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ORIGINAL ARTICLE

The highly conserved negatively charged Glu141 and Asp145 of the G-protein-coupled receptor RXFP3 interact with the highly conserved positively charged arginine residues of relaxin-3

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Abstract Relaxin-3 is a newly identified insulin/relaxin superfamily peptide that plays a putative role in the regulation of food intake and stress response by activating its cognate G-protein-coupled receptor RXFP3. Relaxin-3 has three highly conserved arginine residues, B12Arg, B16Arg and B26Arg. We speculated that these positively charged arginines may interact with certain negatively charged residues of RXFP3. To test this hypothesis, we first replaced the negatively charged residues in the extracellular domain of RXFP3 with arginine, respectively. Receptor activation assays showed that arginine replacement of Glu141 or Asp145, especially Glu141, significantly decreased the sensitivity of RXFP3 to wild-type relaxin-3. In contrast, arginine replacement of other negatively charged extracellular residues had little effect. Thus, we deduced that Glu141 and Asp145, locating at the extracellular end of the second transmembrane domain, played a critical role in the interaction of RXFP3 with relaxin-3. To identify the ligand residues interacting with the negatively charged EXXXD motif of RXFP3, we replaced the three conserved arginines of relaxin-3 with negatively charged glutamate or aspartate, respectively. The mutant relaxin-3s retained the native structure, but their binding and activation potencies towards wild-type RXFP3 were decreased significantly. The compensatory effects of the mutant relaxin-3s towards mutant RXFP3s suggested two probable interaction pairs during ligand–receptor interaction: Glu141 of RXFP3 interacted with B26Arg of relaxin-3, meanwhile Asp145 of RXFP3 interacted with both B12Arg and B16Arg of relaxin-3. Based on these results, we proposed a relaxin-3/RXFP3 interaction model that shed new light on the interaction mechanism of the relaxin family peptides with their receptors.

Keywords Insulin superfamily · Ligand–receptor interaction · Mutagenesis · Relaxin-3 · RXFP3

Abbreviations

CD	Circular dichroism
CRE	cAPM response element
GPCR	G-protein-coupled receptor
HEK293T	Human embryonic kidney 293T
INSL5	Insulin-like peptide 5
INSL7	Insulin-like peptide 7
HPLC	High-performance liquid chromatography
TMD	Transmembrane domain
UV	Ultraviolet

Introduction

Relaxin-3 (also known as INSL7) is a newly identified neuropeptide belonging to the insulin/relaxin superfamily

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(Shabanpoor et al. 2009; Smith et al. 2011; Tanaka 2010). It was first identified in 2002 in a search of the human genome, based on the cysteine pattern of the B-chain of the insulin superfamily (Bathgate et al. 2002). Relaxin-3 is predominantly expressed in the nucleus incertus of the brain (Burazin et al. 2002; Ma et al. 2009; Olucha-Bordonau et al. 2012; Ryan et al. 2011; Silvertown et al. 2010; Smith et al. 2010; Tanaka et al. 2005). Administration of exogenous relaxin-3 in animal models (Banerjee et al. 2010; Hida et al. 2006; McGowan et al. 2005, 2006, 2007, 2008; Watanabe et al. 2011a) and gene knockout experiments (Smith et al. 2012; Watanabe et al. 2011b) revealed that relaxin-3 is involved in the regulation of food intake, stress responses, and arousal/exploratory behaviors. In vitro, relaxin-3 can bind and activate the G-protein-coupled receptors RXFP1 (Sudo et al. 2003), RXFP3 (Liu et al. 2003b) and RXFP4 (Liu et al. 2003a). In vivo, the biological functions of relaxin-3 are mainly mediated by RXFP3, while RXFP1 and RXFP4 are the cognate receptors of relaxin (Hsu et al. 2002) and INSL5 (Liu et al. 2005b), respectively.

Relaxin-3 adopts a characteristic relaxin/insulin-like fold in solution (Rosengren et al. 2006). Its B-chain contains a long α-helical segment (B12Arg–B22Cys), while its A-chain contains two short α-helical segments (A1Arg–A13Ile and A17Lys–A24Cys). The B-chain C-terminus of relaxin-3 folds back, allowing B27Trp to interact with the hydrophobic core. In contrast, the B-chain C-terminus of relaxin-2 and INSL5 forms an α-helical conformation (Eigenbrot et al. 1991; Haugaard-Jonsson et al. 2009), rendering the C-terminus apart from the hydrophobic core. The chimeric R3/I5 peptide (containing the B-chain of relaxin-3 and the A-chain of INSL5) retains full activity towards RXFP3 and RXFP4, although its activity toward RXFP1 was almost abolished (Liu et al. 2005a). Deletion of the A-chain N-terminal (up to nine residues) of relaxin-3 did not result in any changes in the activity towards RXFP3; however, its activity towards RXFP1 was significantly decreased (Hossain et al. 2008). Thus, the B-chain of relaxin-3 plays a critical role in the binding and activation of RXFP3 and RXFP4. Site-directed mutagenesis of the relaxin-3 B-chain revealed some critical residues (Kuei et al. 2007), such as the positively charged B12Arg, B16Arg and B26Arg. Deletion of the B-chain C-terminal B23Gly–B27Trp segment of relaxin-3 was found to abolish its activation potency towards both RXFP3 and RXFP4 (Kuei et al. 2007). Introduction of an additional Arg at the shortened B-chain C-terminus restored its binding affinity towards RXFP3 and RXFP4, but was unable to restore its activation potency towards these two receptors (Kuei et al. 2007). Thus, the B-chain C-terminus of relaxin-3 is essential for the activation of RXFP3 and RXFP4.

Previous mutagenesis and deletion analyses have revealed critical functional determinants of relaxin-3 towards binding and activation of its cognate receptor RXFP3. However, their counterparts in the receptor are still unknown so far. In a previous study, a domain-swapping approach was employed to study the ligand discrimination between the homologous RXFP3 and RXFP4 (Zhu et al. 2008); however, the common functional determinants present in both receptors could not be revealed by this approach. By site-directed mutagenesis of both the ligand and the receptor, in the present study we disclosed two probable interaction pairs between relaxin-3 and its receptor RXFP3, involving a negatively charged extracellular EXXXD segment of the receptor and three highly conserved positively charged arginine residues of the ligand.

Materials and methods

Site-directed mutagenesis of RXFP3 and relaxin-3

Site-directed mutagenesis of human RXFP3 was carried out with the QuikChange methodology using a pcDNA6/RXFP3 construct (Zhang et al. 2012a, b) as the mutagenesis template. The expected mutations were confirmed by DNA sequencing. To ensure the absence of random mutations, the full-length coding region of the RXFP3 mutants was sequenced. Site-directed mutagenesis of relaxin-3 was also carried out using QuikChange approach. The expression construct of a fully active, easily labeled relaxin-3 (Zhang et al. 2012b) was used as the mutagenesis template. The expected mutations were confirmed by DNA sequencing.

