British Journal of Pharmacology

REVIEW ARTICLE THEMED ISSUE

Relaxin-like peptides in male reproduction – a human perspective

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Received 21 July 2016; **Revised** 10 November 2016; **Accepted** 5 December 2016

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The relaxin family of peptide hormones and their cognate GPCRs are becoming physiologically well-characterized in the cardiovascular system and particularly in female reproductive processes. Much less is known about the physiology and pharmacology of these peptides in male reproduction, particularly as regards humans. H2-relaxin is involved in prostate function and growth, while insulin-like peptide 3 (INSL3) is a major product of the testicular Leydig cells and, in the adult, appears to modulate steroidogenesis and germ cell survival. In the fetus, INSL3 is a key hormone expressed shortly after sex determination and is responsible for the first transabdominal phase of testicular descent. Importantly, INSL3 is becoming a very useful constitutive biomarker reflecting both fetal and post-natal development. Nothing is known about roles for INSL4 in male reproduction and only very little about relaxin-3, which is mostly considered as a brain peptide, or INSL5. The former is expressed at very low levels in the testes, but has no known physiology there, whereas the INSL5 knockout mouse does exhibit a testicular phenotype with mild effects on spermatogenesis, probably due to a disruption of glucose homeostasis. INSL6 is a major product of male germ cells, although it is relatively unexplored with regard to its physiology or pharmacology, except that in mice disruption of the INSL6 gene leads to a disruption of spermatogenesis. Clinically, relaxin analogues may be useful in the control of prostate cancer, and both relaxin and INSL3 have been considered as sperm adjuvants for *in vitro* fertilization.

Abbreviations

GEO, gene expression omnibus; INSL3–6, insulin-like peptide 3–6; LGR7, leucine-rich repeat-containing GPCR 7, synonymous with RXFP1; LH, luteinizing hormone; RLN2, H2-relaxin; RLN3, relaxin-3; RXFP1–4, relaxin family peptide receptor 1–4

DOI:10.1111/bph.13689

Tables of Links

TARGETS	
RXFP1 receptor	RXFP3 receptor
RXFP2 receptor	RXFP4 receptor

LIGANDS	
H2 relaxin (RNL2)	Relaxin-1
INSL3	Relaxin-3 (RNL3)
INSL5	

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan *et al.*, 2016), and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/16 (Alexander *et al.*, 2015).

Introduction

The relaxin-like peptides represent a family of peptide hormones, which appear to have evolved in both vertebrate and invertebrate lineages, evidently taking advantage of the rigid peptide scaffold involving multiple cysteine bridges common also to insulin and the insulin-like growth factors (Yegorov and Good, 2012; Yegorov *et al.*, 2014). This scaffold allows the manifestation of a small relatively rigid three-dimensional surface whose specificity of interaction is dictated by the charges and side-groups of component amino acids. Largely based on insulin and early studies on relaxin in the pig, it is assumed that the mature circulating peptides exist mostly as the ca. 6 kDa A–B heterodimers, which appear to exhibit all of the epitopes essential for specific activation of their cognate receptors. These heterodimers never appear to be further modified by glycosylation or other post-translational changes, such that their informational content resides exclusively within the folded primary amino acid sequence. However, it is important to note that at least for relaxin and insulin-like peptide 3 (INSL3), it is likely that a large proportion of the secreted bioactive hormone is in fact represented by the A–B–C unprocessed pro-form (MW 12–18 kDa), which retains the flexible intermediate C (connecting) peptide, which at least in its interaction with receptors does not appear to hinder specificity or binding in any way (Zarreh-Hoshyari-Khah *et al.*, 1999, 2001; Luo *et al.*, 2009; Minagawa *et al.*, 2012). Whether the C-peptide has any function in its own right or contributes to the stability or half-life of the peptides is not known. There is little or no information concerning the other members of the relaxin family, except that for relaxin-3 and INSL5 binding to their respective cognate receptors, relaxin family peptide receptor 3 (RXFP3) and RXFP4, it would appear that cleavage of the C-peptide to reveal the C-terminus of the B-chain is necessary for full bioactivity (Luo *et al.*, 2010a,b). From a technical perspective, immunoassays using antibodies raised against the A–B heterodimer, such as for relaxin or INSL3, will usually recognize both the 6 kDa heterodimer as well as the larger pro-form (e.g. Zarreh-Hoshyari-Khah *et al.*, 1999; Ivell R and Anand-Ivell R, unpublished observations). Western blotting of tissue extracts on the other hand, where successful, will usually visualize the large pro-form only, and only in exceptional circumstances is the smaller heterodimer evident (e.g. Klonisch *et al.*, 2003).

Whilst the principal branches of the vertebrate relaxin-like family have existed since early chordates, notably INSL3 and ovarian H2 relaxin have evolved substantially to accommodate the new functions acquired by mammals in the context of viviparity. This has led to the coining of the term 'neohormones' for all such peptides that have acquired regulatory or modulatory roles specifically linked to mammalian traits, such as internal fertilization, a scrotal testis, lactation and maternal behaviour (Ivell and Bathgate, 2006; Anand-Ivell *et al.*, 2013a). Importantly, the H2 relaxin locus, which also includes relaxin-1 (also known as H1 relaxin), INSL4 and INSL6, is closely linked to the DMRT1 gene, which is recognized as the ancient sex-determination system for all vertebrates, suggesting an important role for these peptides in reproductive function (Ivell and Grutzner, 2009).

