucleus O or ventromedial dorsal tegmental nucleus, has extensive projections to areas rich in relaxin-3 binding sites revealed using the RXFP3-specific tracer ^{125}I-relaxin-3/INSL5 [50]. The strongest autoradiographic localisation of RXFP3 binding sites in the rat is in the superior colliculus, a nucleus best known for the control of saccadic eye movements that combines inputs from the various senses to promote adaptive motor responses. Areas that are rich in RXFP3, such as the olfactory bulb/nucleus, sensory cortex, amygdala, thalamus and spinal trigeminal tract are consistent with a role for relaxin-3 in neuroendocrine, sensory and emotional processing [50]. A potential role for relaxin-3 in stress and anxiety was recognized early since the nucleus incertus also expresses CRF-R1 [117]. In fact, essentially all relaxin-3 neurons in the nucleus incertus also express CRF-R1 [117] and most show glutamic acid decarboxylase-65-like immunoreactivity, suggesting that these cells also produce GABA [116]. A few areas known for nucleus incertus projections also bind relaxin [86], suggesting that relaxin-3 could act through RXFP1 as well as RXFP3: i.v. or i.c.v. injections of relaxin cause responses attributable to RXFP1 activation, such as fluid intake [22]. A subset of efferents from the nucleus incertus terminate in areas associated with memory processing [118] and the nucleus incertus has

been shown to contribute to the generation of hippocampal theta rhythms. Inputs to the nucleus incertus suggest that this nucleus integrates information related to hippocampal function (learning and memory), habenular processing (cognition and vulnerability to stress) and behavioural planning. In summary, the neuroanatomy of relaxin-3 and RXFP3 is consistent with roles in mnemonic processing and behavioural activation related to metabolic and stressful cues.

Physiological and pharmacological data also support a role for RXFP3 in the stress response. Relaxin-3 neurons in the nucleus incertus (nearly all of which co-express CRF-R1) respond to forced swim stress or i.c.v. CRF with increased expression of C-Fos and relaxin-3 [33]. While data published thus far has been predominantly from rodents, recent work confirms that relaxin-3-like immunoreactivity in the macaque is consistent with comparable observations in the rat [119]. In addition, CRF levels are elevated in human post-traumatic stress disorder subjects [120]. These data suggest the potential therapeutic use of RXFP3 antagonists to treat anxiety and perhaps post-traumatic stress disorder.

Recent data also support potential uses for RXFP3 modulators in metabolic disease. As mentioned above, the afferent and efferent projections of the nucleus incertus in the rat involve hypothalamic and sensory areas where RXFP3 mRNA and binding sites are found. Some of the arguments suggesting that RXFP3 is involved in stress responses are also consistent with a role in feeding behaviour that also involves hypothalamic, sensory and memory systems. Bolus injections of relaxin-3 into the third ventricle cause dose-related increases in feeding behaviour of satiated Wistar rats, while similar injections of human relaxin do not affect feeding behaviour [51]. Since RXFP3 lacks affinity for relaxin-1 and relaxin [7,121], this hyperphagic effect has been ascribed to RXFP3. Attribution of the hyperphagic effect of relaxin-3 to RXFP3 is also supported by i.c.v. injection of the selective RXFP3 agonist relaxin-3/INSL5, which causes hyperphagia in satiated rats, and is blocked by prior injection of the selective RXFP3 antagonist relaxin-3(BΔ23–27)R/INSL5[7] [122]. Tissue injections of relaxin-3 to hypothalamic nuclei expressing RXFP3 mRNA and binding sites have the same hyperphagic effect. Chronic dosing of rats with relaxin-3, given either twice daily via iPVN injection [123] or i.c.v. using Alzet minipumps [34], shows that the hyperphagic effect of RXFP3 agonists and corresponding increased body weight growth are sustained through 2 weeks of chronic dosing. These effects are reflected in increased blood leptin levels, greater epididymal fat pad masses and increased blood insulin levels [34]. Additional studies are necessary to test the potential of RXFP3 antagonists, given alone, to reduce feeding behaviour and body weight growth, as well as to investigate the impact of changes in diet composition. These results suggest therapeutic potential for RXFP3 agonists in conditions such as anorexia nervosa or cachexia. RXFP3 antagonists might also be therapeutically useful to reduce feeding and body weight gain in obesity thus potentially improving glycaemic control in type II diabetes.