Preparation of recombinant mutant relaxin-3s

Mutant relaxin-3s were recombinantly expressed in *Escherichia coli* as single-chain precursors and refolded in vitro through the disulfide-reshuffling approach according to the procedure in our previous study (Luo et al. 2010). The folded precursors were subsequently converted to mature two-chain forms through sequential enzymatic treatment as described previously (Zhang et al. 2012b). The analogs were purified to homogeneity using C_{18} reverse-phase HPLC, and their molecular masses were measured by mass spectrometry.

CD analysis

The mature relaxin-3 analogs were dissolved in 1.0 mM aqueous HCl solution (pH 3.0). Their concentrations were determined by UV absorbance at 280 nm using an

extinction coefficient of $\varepsilon_{280nm} = 12{,}490\ M^{-1}\ cm^{-1}$, which was calculated from the number of tryptophan and tyrosine residues in the peptides. Their final concentration was adjusted to 22 μM for CD measurement, which was performed on a Jasco-715 spectrometer at room temperature. The spectra were scanned from 250 to 190 nm using a quartz cell of 0.1 cm path length. J-700 for Windows Secondary Structural Estimation software (version 1.10.00) was used to analyze the secondary structural content from the CD spectra.

RXFP3 binding assays

The competition receptor-binding assays were carried out according to our previous procedure (Zhang et al. 2012a, b). Briefly, transfected HEK293T cells transiently expressing wild-type human RXFP3 were seeded into a 96-well plate and continuously cultured at 37 °C until they reached approximately 80 % confluence (24–48 h). For the receptor-binding assay, the culture medium was removed and 200 μl of binding solution (DMEM medium plus 1 % BSA) containing 2.0 nM of DTPA/Eu^{3+}-labeled R3/I5 tracer and different concentrations of mutant relaxin-3s was added to each well. After binding at 21–22 °C for 2 h, the binding solution was removed and the cells were washed twice with washing solution (200 μl/well, cold DMEM medium). Then, enhancer solution (100 μl/well; PerkinElmer, Waltham, MA, USA) was added to dissociate Eu^{3+} from the DTPA chelator. After shaking for 1–2 h, the solutions (80 μl/well) were transferred to an opaque white 384-well plate for time-resolved fluorescence measurement using a Spectramax M5 plate reader. The specific binding data were fitted with a one-site receptor-binding model using SigmaPlot 10.0 software.

RXFP3 activation assays

The RXFP3 activation assays were carried out as described in our previous study (Zhang et al. 2012b) using firefly luciferase (pGL4.29[luc2P/CRE/Hygro]; Promega, Madison, WI, USA) or the newly developed nanoluciferase as reporter. The new CRE-controlled nanoluciferase reporter was constructed in our laboratory by insertion of a CRE-minimal promoter DNA fragment (PCR amplified from pGL4.29[luc2P/CRE/Hygro]) into the pNL1.2 vector (Promega, Madison, WI, USA). The RXFP3 expression construct and the reporter vector were co-transfected into HEK293T cells. One day after transfection, the cells were trypsinized and seeded into a 96-well plate. After the cells grew to 60–80 % confluence (24–48 h), the medium was removed and 100 μl of activation solution (serum-free DMEM medium plus 1 % BSA) containing 5 μM of forskolin and different concentrations of peptide was added to each well. After the cells were cultured at 37 °C for 5–6 h, the activation solution was removed and the luciferase activity was assayed. The relative luciferase activity data were fitted with sigmoidal curves using SigmaPlot 10.0 software.

Results

Mutagenesis of human RXFP3 and activation of the mutant receptors by wild-type relaxin-3

As reported previously, the three highly conserved Arg residues (B12Arg, B16Arg and B26Arg) of relaxin-3 are critical for binding and activation of receptor RXFP3 (Kuei et al. 2007) (Fig. 1a). We speculated that these positively charged Arg residues may interact with certain negatively charged residues of the receptor during ligand–receptor interaction. To test this hypothesis, we replaced the negatively charged extracellular residues of RXFP3 with positively charged arginine, respectively (Fig. 1b). Some previous work showed that the N-terminal sequence of bovine rhodopsin (rho-tag with the amino acid sequence of MNGTEGPNFYVPFSNKTG) can improve the expression of some GPCRs (Krautwurst et al. 1998; Zhuang and Matsunami 2007). After the N-terminal sequence (MQMADAATIATMNKAAGG) of human RXFP3 was replaced by the rho-tag, the resultant rho-RXFP3 was found to be fully active (Table 1), suggesting that the N-terminal segment is not important for RXFP3 activation by relaxin-3. Thus, Asp5 in the N-terminal of human RXFP3 was not included in the present work. All of the other negatively charged extracellular residues of human RXFP3 were replaced by arginine, and totally 18 mutant RXFP3s were obtained by site-directed mutagenesis. The mutant RXFP3s were transiently expressed in HEK293T cells and their sensitivity to wild-type relaxin-3 was assayed using a CRE-controlled firefly luciferase reporter according to our previous procedure (Zhang et al. 2012b). In this paper, wild-type relaxin-3 refers to the fully active, easily labeled relaxin-3 that was used as the mutagenesis template in the present work. Most of the mutant RXFP3s were activated by wild-type relaxin-3 as effectively as wild-type RXFP3 (Fig. 2a; the calculated pEC_{50} values are summarized in Table 1), suggesting that these negatively charged residues were not involved in the interaction with relaxin-3. However, arginine replacement of Glu141 or Asp145, especially Glu141, significantly decreased the sensitivity of the resultant receptors to wild-type relaxin-3 (Fig. 2b), suggesting that Glu141 and Asp145 were critical for RXFP3 interaction by relaxin-3. Since the mutant [E141R]RXFP3 could be activated by a mutant relaxin-3 as shown below, we thought that its insensitivity to wild-type