It is a feature of mammalian reproduction that the new adaptations to viviparity, such as maternal recognition of pregnancy, mode of placentation and implantation and uterine or vaginal ejaculation, are highly variable. Accordingly, it is not therefore surprising that those neohormones regulating such processes are also very variable in their expression and mode of action between species. The relaxin family of peptide hormones is no exception. For that reason, in the present review, emphasis will be given primarily to the situation in humans, and animal models referred to where they support or diverge from the human pattern.

Relaxin-like peptides and their receptors

Of the relaxin-like family of peptide hormones in humans, relaxin-3 and INSL5 appear to be restricted in their expression to the brain and gastrointestinal tract respectively. In a reproductive context, the major players are H2 relaxin and INSL3. The roles of relaxin-1, INSL4 and INSL6 are less clear. Unlike relaxin-3 and INSL5, which appear to specifically address a class of GPCR with short extracellular domains, RXFP3 and RXFP4, respectively, H2 relaxin and INSL3 are ligands for a quite different class of GPCR, RXFP1 and RXFP2 respectively (Bathgate *et al.*, 2006a). These latter receptors are both characterized by very large extracellular domains, comprising a single LDLa receptor type A module and 10 leucine-rich repeats (Bathgate *et al.*, 2006a) and, at least in transfected cell systems, mostly act by signalling through Gs to activate adenylyl cyclase, unlike RXFP3 and RXFP4, which primarily

activate the PI3-kinase pathway. These act predominantly through Gi and inhibit cAMP generation. The structure and activity of these receptors are discussed in greater detail elsewhere (Halls *et al.,* 2015).

In a physiological context, it is important to note that the EC_{50} for pure recombinant H2 relaxin at human RXFP1 receptors is ca. 0.7 nM and, at human RXFP2 receptors, ca. 50 nM; human INSL3 is unable to bind to RXFP1 receptors at all, but interacts with human RXFP2 receptors with an EC_{50} of ca. 0.6 nM (Halls *et al.,* 2015). Relaxin 3 can also interact with human RXFP1 receptors, with an EC_{50} of ca. 15 nM, but cannot interact with human RXFP2 receptors (Bathgate *et al.,* 2006b). These data are important because, even at the highest concentrations recorded, H2 relaxin in peripheral blood in women during pregnancy does not exceed ca. 200 $pg{\cdot}mL^{-1}$ (equivalent to ca. 35 pM) (Sherwood, 1994). Similarly, INSL3 in blood in women also does not exceed ca. 200 $pg{\cdot}mL^{-1}$ (Anand-Ivell *et al.,* 2013b), whilst in men, it can reach ca. 5 $ng{\cdot}mL^{-1}$ (equivalent to ca. 900 pM) (Anand-Ivell *et al.,* 2006b). Thus, in humans, H2 relaxin and INSL3 acting as endocrine hormones must be absolutely specific for RXFP1 and RXFP2 receptors respectively. This may be different within source tissues where relaxin may be co-located with RXFP1 and/or RXFP2 receptors. For example, in human follicular fluid relaxin has been estimated at 1.8 $ng{\cdot}mg^{-1}$ protein (Bastu *et al.,* 2015), which, by applying a protein content of 56 $mg{\cdot}mL^{-1}$ (Valckx *et al.,* 2012), amounts to approximately 17 nM. The concentration may be higher in other locations. Thus, only in source tissues is there a possibility for a low level activation of RXFP2 receptors by H2 relaxin, although not at all for RXFP1 receptors by INSL3. There is only a single reference to RLN3 levels in human blood (Ghattas *et al.,* 2013), although the source of this RLN3 remains unknown. Moreover, the data provided are evidently erroneous: they exceed the upper limit of detection of the assay, as indicated by its manufacturer, by 1000-fold, and validation of assay cross-reactivity to related peptides is not reported. Neither INSL4 nor INSL6 is able to interact with either human RXFP1 or human RXFP2 receptors (Bogatcheva *et al.,* 2003), and to date, these peptides still do not have identifiable receptors of their own.

Relaxin and RXFP1 receptors in the male reproductive system

H2 relaxin mRNA has been detected in the human testis (Figure 1) using multiple tissue microarrays, although there has been no systematic study of this tissue to identify its precise cellular location. Immunohistochemistry has failed to