Conclusions

The past five years have seen major advances in our understanding of relaxin family peptides, their receptors and signalling mechanisms. Over this time a group of structurally related insulin-like peptides have been defined and four of them, relaxin, relaxin-3, INSL3 and INSL5 have been shown to be the cognate ligands for the four relaxin family peptide receptors (RXFP1-4). Of these, the peptide/receptor pairing of relaxin/RXFP1 has been the most studied. Although the track record of relaxin as a therapeutic agent has been disappointing, with failures of clinical trials investigating its utility for cervical ripening, scleroderma and orthodontics, there is still considerable interest in its experimentally well-established anti-fibrotic properties. Most interesting is the current trial of the potential of relaxin to treat congestive heart failure. INSL3/RXFP2 have highly specialized roles in reproduction in mammals. However, INSL3 may have potential for the treatment of certain types of cryptorchidism and the role of the system in germ cell maturation in males and females may indicate a possible use for the control of fertility. The therapeutic potential for relaxin-3/RXFP3 is much clearer and although this is at a relatively early stage of development, the role of this neuropeptide/receptor system in stress and anxiety and in control of body composition has been clearly demonstrated. The discrete localisation of the relaxin-3/RXFP3 system in the brain holds out the promise of highly selective antagonists that can modulate anxiety and reduce body weight. What is clear at present is that a better understanding of the biological roles of the relaxin family peptides and their receptors will advance their utility as therapeutic targets.

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References

1 Hisaw, F.L. (1926) Experimental relaxation of the pubic ligament of the guinea pig. *Proc. Soc. Exp. Biol. Med.* 23, 661–663

2 Bathgate, R.A.D. *et al.* (2006) Physiology and molecular biology of the relaxin peptide family. In *Knobil and Neill's Physiology of Reproduction* (Neill, J.D., ed.), Academic Press

3 Wilkinson, T.N. *et al.* (2005) Coevolution of the relaxin-like peptides and their receptors. *Ann. N Y Acad. Sci.* 1041, 534–539

4 Hsu, S.Y. (2003) New insights into the evolution of the relaxin-LGR signaling system. *Trends Endocrinol. Metab.* 14, 303–309

5 Hsu, S.Y. *et al.* (2002) Activation of orphan receptors by the hormone relaxin. *Science* 25, 637–638

[7] Relaxin-3(BΔ23–27)R/INSL5, consists of the relaxin-3 B-chain with a replacement of Gly23 to Arg, a truncation at the C terminus (Gly24-Trp27 deleted), and the A-chain of INSL5.