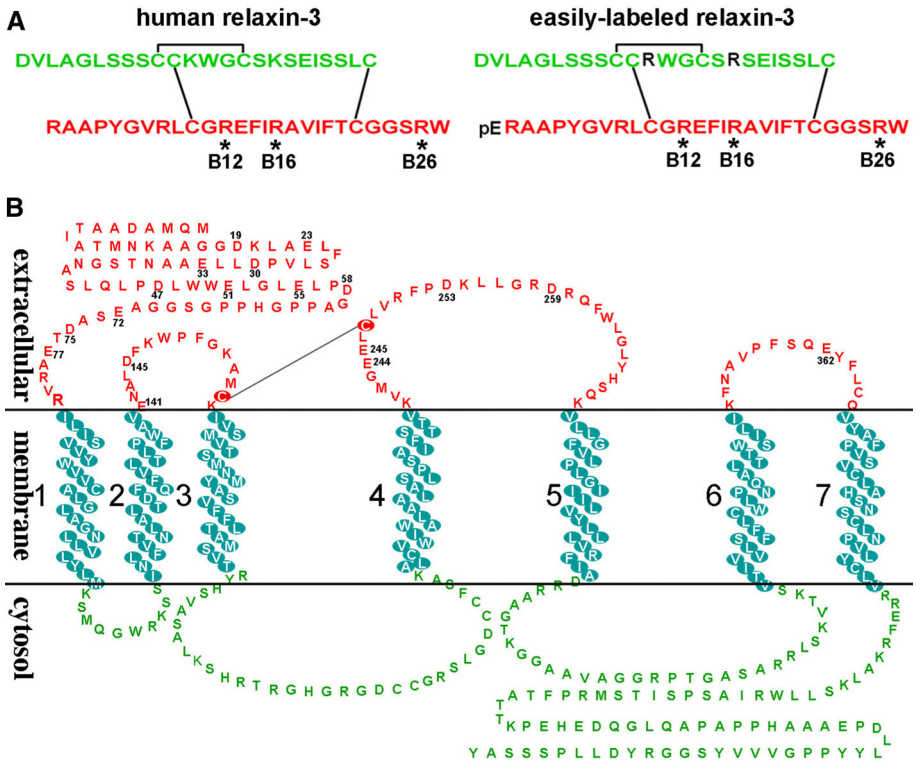

Fig. 1 **a** The amino acid sequence and disulfide linkages of human relaxin-3 and the fully active, easily labeled relaxin-3, which was used as the mutagenesis template in the present study. The B-chain is shown in *red*, and the A-chain in *green*; the disulfide bonds are shown as *lines*. **b** The amino acid sequence and predicted membrane topology of human RXFP3. The negatively charged residues (Asp and Glu) in the extracellular domain are labeled and were replaced by positively charged arginine in the present work. The RXFP3 transmembrane topology was predicted by TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/) and TMpred Server (http://www.ch.embnet.org/cgi-bin/TMPRED) that generated quite consistent model with seven transmembrane domains (colour figure online)

Table 1 Measured pEC_{50} values of wild-type relaxin-3 to wild-type and mutant RXFP3s

RXFP3s	pEC_{50}
Wild-type	10.24 ± 0.21
Rho-RXFP3	10.40 ± 0.15
D19R	10.07 ± 0.10
E23R	10.07 ± 0.21
D30R	10.52 ± 0.09
E33R	10.54 ± 0.14
D47R	10.36 ± 0.10
E51R	10.29 ± 0.09
E55R	10.76 ± 0.10
D58R	9.95 ± 0.09
E72R	10.60 ± 0.40
D75R	10.21 ± 0.19
E77R	10.23 ± 0.17
E141R	N.D.
D145R	8.23 ± 0.16*
E244R	9.79 ± 0.11
E245R	9.66 ± 0.08
D253R	10.11 ± 0.07
D259R	10.34 ± 0.13
E362R	9.99 ± 0.14

A CRE-controlled firefly luciferase reporter was used in these assays. (N.D., not detectable)

* $p < 0.001$ versus wild-type RXFP3

relaxin-3 was mainly caused by lack of essential interaction residue rather than by drastic structural disturbance. Western blot analysis also showed that it had similar expression level compared with wild-type RXFP3 in the transfected HEK293T cells (data not shown). As shown in Fig. 1b, Glu141 and Asp145 are located at the extracellular end of the second predicted transmembrane domain (TMD2) of RXFP3. We deduced that this negatively charged EXXXD segment probably interacted with certain positively charged residues of relaxin-3 during ligand–receptor interaction.

Recombinant preparation and characterization of mutant relaxin-3s

Our above results revealed a negatively charged EXXXD segment in receptor RXFP3 that is essential for interaction with relaxin-3. To identify the relaxin-3 residues that interacted with this EXXXD segment, we replaced the three highly conserved positively charged Arg residues of relaxin-3 with negatively charged Glu or Asp, respectively. The mutant relaxin-3s were recombinantly expressed as single-chain precursors in *E. coli* according to our previous procedure (Luo et al. 2010; Zhang et al. 2012b). The expression levels of [B12D]relaxin-3 and [B16D]relaxin-3 were so low that we could not obtain enough peptide for activity assays. The other four mutant relaxin-3s retained considerable expression levels and could be efficiently refolded in vitro. After sequential enzymatic treatment as described previously (Zhang et al. 2012b), we obtained four homogeneous mutant relaxin-3s whose measured molecular masses were consistent with their theoretical

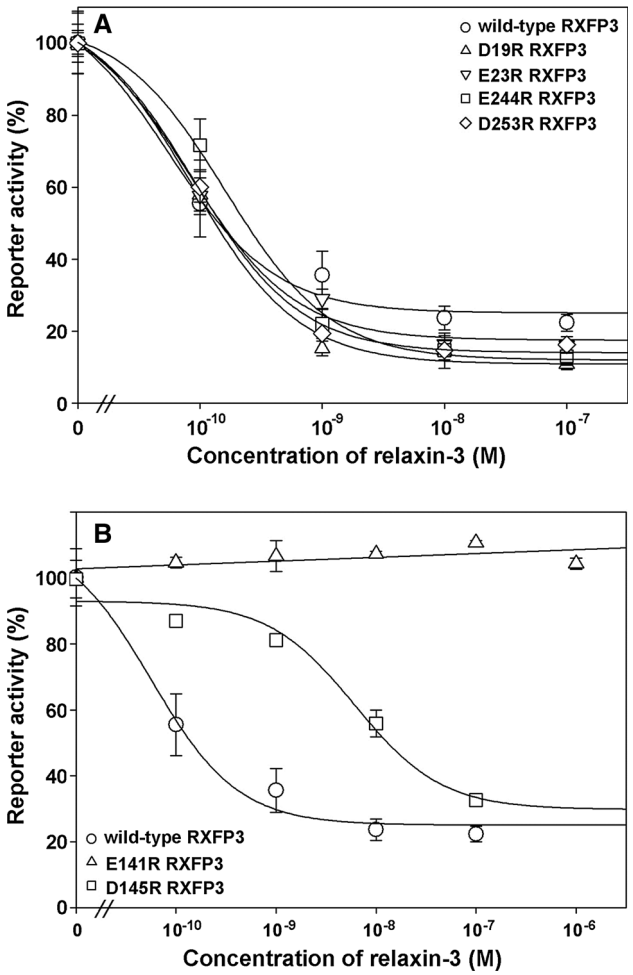

Fig. 2 The activation curves of wild-type relaxin-3 towards wild-type and some mutant RXFP3s. A CRE-controlled firefly luciferase reporter was used in these assays. The data were expressed as mean ± SE ($n = 3$), and fitted with sigmoidal curves using SigmaPlot 10.0 software

values (Table 2). The structural changes of these analogs were assessed by CD (Fig. 3). The spectra of the mutant relaxin-3s were quite similar to that of wild-type relaxin-3, suggesting that the mutation in these analogs did not affect their overall structure. The α-helix contents of the mutant relaxin-3s estimated from CD spectra were also similar to that of the wild-type relaxin-3 (Table 2).