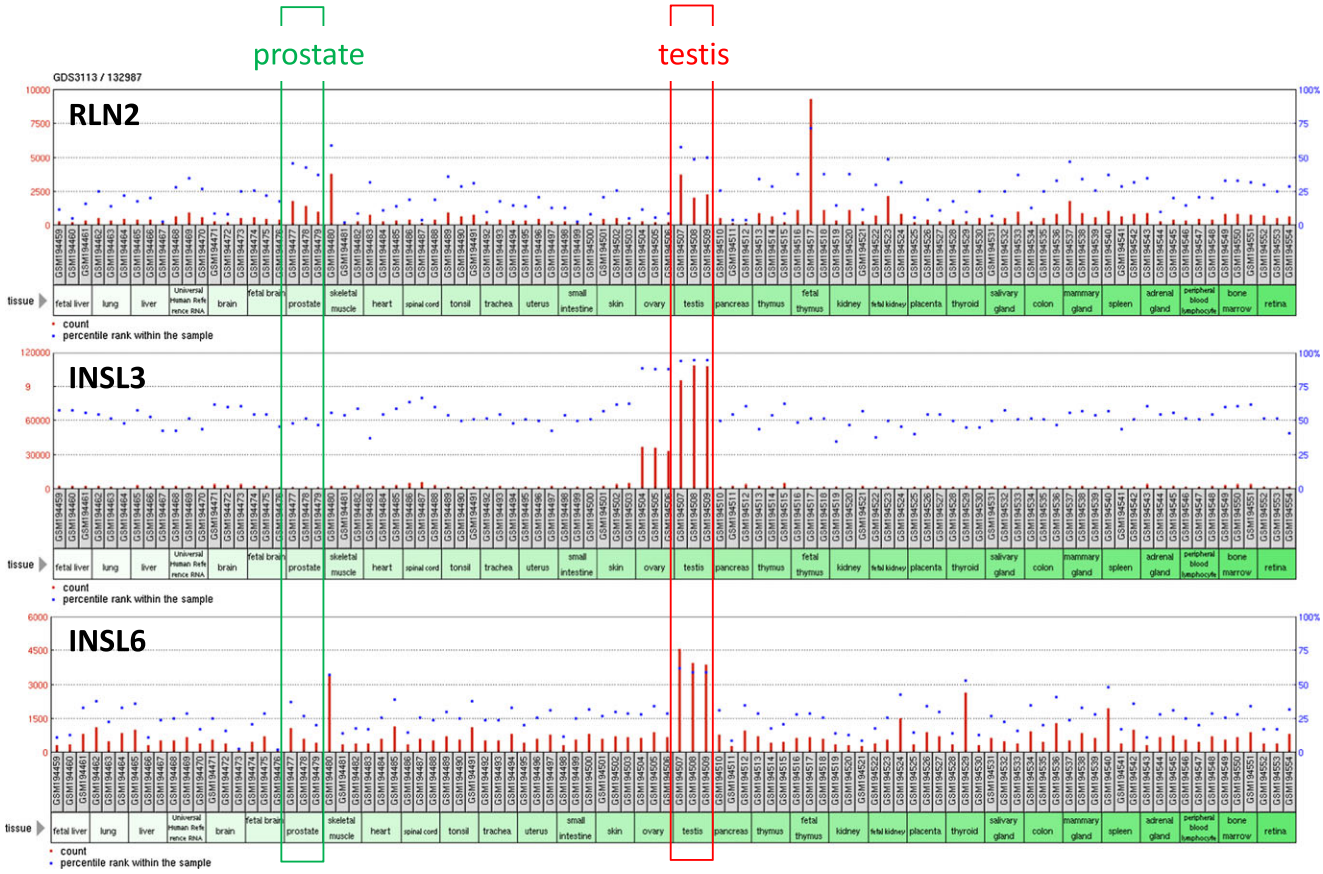

Figure 1

Microarray gene transcript profiles for multiple human tissues for the genes RLN2, INSL3 and INSL6, extracted from the National Centre for Biotechnology Information GEO database. Prostate and testis tissues are highlighted in green and red boxes, respectively.

find specific staining within the human testis using an anti-porcine relaxin antibody (Yki-Järvinen *et al.,* 1983). In contrast, the human prostate gland appears to be a relatively consistent source of H2 relaxin expression, as determined by microarrays (Figure 2) as well as by RT-PCR (Ivell *et al.,* 1989) and at the immunohistochemical level in the secretory epithelium (Yki-Järvinen *et al.*, 1983, Feng *et al.,* 2007). This is reinforced by its identification also in cultured prostatic cells and in prostate carcinoma (Feng *et al.,* 2007; Silvertown *et al.,* 2007; Domińska *et al.,* 2016a). Other tissues of the human male reproductive system have not been assessed. It seems likely that the immunoreactive relaxin identified in human seminal plasma (Winslow *et al.,* 1992; Armbruster *et al.,* 2001), according to its structure and sequence, is H2 relaxin (Winslow *et al.,* 1992) and is of prostatic origin. Although some older studies have reported very high levels of seminal relaxin, this might be due to a cross-reactivity and non-specificity of the antibodies used (reviewed in Ivell *et al.,* 2011). With relaxin present in ejaculated seminal plasma, it is logical to assume that this might react with specific receptors within the distal female tract. The RXFP1 receptor is expressed throughout the female reproductive tract, including the cervix and vagina, although there is some doubt as to its cellular localization with, in addition to smooth muscle cells, both epithelial and stromal cells being implicated (Yao *et al.,* 2009; Soh *et al.,* 2012). Most such studies have been carried out in rodents, where there is a strong cervical response to relaxin; however, clinical trials have failed to show a significant effect of relaxin on cervical softening in women at term of pregnancy, when the greatest response to introduced relaxin might be expected (Weiss *et al.,* 2016).

Relatively little is known about the expression of RXFP1 receptors in the human male tract. The application of various specific anti-RXFP1 antibodies to human testis sections failed to identify specific epitopes (RI, unpublished), and no quantitatively significant hybridization signals for RXFP1 mRNA have been shown for the testis on human tissue microarrays, although it can be detected in human testis by RT-PCR (Silvertown *et al.,* 2010). This is different for the human prostate gland, where consistent RXFP1 (LGR7) mRNA expression is evident in both normal and cancer tissues (Feng *et al.,* 2007). The RXFP1 receptor has also been identified on ejaculated human spermatozoa (Gianesello *et al.,* 2009; Ferlin *et al.,* 2012), and relaxin has been shown to affect *in vitro* human sperm parameters by decreasing apoptosis and increasing mitochondrial activity, hyperactivation, calcium and cAMP levels, as well as the acrosome reaction (Ferlin *et al.,* 2012).