6 Liu, C. *et al.* (2003) Identification of relaxin-3/INSL7 as a ligand for GPCR142. *J. Biol. Chem.* 278, 50765–50770
7 Liu, C. *et al.* (2003) Identification of relaxin-3/INSL7 as an endogenous ligand for the orphan G-protein-coupled receptor GPCR135. *J. Biol. Chem.* 278, 50754–50764
8 Overbeek, P.A. *et al.* (2001) A transgenic insertion causing cryptorchidism in mice. *Genesis* 30, 26–35
9 Kumagai, J. *et al.* (2002) INSL3/Leydig insulin-like peptide activates the LGR8 receptor important in testis descent. *J. Biol. Chem.* 277, 31283–31286
10 Liu, C. *et al.* (2005) INSL5 is a high affinity specific agonist for GPCR142 (GPR100). *J. Biol. Chem.* 280, 292–300
11 Braddon, S.A. (1978) Relaxin-dependent adenosine 6′,5′-monophosphate concentration changes in the mouse pubic symphysis. *Endocrinology* 102, 1292–1299
12 Sanborn, B.M. *et al.* (1980) The interaction of relaxin with the rat uterus. I. Effect on cyclic nucleotide levels and spontaneous contractile activity. *Endocrinology* 106, 1210–1215
13 Parsell, D.A. *et al.* (1996) Relaxin binds to and elicits a response from cells of the human monocytic cell line, THP-1. *J. Biol. Chem.* 271, 27936–27941
14 Hsu, S.Y. *et al.* (2000) The three subfamilies of leucine-rich repeat-containing G protein-coupled receptors (LGR): identification of LGR6 and LGR7 and the signaling mechanism for LGR7. *Mol. Endocrinol.* 14, 1257–1271
15 Bathgate, R.A. *et al.* (2006) International Union of Pharmacology LVII: Recommendations for the nomenclature of receptors for relaxin family peptides. *Pharmacol. Rev.* 58, 7–31
16 Bullesbach, E.E. and Schwabe, C. (2005) The trap-like relaxin-binding site of LGR7. *J. Biol. Chem.* 280, 14051–14056
17 Bullesbach, E.E. and Schwabe, C. (2000) The relaxin receptor-binding site geometry suggests a novel gripping mode of interaction. *J. Biol. Chem.* 275, 35276–35280
18 Samuel, C.S. *et al.* (2006) 'Relaxin' the stiffened heart and arteries: the therapeutic potential for relaxin in the treatment of cardiovascular disease. *Pharmacol. Ther.* 112, 529–552
19 Nistri, S. *et al.* (2007) Relaxin as a cardiovascular hormone: physiology, pathophysiology and therapeutic promises. *Cardiovasc. Hematol. Agents Med. Chem.* 5, 101–108
20 Bani, D. and Bigazzi, M. (2005) Clinical aspects and therapeutic perspectives of relaxin. *Curr. Med. Chem. – IEMA* 5, 403–410
21 Dschietzig, T. *et al.* (2006) Relaxin-a pleiotropic hormone and its emerging role for experimental and clinical therapeutics. *Pharmacol. Ther.* 112, 38–56
22 Sherwood, O.D. (2004) Relaxin's physiological roles and other diverse actions. *Endocr. Rev.* 25, 205–234
23 Conrad, K.P. and Novak, J. (2004) Emerging role of relaxin in renal and cardiovascular function. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 287, R250–261
24 Ivell, R. and Bathgate, R. (2006) Neohormone systems as exciting targets for drug development. *Trends Endocrinol. Metab.* 17, 123
25 Du, X.J. *et al.* (2003) Increased myocardial collagen and ventricular diastolic dysfunction in relaxin deficient mice: a gender-specific phenotype. *Cardiovasc. Res.* 57, 395–404
26 Samuel, C.S. *et al.* (2004) Relaxin-1-deficient mice develop an age-related progression of renal fibrosis. *Kidney Int.* 65, 2054–2064
27 Samuel, C.S. *et al.* (2003) Relaxin deficiency in mice is associated with an age-related progression of pulmonary fibrosis. *FASEB J.* 17, 121–123
28 Samuel, C.S. *et al.* (2005) The relaxin gene knockout mouse: a model of progressive scleroderma. *J. Invest. Dermatol.* 125, 692–699
29 Mookerjee, I. *et al.* (2006) Endogenous relaxin regulates collagen deposition in an animal model of allergic airway disease. *Endocrinology* 147, 754–761
30 Lekgabe, E.D. *et al.* (2006) The effects of relaxin and estrogen deficiency on collagen deposition and hypertrophy of nonreproductive organs. *Endocrinology* 147, 5575–5583
31 Hewitson, T.D. *et al.* (2007) Endogenous relaxin is a naturally occurring modulator of experimental renal tubulointerstitial fibrosis. *Endocrinology* 148, 660–669
32 Bathgate, R.A. *et al.* (2002) Human relaxin gene 3 (H3) and the equivalent mouse relaxin (M3) gene. Novel members of the relaxin peptide family. *J. Biol. Chem.* 277, 1148–1157
33 Tanaka, M. *et al.* (2005) Neurons expressing relaxin 3/INSL 7 in the nucleus incertus respond to stress. *Eur. J. Neurosci.* 21, 1659–1670
34 Hida, T. *et al.* (2006) Chronic intracerebroventricular administration of relaxin-3 increases body weight in rats. *J. Recept. Signal. Transduct. Res.* 26, 147–158
35 Zimmermann, S. *et al.* (1999) Targeted disruption of the INSL3 gene causes bilateral cryptorchidism. *Mol. Endocrinol.* 13, 681–691
36 Nef, S. and Parada, L.F. (1999) Cryptorchidism in mice mutant for INSL3. *Nat. Genet.* 22, 295–299
37 Kawamura, K. *et al.* (2004) Paracrine regulation of mammalian oocyte maturation and male germ cell survival. *Proc. Natl. Acad. Sci. U.S.A.* 101, 7323–7328