Binding and activation of wild-type RXFP3 by mutant relaxin-3s

As shown above, we prepared four mutant relaxin-3s through recombinant expression and in vitro maturation. We first measured their binding affinities to wild-type RXFP3 through competition receptor-binding assay using a DTPA/Eu^{3+}-labeled chimeric R3/I5 peptide as tracer (Fig. 4a; calculated pIC_{50} values are listed in Table 2) (Zhang et al. 2013). Replacement of B26Arg or B12Arg of relaxin-3 with negatively charged Asp or Glu resulted in an affinity decrease to wild-type RXFP3 by over 100-fold (one pIC_{50} decrease is equal to a tenfold affinity decrease), suggesting that both B26Arg and B12Arg of relaxin-3 are important for binding with receptor RXFP3. Replacement of B16Arg of relaxin-3 with Glu also significantly decreased its RXFP3-binding affinity (approximately 60-fold decrease), suggesting that B16Arg is also important for the high-affinity binding of relaxin-3 with receptor RXFP3. We also measured the receptor activation potencies of these mutant relaxin-3s towards wild-type RXFP3 using a CRE-controlled nanoluciferase reporter (Fig. 4b; the calculated pEC_{50} values are listed in Table 3). The newly developed nanoluciferase reporter (Hall et al. 2012) was more sensitive and more reproducible than the conventional firefly luciferase due to its high activity and ATP-independent catalysis mechanism. Thus, the pEC_{50} of wild-type relaxin-3 to wild-type RXFP3 measured using the nanoluciferase reporter was slightly higher than that measured using the firefly luciferase reporter (Table 1). Consistent with the decreased binding affinity, the RXFP3 activation potencies of the four mutant relaxin-3s were also decreased significantly, particularly the B26 and B12 mutants, suggesting the importance of the three highly conserved arginine residues in RXFP3 activation.

Activation of [E141R]RXFP3 and [D145R]RXFP3 by mutant relaxin-3s

In above work, we identified an essential negatively charged EXXXD motif in receptor RXFP3, and found that replacement of the three highly conserved Arg residues of

Table 2 Measured molecular masses, α-helix contents and pIC_{50} values of the mutant relaxin-3s

Relaxin-3s	wild-type	B12E	B16E	B26E	B26D
Molecular mass[a]	5,668.0 (5,667.5)	5,642.0 (5,640.4)	5,642.0 (5,640.4)	5,641.0 (5,640.4)	5,628.0 (5,626.4)
α-helix content[b]	40 %	39 %	39 %	40 %	39 %
pIC_{50}[c]	8.35 ± 0.06	6.09 ± 0.05*	6.56 ± 0.05*	5.95 ± 0.09*	5.73 ± 0.13*

* $p < 0.001$ versus wild-type relaxin-3

[a] Measured by electrospray mass spectrometry. The theoretical values are listed in parentheses

[b] Estimated from the CD spectra shown in Fig. 3

[c] Inhibition of tracer binding to wild-type human RXFP3

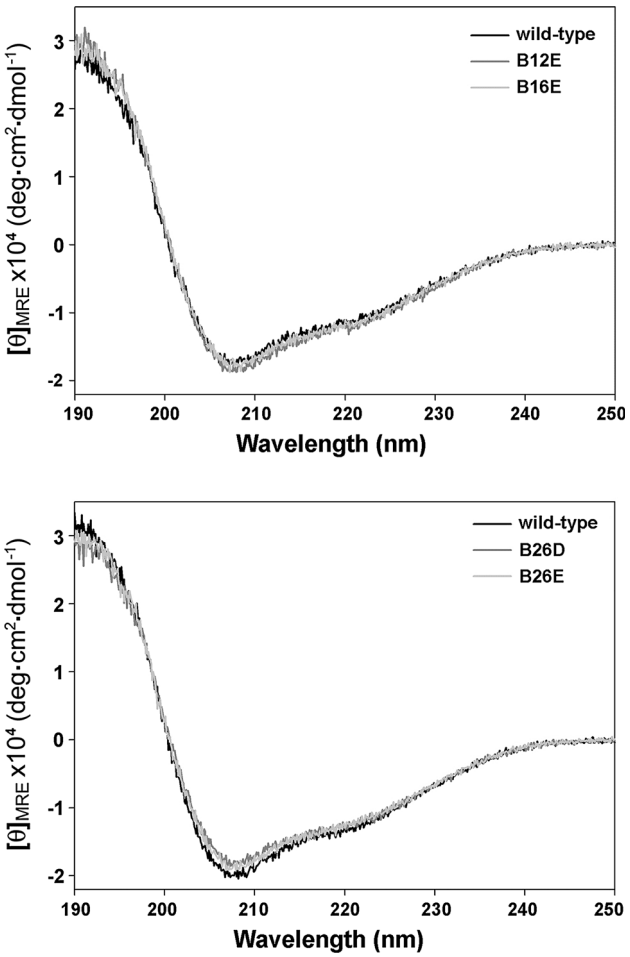

Fig. 3 Circular dichroism spectra of the mutant relaxin-3s

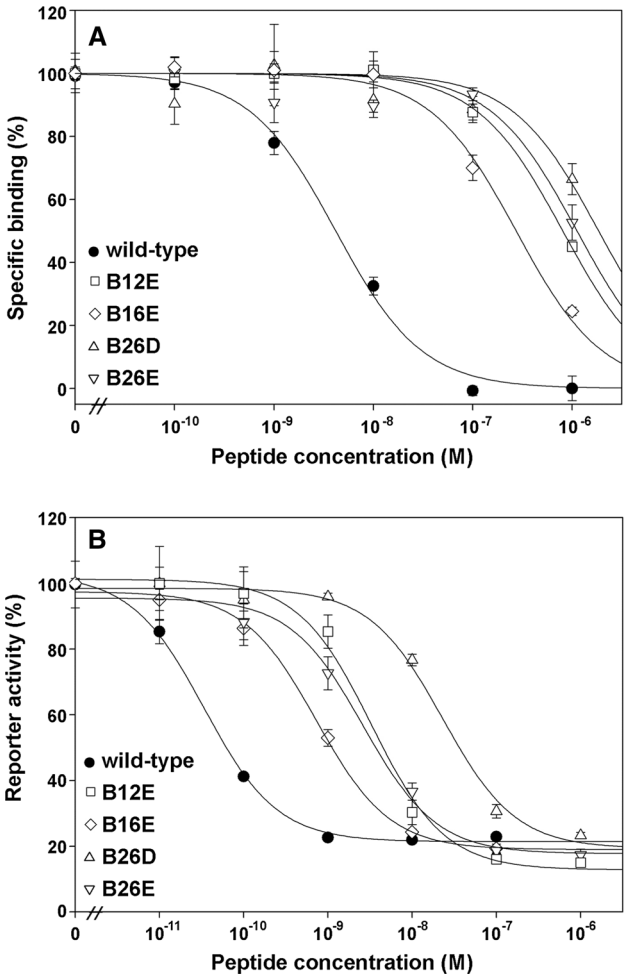

Fig. 4 Competition receptor-binding (**a**) and receptor activation (**b**) of mutant relaxin-3s towards wild-type RXFP3. A DTPA/Eu^{3+}-labeled R3/I5 was used as tracer in the competition receptor-binding assays and a CRE-controlled nanoluciferase was used as reporter in the receptor activation assays. The measured data were expressed as mean ± SE ($n = 3$), and fitted with sigmoidal curves using SigmaPlot 10.0 software