Several studies have looked at the role of relaxin and RXFP1 receptors in human prostate cancer and its progression. In particular, whilst RXFP1 receptor levels do not appear to differ between normal and cancerous tissue, relaxin peptide is more highly expressed in the latter cells (Thompson *et al.*, 2006; Feng *et al.,* 2007; Vinall *et al.,* 2011), and an increased expression of RLN2 was reported during neuroendocrine differentiation of prostate cells (Figueiredo *et al.,* 2005). It was also shown that the mutant P53 regulates RLN2 expression through direct binding to its gene promoter (Vinall *et al.,* 2006). It appears that H2 relaxin can regulate the expression of prostate-specific antigen through an androgen-mediated pathway by activation of the PI3K/Akt/NFκB and components of β-catenin/Wnt signalling (Liu *et al.,* 2008;

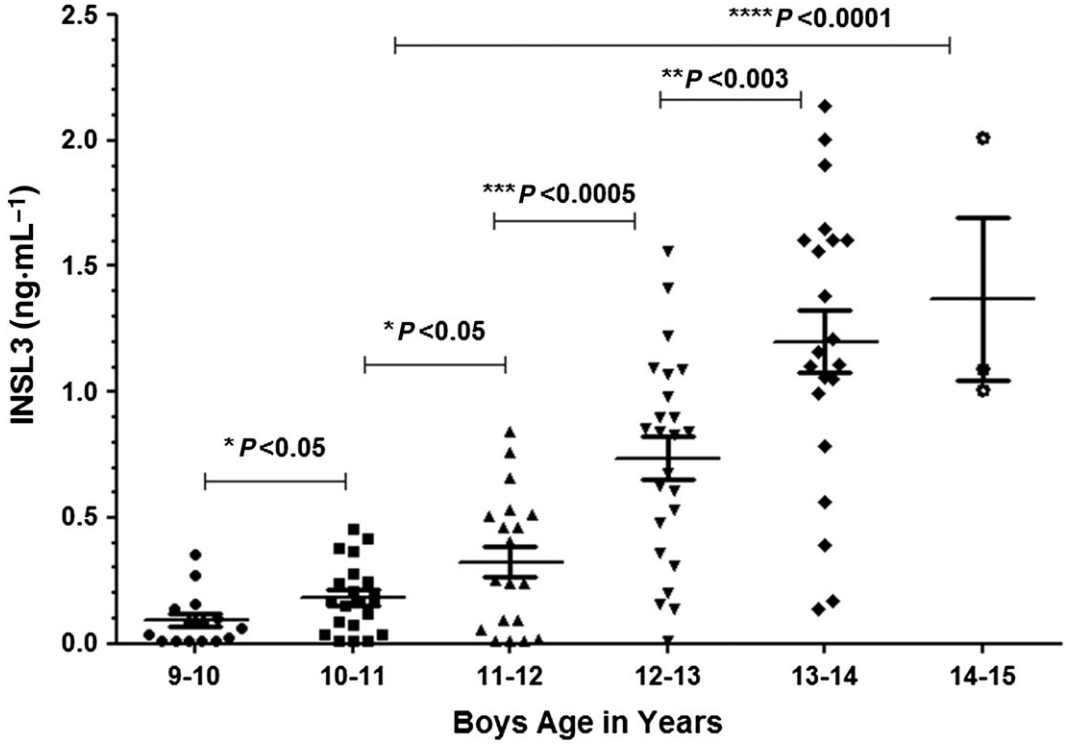

Figure 2

INSL3 peptide in the circulation of boys during pubertal development (based on data published in Johansen *et al.* 2014).

Thompson *et al.,* 2010; Vinall *et al.,* 2011). In cell culture, relaxin acts on prostate cancer lines to increase proliferation, migration and invasiveness, presumably by increasing the turnover of extracellular matrix (Klonisch *et al.,* 2007; Feng *et al.,* 2010; Vinall *et al.,* 2011; Domińska *et al.,* 2016b). Moreover, transgenic overexpression of relaxin causes an increased tumour growth in transgenic adenocarcinoma of the mouse prostate resulting in a shorter survival time of the mice (Feng *et al.,* 2007). Similarly, xenografts with cells overexpressing RLN2 grow faster and are more vascularized (Silvertown *et al.,* 2006). Finally, suppression of RXFP1 with siRNA or peptide antagonists decreases human cancer cell xenograft growth in immunocompromised mice (Silvertown *et al.,* 2007; Feng *et al.,* 2010; Feng and Agoulnik, 2011; Neschadim *et al.,* 2014).