38 (1999) Identification of INSL5, a new member of the insulin superfamily. *Genomics* 60, 50–56
39 Conklin, D. *et al.* (2005) Gene finding for the helical cytokines. *Bioinformatics* 21, 1776–1781
40 Scott, D.J. *et al.* (2005) Characterization of the mouse and rat relaxin receptors. *Ann. N Y Acad. Sci.* 1041, 8–12
41 Scott, D.J. *et al.* (2005) Characterization of the rat INSL3 receptor. *Ann. N Y Acad. Sci.* 1041, 13–16
42 Rosengren, K.J. *et al.* (2006) Solution structure and novel insights into the determinants of the receptor specificity of human relaxin-3. *J. Biol. Chem.* 281, 5845–5851
43 Halls, M.L. *et al.* (2005) Multiple binding sites revealed by interaction of relaxin family peptides with native and chimeric relaxin family peptide receptors 1 and 2 (LGR7 and LGR8). *J. Pharmacol. Exp. Ther.* 313, 677–687
44 Bullesbach, E.E. and Schwabe, C. (2005) LGR8 signal activation by the relaxin-like factor. *J. Biol. Chem.* 280, 14856–14890
45 Sudo, S. *et al.* (2003) H3 relaxin is a specific ligand for LGR7 and activates the receptor by interacting with both the ectodomain and the exoloop 2. *J. Biol. Chem.* 278, 7855–7862
46 Scott, D.J. *et al.* (2007) Defining the LGR8 residues involved in binding insulin-like peptide 3. *Mol. Endocrinol.* 21, 1699–1712
47 Scott, D.J. *et al.* (2006) Characterization of novel splice variants of LGR7 and LGR8 reveals that receptor signaling is mediated by their unique LDLa modules. *J. Biol. Chem.* 281, 34942–34954
48 Del Borgo, M.P. *et al.* (2006) Analogs of insulin-like peptide 3 (INSL3) B-chain are LGR8 antagonists *in vitro* and *in vivo*. *J. Biol. Chem.* 281, 13068–13074
49 Hopkins, E.J. *et al.* (2007) The NMR solution structure of the relaxin (RXFP1) receptor lipoprotein receptor class A module and identification of key residues in the N-terminal region of the module that mediate receptor activation. *J. Biol. Chem.* 282, 4172–4184
50 Sutton, S.W. *et al.* (2004) Distribution of G-protein-coupled receptor (GPCR)135 binding sites and receptor mRNA in the rat brain suggests a role for relaxin-3 in neuroendocrine and sensory processing. *Neuroendocrinology* 80, 298–307
51 McGowan, B.M. *et al.* (2005) Central relaxin-3 administration causes hyperphagia in male Wistar rats. *Endocrinology* 146, 3295–3300
52 Liu, C. *et al.* (2005) Relaxin-3/insulin-like peptide 5 chimeric peptide, a selective ligand for G protein-coupled receptor (GPCR)135 and GPCR142 over leucine-rich repeat-containing G protein-coupled receptor 7. *Mol. Pharmacol.* 67, 231–240
53 Halls, M.L. *et al.* (2006) Relaxin family peptide receptors RXFP1 and RXFP2 modulate cAMP signaling by distinct mechanisms. *Mol. Pharmacol.* 70, 214–226
54 Nguyen, B.T. and Dessauer, C.W. (2005) Relaxin stimulates protein kinase C zeta translocation: Requirement for cyclic adenosine 3′,5′-monophosphate production. *Mol. Endocrinol.* 19, 1012–1023
55 Nguyen, B.T. *et al.* (2003) Phosphoinositide 3-kinase activity is required for biphasic stimulation of cyclic adenosine 3′,5′-monophosphate by relaxin. *Mol. Endocrinol.* 17, 1075–1084
56 Tabanelli, S. *et al.* (1992) In vitro decidualization of human endometrial stromal cells. *J. Steroid Biochem. Mol. Biol.* 42, 337–344
57 Kompa, A.R. *et al.* (2002) Inotropic responses to human gene 2 (B29) relaxin in a rat model of myocardial infarction (MI): effect of pertussis toxin. *Br. J. Pharmacol.* 137, 710–718
58 Zhang, Q. *et al.* (2002) Relaxin activates the MAP kinase pathway in human endometrial stromal cells. *J. Cell Biochem.* 85, 536–544
59 Moore, X.L. *et al.* (2007) Relaxin antagonizes hypertrophy and apoptosis in neonatal rat cardiomyocytes. *Endocrinology* 148, 1582–1589
60 Nistri, S. and Bani, D. (2003) Relaxin receptors and nitric oxide synthases: search for the missing link. *Reprod. Biol. Endocrinol.* 1, 5
61 Cosen-Binker, L.I. *et al.* (2006) Relaxin prevents the development of severe acute pancreatitis. *World J. Gastroenterol.* 12, 1558–1568
62 Masini, E. *et al.* (2006) Protective effects of relaxin in ischemia/reperfusion-induced intestinal injury due to splanchnic artery occlusion. *Br. J. Pharmacol.* 148, 1124–1132
63 Zhang, J. *et al.* (2005) Effect of relaxin on myocardial ischemia injury induced by isoproterenol. *Peptides* 26, 1632–1639
64 Boehnert, M.U. *et al.* (2005) Relaxin as an additional protective substance in preserving and reperfusion solution for liver transplantation, shown in a model of isolated perfused rat liver. *Ann. N Y Acad. Sci.* 1041, 434–440
65 Perna, A.M. *et al.* (2005) Novel drug development opportunity for relaxin in acute myocardial infarction: evidences from a swine model. *FASEB J.* 19, 1525–1527
66 Bani, D. *et al.* (1998) Relaxin protects against myocardial injury caused by ischemia and reperfusion in rat heart. *Am. J. Pathol.* 152, 1367–1376