relaxin-3 by negatively charged residue significantly decreased the ligand activity. Next, we attempted to reveal probable interactions between the essential EXXXD motif of RXFP3 and the three highly conserved Arg residues of relaxin-3. Since the labeled tracer used in receptor-binding assays was unsuitable for the mutant receptors due to its low binding affinity, we could not measure the binding affinities of [E141R]RXFP3 and [D145R]RXFP3 to wild-type and mutant relaxin-3s. However, we could measure their activation sensitivity to wild-type and mutant relaxin-3s (Fig. 5; calculated pEC_{50} values are summarized in Table 3). As shown in Fig. 5a, [E141R]RXFP3 could not be activated by wild-type relaxin-3 or the mutant relaxin-3s carrying B12 or B16 mutation, due to the arginine replacement of Glu141. However, this mutant RXFP3 could be activated by high concentrations of the mutant relaxin-3s carrying B26 mutation, suggesting that mutation of Glu141 rendered RXFP3 insensitive to relaxin-3 mutation at the B26 position. Thus, we deduced that Glu141 of RXFP3 probably interacted with B26Arg of relaxin-3 during ligand–receptor interaction. In the case of [D145R]RXFP3 (Fig. 5b), wild-type relaxin-3 and mutant relaxin-3s carrying B12 or B16 mutation had similar activation potencies to this mutant receptor, suggesting that mutation of Asp145 rendered RXFP3 insensitive to relaxin-3 mutation at the B12 or B16 positions once Asp145 was replaced. Thus, we deduced that Asp145 of RXFP3 probably interacted with both B12Arg and B16Arg of relaxin-3 during ligand–receptor interaction. As shown in Fig. 5b, [D145R]RXFP3 could not be activated by the B26 mutant relaxin-3s. In contrast, wild-type RXFP3 and [E141R]RXFP3 could be activated by B26 mutant relaxin-3s although high peptide concentrations were needed. This phenomenon was consistent with our above two-site interaction model: in this ligand–receptor pair, the interaction between Asp145 of RXFP3 and B12Arg/B16Arg of

Table 3 Measured pEC_{50} values of wild-type and mutant relaxin-3s to wild-type and mutant RXFP3s

Receptor RXFP3s	Ligand relaxin-3s				
	Wild-type	B12E	B16E	B26E	B26D
Wild-type	10.47 ± 0.09	8.48 ± 0.15*	9.14 ± 0.07*	8.58 ± 0.11*	7.64 ± 0.11*
E141R	N.D.	N.D.	N.D.	7.13 ± 0.18	6.69 ± 0.40
D145R	8.40 ± 0.20	8.09 ± 0.11	8.75 ± 0.13	N.D.	N.D.

A CRE-controlled nanoluciferase reporter was used in these assays (N.D., not detectable)

* $p < 0.001$ versus wild-type relaxin-3 towards wild-type RXFP3

relaxin-3 was abolished due to mutation of Asp145; meanwhile, the interaction between Glu141 of RXFP3 and B26Arg of relaxin-3 was also abolished due to mutation of B26Arg. Thus, [D145R]RXFP3 could not be activated by the mutant relaxin-3s carrying a mutation at the B26 position due to "accumulation effect" of the receptor mutation and the ligand mutation. The activity compensation between the mutant ligands and the mutant receptors could also be seen from the view of the ligand (Table 3, row comparison). The activation efficiencies of wild-type relaxin-3 and the B12 or B16 mutant relaxin-3s were considerably higher towards wild-type RXFP3 than towards [E141R]RXFP3 (estimated to be over 1,000-fold higher, since the pEC_{50} values towards the E141R mutant were below detectable levels). However, the activation efficiency of the B26 mutant relaxin-3s was only 10- to 30-fold higher towards wild-type RXFP3 than towards [E141R]RXFP3. Thus, mutation of B26Arg, rather than B12Arg or B16Arg, could attenuate the activity loss of relaxin-3 towards [E141R]RXFP3, suggesting that B26Arg of relaxin-3 and Glu141 of RXFP3 formed an interaction pair during ligand–receptor interaction. The activation efficiencies of wild-type relaxin-3 and the B26 mutants were approximately 100-fold higher towards wild-type RXFP3 than towards [D145R]RXFP3 (estimated value, since the pEC_{50} value of B26 mutant relaxin-3 towards D145R mutant receptor was below the detectable levels). However, the activation efficiencies of the mutant relaxin-3s carrying B12 or B16 mutation were only approximately two- to three-fold higher towards the wild-type RXFP3 than towards [D145R]RXFP3. Thus, mutation of B12Arg or B16Arg, rather than B26Arg, could attenuate the activity loss of relaxin-3 towards [D145R]RXFP3, suggesting that B12Arg and B16Arg of relaxin-3 probably interacted with Asp145 of RXFP3 during ligand–receptor interaction. Thus, our present results suggested that the positively charged B12Arg, B16Arg and B26Arg of relaxin-3 interacted with the negatively charged EXXXD motif of RXFP3 in a pairwise manner: B26Arg of relaxin-3 probably interacted with Glu141 of RXFP3, meanwhile both B12Arg and B16Arg of relaxin-3 probably interacted with Asp145 of RXFP3.

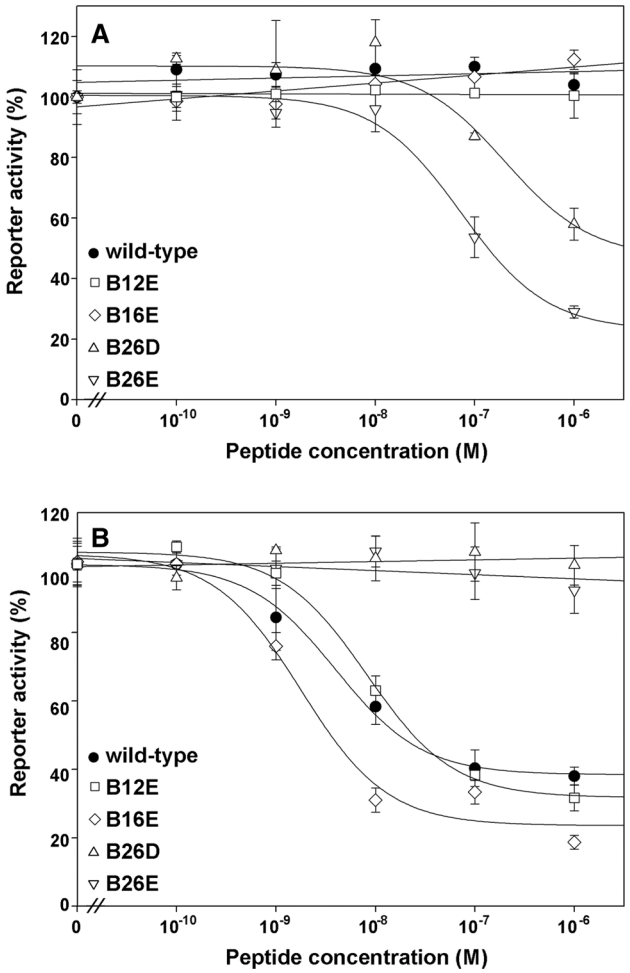

Fig. 5 Receptor activation of mutant relaxin-3s towards [E141R]RXFP3 (**a**) and [D145R]RXFP3 (**b**). A CRE-controlled nanoluciferase reporter was used in these assays. The measured data were expressed as mean ± SE ($n = 3$), and fitted with sigmoidal curves using SigmaPlot 10.0 software

Discussion

In the present study, we first identified an essential negatively charged extracellular EXXXD segment in receptor RXFP3 by replacing the negatively charged extracellular residues of the receptor with positively charged arginine.