Relaxin and RXFP1 receptors have been more intensively studied in various animal models, including rats, mice and pigs. Whilst similar in some ways to the human, each of these species also exhibits significant differences from the human situation. The boar testis expresses substantial amounts of relaxin exclusively from the interstitial Leydig cells, confirming an earlier immunohistochemical study (Dubois and Dacheux, 1978), and it appears to act on RXFP1 receptors located both on Leydig cells as well as on germ cells within the seminiferous epithelium (Min and Sherwood, 1998; Kato *et al.,* 2010). Another immunohistochemical analysis using the pig, however, failed to identify any specific relaxin signal not only in testis but also in the prostate, epididymis or bulbo-urethral gland (Kohsaka *et al.,* 1992). This study only showed relaxin immunoreactivity in the seminal vesicles. In mice, microarray hybridization [gene expression omnibus (GEO) database] generally fails to indicate relaxin mRNA signals in the testes, although specific RT-PCR does identify relaxin mRNA both here and in the epididymis, but not in any other tissues of the male tract (RI, unpublished observations). RXFP1 mRNA was identified by RT-PCR in diverse somatic cells of the mouse testis (Ivell *et al.,* 2011). An earlier study revealed the expression of both relaxin and RXFP1 receptors in the mouse testis (Samuel *et al.,* 2003). The rat testis also expresses relaxin mRNA but apparently in the Sertoli cells (Cardoso *et al.,* 2010), rather than in the interstitial compartment. Also, the prostate gland of this species appears to express relaxin mRNA (Cardoso *et al.,* 2010). RXFP1 receptor mRNA has also been identified in rat Sertoli cells as well as in the prostate gland and in the vas deferens (Cardoso *et al.,* 2010), supporting functional studies suggesting that relaxin affects Sertoli cell signalling and the vas deferens (Cardoso *et al.,* 2010; Nascimento *et al.,* 2012).

Knockout mice have been generated with deletions in both the relaxin gene as well as in the gene for RXFP1 receptors (Zhao *et al.,* 1999; Kamat *et al.,* 2004; Kaftanovskaya *et al.,* 2015a). The resulting phenotype in the male and its interpretation is somewhat controversial. Whereas the initial descriptions indicated a modest disruption of spermatogenesis in early generations, it appears that this phenotype is largely lost in later generations. In agreement with studies on different organ systems (Bennett, 2009), the loss of relaxin production appeared to induce a local fibrosis in both the testes and prostate gland (Samuel *et al.,* 2003). This, however, was not evident in studies carried out on similar animals several years later (Ganesan *et al.,* 2009; Ivell *et al.,* 2011).

In conclusion, there appear to be substantial differences between species in the relative levels of expression, particularly of relaxin, in different organs of the male reproductive system, and caution is therefore warranted in translating findings from animal models to humans.

INSL3 in the male reproductive system

INSL3, because of its similarity to relaxin, was originally referred to as the relaxin-like factor. It was independently identified, using differential cloning techniques, as a highly expressed transcript in the testes of boars (Adham *et al.,* 1993) and mice (Pusch *et al.,* 1996). Subsequently, it was also shown to be expressed at high levels in the human testis (Ivell *et al.,* 1997). In all mammalian species so far examined, INSL3 is a major secretory product of mature interstitial Leydig cells. Moreover, INSL3 in the circulation derives exclusively from the Leydig cells and from nowhere else, making INSL3 a unique biomarker for the functional capacity of the Leydig cell (Ivell *et al.,* 2013). In normal men, the circulating INSL3 concentration is approximately 1 $ng{\cdot}mL^{-1}$ (range 0.5–2.0-$ng{\cdot}mL^{-1}$), whereas in Klinefelter patients, those with severe infertility, in uniorchid men or in those with a suppressed hypothalamo-pituitary-gonadal (HPG) axis, the INSL3 concentration is significantly less or undetectable (Foresta *et al.,* 2004; Bay *et al.,* 2005). Importantly, it is expressed constitutively and is acutely independent of the HPG axis. What this means is that while the other major Leydig cell product, testosterone, can be acutely (within minutes) up-regulated by pituitary luteinizing hormone (LH), INSL3 is not affected. Nevertheless, since INSL3 is a product of more differentiated Leydig cells, factors like LH, which may chronically (days) affect the differentiation status of Leydig cells, can in the long term alter INSL3 expression (Ivell *et al.,* 2014). This helps to explain, for example, why hCG treatment over several weeks in men with hypothalamic hypogonadism can improve circulating INSL3 (Foresta *et al.,* 2004); whereas uniorchid subjects similarly treated but for only 3–4 days showed no such increase in INSL3 (Bay *et al.,* 2005). In the human male, circulating INSL3 is at the limit of detection before puberty and increases concomitantly with the differentiation of the Leydig cells (Ferlin *et al.,* 2006b; Wikström *et al.,* 2006; Johansen *et al.,* 2014; Figure 2). As shown by the longitudinal data in Figure 2 and illustrated in other studies (Ferlin *et al.,* 2006a,b; Wikström *et al.,* 2006), there is substantial variation in INSL3 levels during puberty, the origin of which is still largely unknown. In adult men, peripheral INSL3 concentration is generally in the range 0.5–2.0 $ng{\cdot}mL^{-1}$, declining steadily with age (ca. 12% per decade) (Anand-Ivell *et al.,* 2006a). Because there appears to be no compensatory feedback via the HPG axis, as for gonadal steroids, this age-dependent decline is greater than that for testosterone, which only declines at ca. 6% per decade, because of the steady increase in LH secretion with age (Anand-Ivell *et al.,* 2006b). Importantly, INSL3 appears to be highly consistent as a clinical parameter within an individual over periods of several years and is not subject to short-term variations like testosterone (Anand-Ivell, unpublished observations). Because INSL3 is only dependent on Leydig cell numbers (which do not significantly change with age within an individual) and their

differentiation status – together referred to as Leydig cell functional capacity – in men who have had one testis removed because of seminoma, circulating INSL3 levels are significantly and permanently reduced, unlike testosterone, which, through a compensatory increased LH, is restored to normal intact values in these men (Anand-Ivell *et al.,* 2006b). Although, it should be noted that over time, uniorchid men do show some increase in INSL3 levels above the expected 50% of the intact level, due to the chronically increased LH secretion and consequently increased differentiation status of the uniorchid Leydig cells.