67 Nistri, S. *et al.* (2003) Relaxin inhibits lipopolysaccharide-induced adhesion of neutrophils to coronary endothelial cells by a nitric oxide-mediated mechanism. *FASEB J.* 17, 2109–2111

68 Failli, P. *et al.* (2002) Relaxin upregulates inducible nitric oxide synthase expression and nitric oxide generation in rat coronary endothelial cells. *FASEB J.* 16, 252–254

69 Masini, E. *et al.* (1997) Relaxin counteracts myocardial damage induced by ischemia-reperfusion in isolated guinea pig hearts: evidence for an involvement of nitric oxide. *Endocrinology* 138, 4713–4720

70 BaniSacchi, T. *et al.* (1995) Relaxin-induced increased coronary flow through stimulation of nitric oxide production. *Br. J. Pharmacol.* 116, 1589–1594

71 Wilson, B.C. *et al.* (2006) Relaxin-induced reduction of infarct size in male rats receiving MCAO is dependent on nitric oxide synthesis and not estrogenic mechanisms. *Neurosci. Lett.* 393, 160–164

72 van der Westhuizen, E.T. *et al.* (2007) The relaxin family peptide receptor 3 (RXFP3) activates ERK1/2 through a PKC dependent mechanism. *Mol. Pharmacol.* 71, 1618–1629

73 Morikawa, Y. *et al.* (2004) Fasting-induced activation of mitogen-activated protein kinases (ERK/p38) in the mouse hypothalamus. *J. Neuroendocrinol.* 16, 105–112

74 Shen, C.P. *et al.* (2004) Activation of Erk and JNK MAPK pathways by acute swim stress in rat brain regions. *BMC Neurosci.* 5, 36

75 Apergis-Schoute, A.M. *et al.* (2005) Auditory fear conditioning and long-term potentiation in the lateral amygdala require ERK/MAP kinase signaling in the auditory thalamus: a role for presynaptic plasticity in the fear system. *J. Neurosci.* 25, 5730–5739

76 Carrasquillo, Y. and Gereau, R.W.t. (2007) Activation of the extracellular signal-regulated kinase in the amygdala modulates pain perception. *J. Neurosci.* 27, 1543–1551

77 Zink, A. *et al.* (1992) Inhibitory effect of somatostatin on cAMP accumulation and calcitonin secretion in C-cells: involvement of pertussis toxin-sensitive G-proteins. *Mol. Cell Endocrinol.* 86, 213–219

78 Kohsaka, T. *et al.* (1998) Identification of specific relaxin-binding cells in the human female. *Biol. Reprod.* 59, 991–999

79 Kuenzi, M.J. and Sherwood, O.D. (1995) Immunohistochemical localization of specific relaxin-binding cells in the cervix, mammary glands, and nipples of pregnant rats. *Endocrinology* 136, 1367–1373