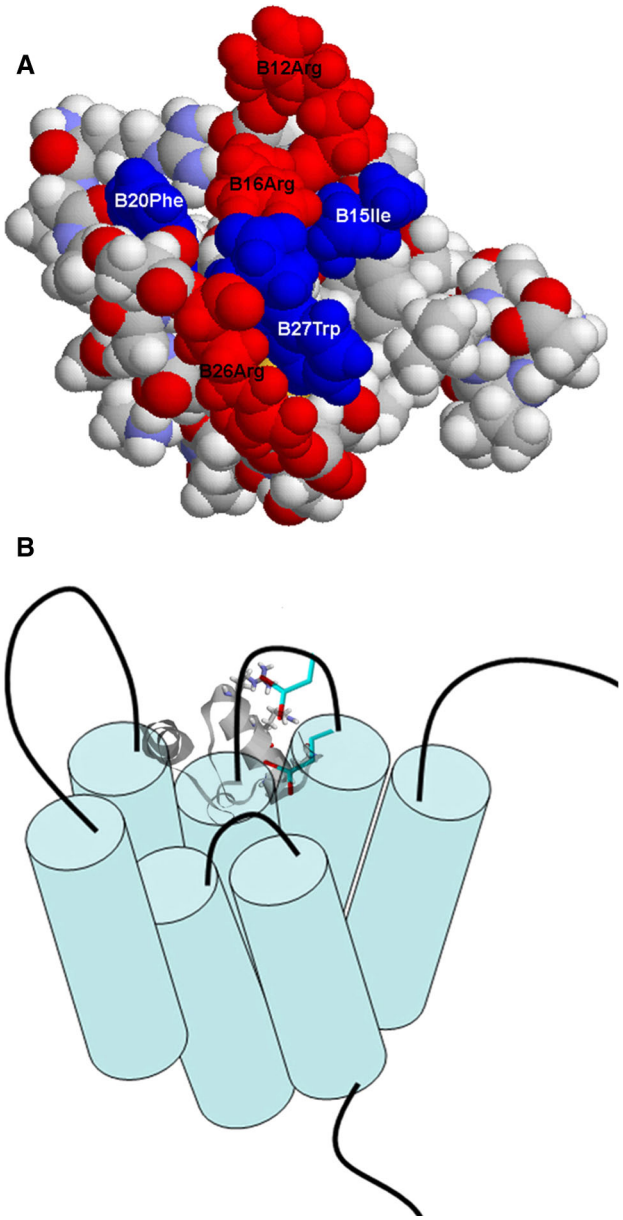

Fig. 6 **a** The critical positively charged arginine patches (*red*) and critical hydrophobic patch (*blue*) on the surface of human relaxin-3. The relaxin-3 structure was generated according to the previously reported NMR structure (PDB ID: 2FHW). **b** The proposed working model for the interaction between RXFP3 and relaxin-3, involving the negatively charged EXXXD motif of RXFP3 and the three highly conserved B12Arg, B16Arg and B26Arg of relaxin-3 (colour figure online)

Charged polar residues are generally located at the protein surface, thus their replacement might result in fewer disturbances on the protein structure than the replacement of hydrophobic residues, which are generally buried in the interior of the protein structures. The negatively charged Glu141 and Asp145 are highly conserved in RXFP3s from different species (supplementary Fig. s1), suggesting that this motif is critical for RXFP3.

By replacing the highly conserved positively charged Arg residues of relaxin-3, we identified two probable interaction pairs between RXFP3 and relaxin-3: Glu141 of RXFP3 with B26Arg of relaxin-3, and Asp145 of RXFP3 with both B12Arg and B16Arg of relaxin-3. Since Glu141 is near the membrane region, we deduced that it was located in a deep part of the ligand-binding pocket. B26Arg of relaxin-3 was located at the flexible B-chain C-terminus, so it could insert into the deep part of the ligand-binding pocket and interact with Glu141 of RXFP3. Since Asp145 is located at a distance from the membrane region, we deduced it was located at a shallow part of the receptor-binding pocket. Both B12Arg and B16Arg are located in a rigid helical segment of relaxin-3, thus they could only interact with the shallowly located Asp145 of RXFP3. According to the NMR structure of relaxin-3 (Rosengren et al. 2006), B12Arg and B16Arg form a positively charged hydrophilic patch on the surface of relaxin-3, while B26Arg forms another positively charged patch on the surface (Fig. 6a, shown in red). These two positively charged patches are spatially near each other due to folding back of the B-chain C-terminus. Since these two arginine patches are adjacent, we deduced that they could interact with both Glu141 and Asp145 of RXFP3 in a pairwise manner (Fig. 6b).

During this manuscript preparation and submission, similar results were reported by another laboratory (Bathgate et al. 2013). In their work, the negatively charged extracellular residues of RXFP3 were replaced by hydrophobic alanine residue and the ligand-binding activities of the resultant RXFP3 mutants were measured. In our present work, the negatively charged extracellular residues of RXFP3 were replaced by positively charged hydrophilic arginine residue and the activation potencies of the resultant RXFP3 mutants were measured. Thus, complementary approaches were used in our present work and the reported work. Together with the results of receptor-binding assay and molecular modeling, in the reported work the authors proposed a ligand–receptor interaction model in which B26Arg of relaxin-3 interacted with Glu141 of RXFP3, B16Arg of relaxin-3 interacted with Asp145 of RXFP3, while B12Arg of relaxin-3 interacted with Glu244 of RXFP3. The reported interaction model was consistent with our present model except that B12Arg of relaxin-3 interacted with Glu244 of RXFP3 in their model rather than interacted with Glu145 in our model. When Glu244 of RXFP3 was replaced by hydrophobic alanine, the resultant mutant receptor lost most of the ligand-binding potency (Bathgate et al. 2013), suggesting Glu244 was important for RXFP3. In contrast, when Glu244 was replaced by positively charged arginine in our present work, the resultant receptor retained almost full sensitivity to relaxin-3 (Table 1), suggesting Glu244 was not important for the receptor. It was well known that sometimes mutagenesis

could cause unexpected structural disturbance, so high-resolution structure of relaxin-3/RXFP3 complex would be needed to finally solve this difference in future studies.