INSL3 acts uniquely at the RXFP2 receptor and, in adult men, appears to have two functions. As an endocrine hormone, INSL3 probably modulates bone metabolism, since individuals with a dysfunctionally mutated RXFP2 receptor exhibit significant osteopaenia and osteoporosis (Ferlin *et al.,* 2008a). INSL3 also appears to act locally within the testis on germ cells. Within the human testis, the RXFP2 receptor is expressed both on Leydig cells themselves as well as on predominantly post-meiotic germ cells, as demonstrated by specific immunohistochemistry (Anand-Ivell *et al.,* 2006a). In a study looking at a steroidal male contraception regimen, it was shown that men with greater circulating INSL3 levels showed higher residual spermatogenesis (Amory *et al.,* 2007), suggesting that INSL3 was acting as an anti-apoptotic survival agent vis-à-vis germ cells.

All male mammals so far investigated show substantial expression of INSL3 in the interstitial Leydig cells of the testis and nowhere else. As in humans, studies in boars, bulls, deer, dogs, cats and rodents (Bathgate *et al.,* 1996; Pusch *et al.,* 1996; Spiess *et al.,* 1999; Hombach-Klonisch *et al.,* 2004; Pathirana *et al.,* 2011; Minagawa *et al.,* 2012; Braun *et al.,* 2015) all show negligible prepubertal expression of INSL3, which increases with pubertal development to reach a maximum in early adulthood. Studies in rats and boars using either a specific RXFP2 antagonist or a passive immunization protocol, respectively, both confirm the importance of INSL3 as a germ cell survival factor (Del Borgo *et al.,* 2006; Sagata *et al.,* 2015). Studies in mice in which the RXFP2 gene has been deleted also show significant osteopenia, supporting a role for it in bone metabolism (Ferlin *et al.,* 2008a,b). However, it should be noted that in adult mice in which the RXFP2 gene has been selectively deleted in germ cells, there is no evidence for an effect of INSL3 on germ cell survival (Huang *et al.,* 2012). In order for INSL3 to act on the germ cell receptors, the peptide hormone needs to cross the blood-testis-barrier comprising Sertoli cell tight junctions from the interstitial compartment into the seminiferous tubule lumen. Careful measurements of INSL3 peptide within different testicular compartments in both rats and boars indicate indeed that this occurs, presumably by some specific transport mechanism (Anand-Ivell *et al.,* 2009). Taken together, and unlike the situation for relaxin in the male tract, there appears to be much more consistency between species in the expression and physiology of INSL3 and its receptor, RXFP2, in the adult male mammal.

INSL3 in the fetus

INSL3 is also expressed by the Leydig cells of the testes in the male fetus. These Leydig cells represent a separate population from the adult-type Leydig cells, which differentiate after puberty. Whereas it appears that in rodents, fetal and adult Leydig cells represent discrete cell populations (Kaftanovskaya *et al.,* 2015b), for other species, this is less clear. It seems possible for a human that there is a perinatal involution of the fetal population, which then redifferentiates in late infancy (Teerds and Huhtaniemi, 2015). Fetal Leydig cells begin to differentiate from undifferentiated mesenchyme cells immediately following SRY-driven sex determination in order to provide androgens, which are needed to promote gender-specific differentiation of tissues and behaviour. The major phenotype of mice, in which either INSL3 or the RXFP2 receptor have been deleted, is the failure of the first phase of testicular descent within the abdomen (Nef and Parada, 1999; Zimmermann *et al.,* 1999; Overbeek *et al.,* 2001; Gorlov *et al.,* 2002). This occurs at different times during gestation in different mammalian species (Figure 3), but occurs shortly after testis formation and is concomitant with the differentiation of the Leydig cells. From studies in rodents, we know that INSL3 is essential for this process by acting on RXFP2 receptors expressed within the gubernacular ligaments, causing these to expand and anchor the testes in the inguinal region, while the other organs including the kidneys grow away in an antero-dorsal direction. In a second inguino-scrotal phase, androgens under the influence of the developing HPG axis then promote the movement of the testes through the inguinal ring into the scrotum (Kaftanovskaya *et al.,* 2012). Studies measuring INSL3 in human amniotic fluid collected at routine amniocentesis (Anand-Ivell *et al.,* 2008; Jensen *et al.,* 2015), or looking at INSL3 in amniotic or allantoic fluids as well as fetal serum from pigs (Vernunft *et al.,* 2016), cows (Anand-Ivell *et al.,* 2011) and rats (Anand-Ivell and Ivell, 2014), all confirm the high expression of INSL3 at precisely that time accompanying the first tranbsabdominal phase of testicular descent. Because INSL3 is only expressed by the male fetus, it also acts as a useful biomarker specific for fetal gender and has been used to show transfer of the fetal hormone in calves across the placenta to the maternal circulation (Anand-Ivell *et al.,* 2011) or in pigs between male and female fetuses (Vernunft *et al.,* 2016).