80 Goldsmith, L.T. *et al.* (2004) Relaxin regulation of endometrial structure and function in the rhesus monkey. *Proc. Natl. Acad. Sci. U.S.A.* 101, 4685–4689

81 Einspanier, A. *et al.* (2001) Characterization of relaxin binding in the uterus of the marmoset monkey. *Mol. Hum. Reprod.* 7, 963–970

82 (1991) The effect of human chorionic gonadotropin and pregnancy on the circulating level of relaxin. *J. Clin. Endocrinol. Metab.* 72, 1042–1047

83 Weiss, G. (1989) Relaxin in the male. *Biol. Reprod.* 40, 197–200

84 Anand-Ivell, R.J. *et al.* (2006) Expression of the insulin-like peptide 3 (INSL3) hormone-receptor (LGR8) system in the testis. *Biol. Reprod.* 74, 945–953

85 Tan, Y.Y. *et al.* (1999) Quantitative autoradiographic studies of relaxin binding in rat atria, uterus and cerebral cortex: characterization and effects of oestrogen treatment. *Br. J. Pharmacol.* 127, 91–98

86 Osheroff, P.L. and Phillips, H.S. (1991) Autoradiographic localization of relaxin binding sites in rat brain. *Proc. Natl. Acad. Sci. U.S.A.* 88, 6413–6417

87 (1999) Water drinking in rats resulting from intravenous relaxin and its modification by other dipsogenic factors. *Endocrinology* 140, 5082–5086

88 Sunn, N. *et al.* (2002) Circulating relaxin acts on subfornical organ neurons to stimulate water drinking in the rat. *Proc. Natl. Acad. Sci. U.S.A.* 99, 1701–1706

89 Shen, P.J. *et al.* (2005) Restricted expression of LGR8 in intralaminar thalamic nuclei of rat brain suggests a role in sensorimotor systems. *Ann. N Y Acad. Sci.* 1041, 510–515

90 Novak, J. *et al.* (2001) Relaxin is essential for renal vasodilation during pregnancy in conscious rats. *J. Clin. Invest.* 107, 1469–1475

91 Danielson, L.A. *et al.* (1999) Relaxin is a potent renal vasodilator in conscious rats. *J. Clin. Invest.* 103, 525–533

92 Fu, P. *et al.* (2005) Detection, localization, and action of the INSL3 receptor, LGR8, in rat kidney. *Ann. N Y Acad. Sci.* 1041, 516–519

93 Unemori, E.N. and Amento, E.P. (1990) Relaxin modulates synthesis and secretion of procollagenase and collagen by human dermal fibroblasts. *J. Biol. Chem.* 265, 10681–10685

94 Unemori, E.N. *et al.* (1996) Relaxin induces an extracellular matrix-degrading phenotype in human lung fibroblasts *in vitro* and inhibits lung fibrosis in a murine model *in vivo*. *J. Clin. Invest.* 98, 2739–2745

95 Samuel, C.S. *et al.* (2004) Relaxin modulates cardiac fibroblast proliferation, differentiation, and collagen production and reverses cardiac fibrosis *in vivo*. *Endocrinology* 145, 4125–4133

96 Unemori, E.N. *et al.* (1993) Human relaxin decreases collagen accumulation *in vivo* in two rodent models of fibrosis. *J. Invest. Dermatol.* 101, 280–285

97 Garber, S.L. *et al.* (2001) Relaxin decreases renal interstitial fibrosis and slows progression of renal disease. *Kidney Int.* 59, 876–882

98 Amento, E.P. *et al.* (2001) Deletion of the relaxin gene causes age-related progressive dermal fibrosis. *Arthritis Rheum.* 44, A847

99 Krajnc-Franken, M.A. *et al.* (2004) Impaired nipple development and parturition in LGR7 knockout mice. *Mol. Cell Biol.* 24, 687–696

100 Zhao, L. *et al.* (1999) Mice without a functional relaxin gene are unable to deliver milk to their pups. *Endocrinology* 140, 445–453

101 (1995) Relaxin acts directly on rat mammary nipples to stimulate their growth. *Endocrinology* 136, 2943–2947

102 Samuel, C.S. *et al.* (2003) Relaxin is a key mediator of prostate growth and male reproductive tract development. *Lab Invest.* 83, 1055–1067