In addition to interactions between the EXXXD motif of RXFP3 and the B-chain arginine residues of relaxin-3, hydrophobic interactions must also be involved in the relaxin-3/RXFP3 interaction. Previous work has revealed three critical hydrophobic residues in relaxin-3: B27Trp, B20Phe and B15Ile (Kuei et al. 2007). As shown in Fig. 6a, they form a hydrophobic patch on the surface of relaxin-3 (blue) adjacent to the positively charged arginine patches. This hydrophobic patch of relaxin-3 probably interacts with the hydrophobic residues of RXFP3, thereby contributing to the ligand–receptor interaction. Consistent with this hypothesis, highly conserved hydrophobic residues are present around the EXXXD motif of RXFP3 (supplementary Fig. s1). In future studies, we will try to reveal the critical hydrophobic residues in RXFP3 that interact with the critical hydrophobic patch of relaxin-3.

To activate RXFP3, the binding of relaxin-3 should lead to a change in the orientation of the transmembrane helices, thus activating downstream signaling (Hulme 2013; Lebon et al. 2011; Xu et al. 2011). It is plausible that relaxin-3 inserts into the ligand-binding pocket of RXFP3 like a wedge, pushing the extracellular end of TMD2 or other TMDs by the lever principle (Fig. 6b). To cause enough movement of the TMDs to activate RXFP3, the B-chain C-terminus of relaxin-3 should be large sufficiently. For example, replacement of B27Trp with a small alanine residue resulted in a mutant that retained the high RXFP3-binding affinity but had low activation potency (Kuei et al. 2007). Furthermore, the B-chain C-terminus-shortened analogs R3(BΔ23-27)R and R3(BΔ23-27)R/I5 also retained high-binding affinities for RXFP3, but were unable to activate the receptor (Kuei et al. 2007). The additional Arg introduced to the shortened B-chain C-terminus was critical for high-affinity receptor binding (Hossain et al. 2009), and we deduced it probably interacted with Glu141 of RXFP3 to enhance the binding affinity. However, this binding could not activate the receptor since the small-size B-chain C-terminus could not cause enough movement of the helices of the receptor.

The EXXXD motif is also present and highly conserved in RXFP4 s from different species (supplementary Fig. s1), suggesting this negatively charged segment is also important for RXFP4. This motif is also located at the extracellular end of the predicted TMD2 of RXFP4. RXFP4 can be activated by both relaxin-3 and INSL5. INSL5 has only two conserved arginine residues (B13Arg and B23Arg), corresponding to B16Arg and B26Arg of relaxin-3 (supplementary Fig. s2). A previous study has demonstrated that B12Arg of relaxin-3 is not important for RXFP4 activation (Kuei et al. 2007); thus, activation of RXFP4 requires only two arginine residues of relaxin-3 or INSL5. We deduced that, during RXFP4 activation, the B-chain C-terminal arginine residue of relaxin-3 or INSL5 interacted with Glu100 of RXFP4, while the B-chain central arginine of relaxin-3 or INSL5 interacted with Asp104 of RXFP4. In the future, we will attempt to reveal the role of the negatively charged EXXXD motif in RXFP4 by site-directed mutagenesis of both the receptor and the ligand.

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Conflict of interest The authors declare that they have no conflict of interest.

References

Banerjee A, Shen PJ, Ma S, Bathgate RA, Gundlach AL (2010) Swim stress excitation of nucleus incertus and rapid induction of relaxin-3 expression via CRF1 activation. Neuropharmacology 58:145–155

Bathgate RA, Samuel CS, Burazin TC, Layfield S, Claasz AA, Reytomas IG, Dawson NF, Zhao C, Bond C, Summers RJ, Parry LJ, Wade JD, Tregear GW (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

Bathgate RA, Oh MH, Ling WJ, Kaas Q, Hossain MA, Gooley PR, Rosengren KJ (2013) Elucidation of relaxin-3 binding interactions in the extracellular loops of RXFP3. Front Endocrinol (Lausanne) 4:13

Burazin TC, Bathgate RA, Macris M, Layfield S, Gundlach AL, Tregear GW (2002) Restricted, but abundant, expression of the novel rat gene-3 (R3) relaxin in the dorsal tegmental region of brain. J Neurochem 82:1553–1557

Eigenbrot C, Randal M, Quan C, Burnier J, O'Connell L, Rinderknecht E, Kossiakoff AA (1991) X-ray structure of human relaxin at 1.5 Å. Comparison to insulin and implications for receptor binding determinants. J Mol Biol 221:15–21

Hall MP, Unch J, Binkowski BF, Valley MP, Butler BL, Wood MG, Otto P, Zimmerman K, Vidugiris G, Machleidt T, Robers MB, Benink HA, Eggers CT, Slater MR, Meisenheimer PL, Klaubert DH, Fan F, Encell LP, Wood KV (2012) Engineered luciferase reporter from a deep sea shrimp utilizing a novel imidazopyrazinone substrate. ACS Chem Biol 7:1848–1857

Haugaard-Jonsson LM, Hossain MA, Daly NL, Craik DJ, Wade JD, Rosengren KJ (2009) Structure of human insulin-like peptide 5 and characterization of conserved hydrogen bonds and electrostatic interactions within the relaxin framework. Biochem J 419:619–627

Hida T, Takahashi E, Shikata K, Hirohashi T, Sawai T, Seiki T, Tanaka H, Kawai T, Ito O, Arai T, Yokoi A, Hirakawa T, Ogura H, Nagasu T, Miyamoto N, Kuromitsu J (2006) Chronic intracerebroventricular administration of relaxin-3 increases body weight in rats. J Recept Signal Transduct Res 26:147–158

Hossain MA, Rosengren KJ, Haugaard-Jonsson LM, Zhang S, Layfield S, Ferraro T, Daly NL, Tregear GW, Wade JD, Bathgate RA (2008) The A-chain of human relaxin family peptides has distinct roles in the binding and activation of the different relaxin family peptide receptors. J Biol Chem 283:17287–17297

Hossain MA, Bathgate RA, Rosengren KJ, Shabanpoor F, Zhang S, Lin F, Tregear GW, Wade JD (2009) The structural and functional role of the B-chain C-terminal arginine in the relaxin-3 peptide antagonist, R3(BD23-27)R/I5. Chem Biol Drug Des 73:46–52

Hsu SY, Nakabayashi K, Nishi S, Kumagai J, Kudo M, Sherwood OD, Hsueh AJ (2002) Activation of orphan receptors by the hormone relaxin. Science 295:671–674

Hulme EC (2013) GPCR activation: a mutagenic spotlight on crystal structures. Trends Pharmacol Sci 34:67–84

Krautwurst D, Yau KW, Reed RR (1998) Identification of ligands for olfactory receptors by functional expression of a receptor library. Cell 95:917–926

Kuei C, Sutton S, Bonaventure P, Pudiak C, Shelton J, Zhu J, Nepomuceno D, Wu J, Chen J, Kamme F, Seierstad M, Hack MD, Bathgate RA, Hossain MA, Wade JD, Atack J, Lovenberg TW, Liu C (2007) R3(BΔ23–27)R/I5 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

Lebon G, Warne T, Edwards PC, Bennett K, Langmead CJ, Leslie AG, Tate CG (2011) Agonist-bound adenosine A2A receptor structures reveal common features of GPCR activation. Nature 474:521–525