Several studies have attempted to link fetal INSL3 expression with the common congenital condition of cryptorchidism, whereby one or both testes fail to descend into the scrotum. Firstly, it is important to understand that cryptorchidism is a very complex and multifactorial condition (Hutson *et al.,* 2010); secondly, a recent meta-analysis of rat studies showed that fetal INSL3 mRNA expression probably needs to be reduced by at least 40% before there is any impact on phenotype (Gray *et al.,* 2016). Some studies in human babies have used cord blood, where it is evident that the INSL3 measured can only be a residual of what may have been present during the first transabdominal phase of testis descent in the transition between first and second trimester, some 4–6 months earlier (Bay *et al.,* 2007; Chevalier *et al.,* 2015; Fenichel *et al.,* 2015). Cord blood INSL3 concentration is usually around 50–100 $pg{\cdot}mL^{-1}$, compared with a probable blood concentration (based on extrapolations from other species and from human amniotic fluid collected in the early second trimester) closer to 1 $ng{\cdot}mL^{-1}$. Nevertheless, such studies do suggest that INSL3 concentration may have been

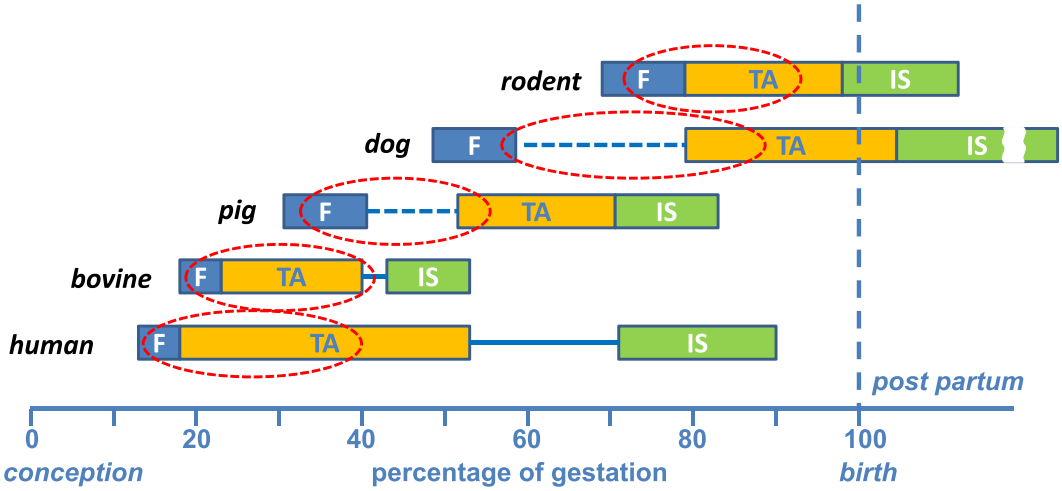

Figure 3

Testis development and testicular descent in various mammal species. Gestation is expressed as a percentage with conception at 0% and term of pregnancy at 100%. F, testis formation; TA, transabdominal phase of testis descent; IS, inguino-scrotal phase of testis descent. The red ovals indicate the periods of maximum INSL3 expression and correspond approximately to the 'male programming window' for each species (modified after Anand-Ivell and Ivell, 2014).

significantly reduced in cases of cryptorchidism. However, a recent very large case–control study of human amniotic fluid samples collected throughout the second trimester did not show evidence of any reduction of INSL3 in cryptorchids compared with controls (Jensen *et al.*, 2015).

Studies have been performed to determine whether there is an association between cryptorchidism in human patients with the presence of mutant INSL3 and RXFP2 alleles (Bogatcheva and Agoulnik, 2005). The frequency of such mutations is usually low (<1–3%) and accounts for only a small portion of all cryptorchidism cases. This is not unexpected as any mutation affecting fertility will be under strong negative selection in the population. Infertility in carrier fathers caused by undescended testis will prevent transmission of the mutant allele to the next generation. Thus, the population spread of the male-limited mutation can be achieved either through female transmission or through an accumulation in the population of genetic modifiers suppressing the harmful phenotype. Both of these mechanisms have been found in the case of the T222P variant allele of the RXFP2 receptor (Gorlov *et al.*, 2002). Functional assays have demonstrated that the mutant T222P receptor failed to be expressed on the cell surface membrane (Bogatcheva *et al.*, 2007). While in the Italian population, heterozygosity for T222P was strongly linked to cryptorchidism, and in all the cases analysed there was a maternal transmission of this allele; in Spain and Northern Africa, T222P heterozygotes were equally present in patient and control groups (El Houate *et al.*, 2008; Ferlin *et al.*, 2008a,b; Feng *et al.*, 2009; Ars *et al.*, 2010). Similarly, while some mutations in INSL3 resulted in its functional deficiency and or were exclusively present in cryptorchid patients (Tomboc *et al.*, 2000; Bogatcheva *et al.*, 2003; Ferlin *et al.*, 2006a; El Houate *et al.*, 2007), other mutant alleles were also detected in the control population (Koskimies *et al.*, 2000; Yamazawa *et al.*, 2007; Mamoulakis *et al.*, 2014; Huang *et al.*, 2016).