103 Kamat, A.A. *et al.* (2004) Genetic targeting of relaxin and insulin-like factor 3 receptors in mice. *Endocrinology* 145, 4712–4720

104 Adham, I.M. *et al.* (2002) The overexpression of the insl3 in female mice causes descent of the ovaries. *Mol. Endocrinol.* 16, 244–252

105 Seibold, J.R. *et al.* (2000) Recombinant human relaxin in the treatment of scleroderma. A randomized, double-blind, placebo-controlled trial. *Ann. Intern. Med.* 132, 871–879

106 (1993) A randomized, double-blind, placebo-controlled trial of the safety of vaginal recombinant human relaxin for cervical ripening. *Obstet. Gynecol.* 82, 328–333

107 Erikson, M.S. and Unemori, E.N. (2001) Relaxin clinical trials in systemic sclerosis. In *Relaxin 2000: Proceedings of the Third International Conference on Relaxin and Related Peptides* (Tregear, G.W. *et al.* eds), pp. 373–382, Kluwer

108 Capeless, E.L. and Clapp, J.F. (1989) Cardiovascular changes in early phase of pregnancy. *Am. J. Obstet. Gynecol.* 161 (6 Pt 1), 1449–1453

109 Stewart, D.R. *et al.* (1990) Relaxin in the peri-implantation period. *J. Clin. Endocrinol. Metab.* 70, 1771–1773

110 Dschietzig, T. *et al.* (2007) A pilot safety and dose-finding trial of intravenous recombinant human relaxin (rhRlx) in compensated congestive heart failure. *Eur. Heart J.* 28, 387

111 Maynard, S.E. *et al.* (2003) Excess placental soluble fms-like tyrosine kinase 1 (sFlt1) may contribute to endothelial dysfunction, hypertension, and proteinuria in preeclampsia. *J. Clin. Invest.* 111, 649–658

112 (1967) Effects of relaxin on the endothelium of endometrial blood vessels in monkeys (Macaca mulatta). *Endocrinology* 81, 375–385

113 (1995) Relaxin depresses platelet aggregation: *in vitro* studies on isolated human and rabbit platelets. *Lab Invest.* 73, 709–716

114 Bani, D. *et al.* (2007) A novel, simple bioactivity assay for relaxin based on inhibition of platelet aggregation. *Regul. Pept.* 144, 10–16

115 Unemori, E.N. *et al.* (1999) Relaxin stimulates expression of vascular endothelial growth factor in normal human endometrial cells *in vitro* and is associated with menometrorrhagia in women. *Hum.Reproduct.* 14, 800–806

116 Ma, S. *et al.* (2007) Relaxin-3 in GABA projection neurons of nucleus incertus suggests widespread influence on forebrain circuits via G-protein-coupled receptor-135 in the rat. *Neuroscience* 144, 165–190

117 Potter, E. *et al.* (1994) Distribution of corticotropin-releasing factor receptor mRNA expression in the rat brain and pituitary. *Proc. Natl. Acad. Sci. U.S.A.* 91, 8777–8781

118 Olucha-Bordonau, F.E. *et al.* (2003) Cytoarchitecture and efferent projections of the nucleus incertus of the rat. *J. Comp. Neurol.* 464, 62–97

119 Ma, S. *et al.* (2007) Distribution of relaxin-3 immunoreactivity in primate brain. *7th IBRO World Congress of Neuroscience* pp. POS-SAT-045

120 Bremner, J.D. *et al.* (1997) Elevated CSF corticotropin-releasing factor concentrations in posttraumatic stress disorder. *Am. J. Psychiatry* 154, 624–629

121 Van Der Westhuizen, E. *et al.* (2005) Responses of GPCR135 to human gene 3 (H3) relaxin in CHO-K1 cells determined by microphysiometry. *Ann. N Y Acad. Sci.* 1041, 332–337

122 Kuei, C. *et al.* (2007) R3(Δ23-27)R/15 chimeric peptide, a selective antagonist for GPCR135 and GPCR142 over relaxin receptor LGR7: *in vitro* and *in vivo* characterization. *J. Biol. Chem.* 282, 25425–25435

123 McGowan, B.M. *et al.* (2006) Effects of acute and chronic relaxin-3 on food intake and energy expenditure in rats. *Regul. Pept.* 136, 72–77