Liu C, Chen J, Sutton S, Roland B, Kuei C, Farmer N, Sillard R, Lovenberg TW (2003a) Identification of relaxin-3/INSL7 as a ligand for GPCR142. J Biol Chem 278:50765–50770

Liu C, Eriste E, Sutton S, Chen J, Roland B, Kuei C, Farmer N, Jörnvall H, Sillard R, Lovenberg TW (2003b) Identification of relaxin-3/INSL7 as an endogenous ligand for the orphan G-protein-coupled receptor GPCR135. J Biol Chem 278:50754–50764

Liu C, Chen J, Kuei C, Sutton S, Nepomuceno D, Bonaventure P, Lovenberg TW (2005a) 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

Liu C, Kuei C, Sutton S, Chen J, Bonaventure P, Wu J, Nepomuceno D, Kamme F, Tran DT, Zhu J, Wilkinson T, Bathgate RA, Eriste E, Sillard R, Lovenberg TW (2005b) INSL5 is a high affinity specific agonist for GPCR142 (GPR100). J Biol Chem 280:292–300

Luo X, Liu YL, Layfield S, Shao XX, Bathgate RA, Wade JD, Guo ZY (2010) A simple approach for the preparation of mature human relaxin-3. Peptides 31:2083–2088

Ma S, Sang Q, Lanciego JL, Gundlach AL (2009) Localization of relaxin-3 in brain of Macaca fascicularis: identification of a nucleus incertus in primate. J Comp Neurol 517:856–872

McGowan BM, Stanley SA, Smith KL, White NE, Connolly MM, Thompson EL, Gardiner JV, Murphy KG, Ghatei MA, Bloom SR (2005) Central relaxin-3 administration causes hyperphagia in male Wistar rats. Endocrinology 146:3295–3300

McGowan BM, Stanley SA, Smith KL, Minnion JS, Donovan J, Thompson EL, Patterson M, Connolly MM, Abbott CR, Small CJ, Gardiner JV, Ghatei MA, Bloom SR (2006) Effects of acute and chronic relaxin-3 on food intake and energy expenditure in rats. Regul Pept 136:72–77

McGowan BM, Stanley SA, White NE, Spangeus A, Patterson M, Thompson EL, Smith KL, Donovan J, Gardiner JV, Ghatei MA, Bloom SR (2007) Hypothalamic mapping of orexigenic action and Fos-like immunoreactivity following relaxin-3 administration in male Wistar rats. Am J Physiol Endocrinol Metab 292:E913–E919

McGowan BM, Stanley SA, Donovan J, Thompson EL, Patterson M, Semjonous NM, Gardiner JV, Murphy KG, Ghatei MA, Bloom SR (2008) Relaxin-3 stimulates the hypothalamic-pituitary-gonadal axis. Am J Physiol Endocrinol Metab 295:E278–E286

Olucha-Bordonau FE, Otero-García M, Sánchez-Pérez AM, Núñez A, Ma S, Gundlach AL (2012) Distribution and targets of the relaxin-3 innervation of the septal area in the rat. J Comp Neurol 520:1903–1939

Rosengren KJ, Lin F, Bathgate RA, Tregear GW, Daly NL, Wade JD, Craik DJ (2006) Solution structure and novel insights into the determinants of the receptor specificity of human relaxin-3. J Biol Chem 281:5845–5851

Ryan PJ, Ma S, Olucha-Bordonau FE, Gundlach AL (2011) Nucleus incertus–an emerging modulatory role in arousal, stress and memory. Neurosci Biobehav Rev 35:1326–1341

Shabanpoor F, Separovic F, Wade JD (2009) The human insulin superfamily of polypeptide hormones. Vitam Horm 80:1–31

Silvertown JD, Neschadim A, Liu HN, Shannon P, Walia JS, Kao JC, Robertson J, Summerlee AJ, Medin JA (2010) Relaxin-3 and receptors in the human and rhesus brain and reproductive tissues. Regul Pept 159:4–53

Smith CM, Shen PJ, Banerjee A, Bonaventure P, Ma S, Bathgate RA, Sutton SW, Gundlach AL (2010) Distribution of relaxin-3 and RXFP3 within arousal, stress, affective, and cognitive circuits of mouse brain. J Comp Neurol 518:4016–4045

Smith CM, Ryan PJ, Hosken IT, Ma S, Gundlach AL (2011) Relaxin-3 systems in the brain—the first 10 years. J Chem Neuroanat 42:262–275

Smith CM, Hosken IT, Sutton SW, Lawrence AJ, Gundlach AL (2012) Relaxin-3 null mutation mice display a circadian hypoactivity phenotype. Genes Brain Behav 11:94–104

Sudo S, Kumagai J, Nishi S, Layfield S, Ferraro T, Bathgate RA, Hsueh AJ (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

Tanaka M (2010) Relaxin-3/insulin-like peptide 7, a neuropeptide involved in the stress response and food intake. FEBS J 277:4990–4997

Tanaka M, Iijima N, Miyamoto Y, Fukusumi S, Itoh Y, Ozawa H, Ibata Y (2005) Neurons expressing relaxin 3/INSL 7 in the nucleus incertus respond to stress. Eur J Neurosci 21:1659–1670

Watanabe Y, Miyamoto Y, Matsuda T, Tanaka M (2011a) Relaxin-3/INSL7 regulates the stress-response system in the rat hypothalamus. J Mol Neurosci 43:169–174

Watanabe Y, Tsujimura A, Takao K, Nishi K, Ito Y, Yasuhara Y, Nakatomi Y, Yokoyama C, Fukui K, Miyakawa T, Tanaka M (2011b) Relaxin-3-deficient mice showed slight alteration in anxiety-related behavior. Front Behav Neurosci 5:50

Xu F, Wu H, Katritch V, Han GW, Jacobson KA, Gao ZG, Cherezov V, Stevens RC (2011) Structure of an agonist-bound human A2A adenosine receptor. Science 332:322–327

Zhang WJ, Luo X, Liu YL, Shao XX, Wade JD, Bathgate RA, Guo ZY (2012a) Site-specific DOTA/europium-labeling of recombinant human relaxin-3 for receptor-ligand interaction studies. Amino Acids 43:983–992

Zhang WJ, Luo X, Song G, Wang XY, Shao XX, Guo ZY (2012b) Design, recombinant expression and convenient A-chain N-terminal europium-labelling of a fully active human relaxin-3 analogue. FEBS J 279:1505–1512

Zhang WJ, Jiang Q, Wang XY, Song G, Shao XX, Guo ZY (2013) A convenient method for europium-labeling of a recombinant chimeric relaxin family peptide R3/I5 for receptor-binding assays. J Pept Sci 19:350–354

Zhu J, Kuei C, Sutton S, Kamme F, Yu J, Bonaventure P, Atack J, Lovenberg TW, Liu C (2008) Identification of the domains in RXFP4 (GPCR142) responsible for the high affinity binding and agonistic activity of INSL5 at RXFP4 compared to RXFP3 (GPCR135). Eur J Pharmacol 590:43–52

Zhuang H, Matsunami H (2007) Synergism of accessory factors in functional expression of mammalian odorant receptors. J Biol Chem 282:15284–15293