Similar to INSL3 in the adult male, there also do not appear to be major differences in fetal INSL3 between mammalian species, except in timing of expression to coincide with the main period of transabdominal testicular descent, and in the hormonal control of Leydig cell differentiation, which appears to differ particularly between rodents and other species (Teerds and Huhtaniemi, 2015). Because of its highly specific expression by Leydig cells only in the male fetus, INSL3 is proving to be a powerful sentinel biomarker for the effects of gestational exposure to environmental endocrine disruptors (Anand-Ivell and Ivell, 2014). These are substances often with steroid-like properties, which are widespread in the environment and have been shown to alter endocrine parameters during fetal life with consequences for adult physiology and possibly subsequent generations (Bergman *et al.*, 2013). In this context, it appears that not all species are similar, with rodent Leydig cells seeming to be more susceptible to exposure to certain such xenobiotics than, for example, human cells (Habert *et al.*, 2014).

Other relaxin-like peptides in male reproduction

INSL6 is highly expressed in post-meiotic germ cells within the testes (Figure 1) and possibly at lower levels in other tissues, such as skeletal muscle, and at least *in vitro* has been shown to be a secretory peptide, presumably contributing to the seminal fluid leaving the testis. Originally identified as an expressed sequence tag from the human testis (Lok *et al.*, 2000), it has since also been identified as a germ-cell-specific transcript in rodents (Lok *et al.*, 2000; Burnicka-Turek *et al.*, 2009), as well as being present in the genomes of all sequenced mammals. Mice in which the INSL6 gene has been deleted show a marked disruption of spermatogenesis with meiotic arrest (Burnicka-Turek *et al.*, 2009), suggesting an

important role in sperm formation. In support of this idea, a single missense mutation in the human INSL6 gene was recently identified in an infertile male patient, though no causative studies were undertaken (Chen *et al.,* 2011). To date, however, no specific receptor for human INSL6 has been discovered, and it appears incapable of interacting with any of the known relaxin and insulin-family peptide receptors.

RLN3 is best known as a brain neuropeptide (Bathgate *et al.,* 2013) but has also been identified at very low transcript levels within the interstitial cells of testes from monkeys (Silvertown *et al.,* 2010) and mice (Ivell *et al.,* 2011). Also, transcripts for its cognate receptors RXFP3 and RXFP4 have been identified within the testes (Silvertown *et al.,* 2010; Ivell *et al.,* 2011). In contrast, the closely related gut peptide INSL5 is not significantly detectable in any part of the male tract (GEO database). However, whereas the phenotype of RLN3 knockout mice does not suggest any essential male reproductive function (Smith *et al.,* 2009), that for INSL5 indicates a mildly reduced fertility in both males and females possibly due to an impact on glucose metabolism (Burnicka-Turek *et al.,* 2012). INSL4 is only known to be expressed in the human placenta, and is not detectable in any tissue of the male reproductive tract (GEO database).

Clinical applications of relaxin-like peptides

To date, only H2 relaxin has been trialled in a clinical/therapeutic context and then for indications generally unrelated to male reproduction (Tietjens and Teerlink, 2016). Importantly, H2 relaxin has passed essential safety criteria for use in humans. However, a potential use of relaxin in male fertility treatment was analysed with regard to sperm survival and fertilization, through its effects on motility, capacitation and the acrosome reaction (Agoulnik, 2007; Ferlin *et al.,* 2012). This would be beneficial in an *in vitro* fertilization setting, especially when dealing with frozen or functionally deficient sperm, or when there is a limited number of sperm from the infertile patients (Lessing *et al.,* 1986). Similarly, the application of relaxin might have even larger use in commercial farm animal breeding where assisted reproduction procedures are widely used. Again, the sperm-activating properties of relaxin would be beneficial especially in situations of reduced sperm motility or unanticipated delayed insemination, using either fresh or frozen–thawed semen (Feugang *et al.,* 2015). However, the somewhat modest and variable effects of relaxin on sperm coupled with the high cost of the recombinant peptide can be limiting factors in these applications.

As with other peptide-based drugs, there are well-recognized challenges in using relaxin as a therapeutic agent especially in a chronic setting. The half-life of the unmodified relaxin peptide is short (Yoshida *et al.,* 2012) and thus has to be administered i.v. The cost of production for recombinant peptide is significant. An additional and still understudied issue is the potential immune response to injections. The use of recently discovered small molecule agonists of RXFP1 receptors might overcome these problems (Xiao *et al.,* 2013). Such small compounds are easy to synthesize; they are stable *in vivo*, and they can be delivered through different routes, including oral or ectopic application. It was shown, however, that the compounds activate the relaxin receptor through allosteric mechanisms, suggesting that downstream cellular signalling might be biased (Huang *et al.,* 2015; Hu *et al.,* 2016). Further investigation of the utility of these agonists, especially in animal models, is warranted.

Concluding remarks

Theoretically, the relaxin family of peptide hormones and their cognate receptors lend themselves ideally for use as therapeutic or pharmacological targets. As neohormones, they subserve a set of properties, particularly related to mammalian reproduction and ageing, which are modulatory rather than being ancient and essential. This is reflected in the safety record for relaxin, which indicates few if any negative side-effects. However, we still understand very little about the physiology of these peptides, especially with respect to human, as opposed to animal models. Being evolutionarily modern hormones, as discussed, there is likely to be considerable between-species variation in their physiology, which still needs to be properly addressed. For relaxin-like peptides, male reproduction is relatively unexplored, and we can expect some exciting findings in the next few years.

Author contributions

All authors have contributed equally to the conception and writing of this review. R.I. had overall management.

Conflict of interest

The authors declare no conflicts of interest.

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