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Minimization of Human Relaxin-3 Leading to High-Affinity Analogues with Increased Selectivity for Relaxin-Family Peptide 3 Receptor (RXFP3) over RXFP1

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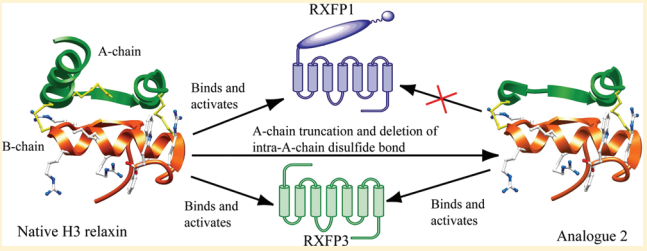
ABSTRACT: Relaxin-3 is a neuropeptide that is implicated in the regulation of stress responses and memory. The elucidation of its precise physiological role(s) has, however, been hampered by cross-activation of the relaxin-2 receptor, RXFP1, in the brain. The current study undertook to develop analogues of human relaxin-3 (H3 relaxin) that can selectively bind and activate its receptor, RXFP3. We developed a high-affinity selective agonist (analogue **2**) by removal of the intra-A chain disulfide bond and deletion of 10 residues from the N terminus of the A chain. Further truncation of this analogue from the C terminus of the B chain to Cys^{B22} and addition of an Arg^{B23} led to a high-affinity, RXFP3-selective, competitive antagonist (analogue **3**). Central administration of analogue **2** in rats increased food intake, which was blocked by prior coadministration of analogue **3**. These novel RXFP3-selective peptides represent valuable pharmacological tools to study the physiological roles of H3 relaxin/RXFP3 systems in the brain and important leads for the development of novel compounds for the treatment of affective and cognitive disorders.



INTRODUCTION

The human relaxin-family of polypeptides consists of seven members, relaxins-1−3 and insulin-like peptides (INSLs) 3−6, which have different tissue expression profiles and distinct physiological roles.[1−4] Relaxin-3 (also known as INSL7) is the most recently identified member[5] and, on the basis of phylogenetic analyses, is the ancestral gene from which the genes for relaxin-1 and -2 and INSLs 3−6 have evolved.[6]

The native receptor for relaxin-3 was recently identified as G-protein-coupled receptor (GPCR) 135 and reclassified according to IUPHAR convention as relaxin-family peptide 3 receptor (RXFP3).[7,8] RXFP3 is a typical, class A neuropeptide receptor with seven transmembrane helices and is similar in structure to the receptor for INSL5, RXFP4 (formerly GPCR142).[7,9] Interestingly though, in cell-based systems, in addition to RXFP3, human H3 relaxin also binds to RXFP4 and RXFP1,[8] the cognate receptor for relaxin-2.[10]

The tertiary structure of H3 relaxin as determined using solution NMR spectroscopy[11] has a core insulin structure that is similar to other members of the relaxin family analyzed to date. Its intramolecular disulfide-bonded A chain contains two terminal α-helices that are separated by a turn, and the chain is linked via two interchain disulfide bonds to the B chain, which contains a long central α-helix that terminates with a prominent turn (Figure 1). Structure−activity studies using Ala scanning have revealed that H3 relaxin binds to its receptor via residues arginine^{B8,12,16} (Arg^{B8,12,16}), Ile^{B15}, and Phe^{B20}, which are located in the helical region of the B chain. In addition to these residues, H3 relaxin requires Arg^{B26} and Trp^{B27} located toward the C terminus of its B chain, to effectively activate RXFP3.[12] In fact, the H3 relaxin B chain alone has also been shown to bind and activate RXFP3, albeit with much lower affinity than native H3 relaxin.[9,13]

In situ hybridization and ligand autoradiography studies have revealed that RXFP3 is strongly expressed by neurons in brain areas containing relaxin-3 immunoreactive nerve fibers, including olfactory bulb, cerebral cortex, major limbic regions, midline thalamus, hypothalamus, and brainstem.[14−16] The physiological functions of relaxin-3 remain to be conclusively identified, but the expression pattern of the peptide and of RXFP3, together with data from initial functional and other anatomical studies, suggests an important and broad modulatory role in arousal, stress responses, and metabolic homeostasis.[3,4] The first pharmacological action of relaxin-3 reported was an increase in food intake in rats after injection of the native peptide into the lateral cerebral ventricle or directly into the hypothalamus.[17,18] This effect was also observed following

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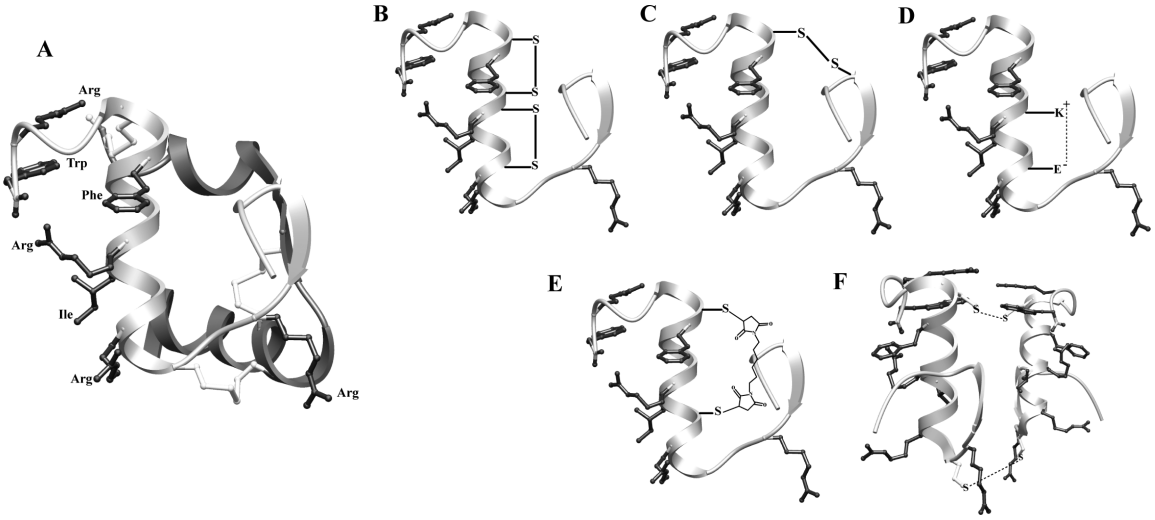

Figure 1. Solution NMR structure of H3 relaxin with receptor binding and activation residues ($R^{8,12,16,26}$, I^{15}, F^{20}, and W^{27}) highlighted (A), two disulfide bonds incorporated along the B chain helix at C^{10-14} and C^{18-22} (B), a disulfide bond incorporated between the strand and the helix at position $C^{5}-C^{21}$ (C), a Glu^{-} to Lys^{+} salt bridge added in the middle of the B chain helix (D), the B chain helix was constrained at position *i* and *i* + 7 with 1,6-bismaleimidohexane (E), and dimer of the B chain (F).

chronic administration.[19,20] Additionally, subsequent studies reported effects of these treatments on the central and peripheral neuroendocrine profile in rats and also confirmed the ability of relaxin-3 to mimic some of the actions of relaxin in the brain,[21,22] making these findings difficult to interpret.

Despite this relative wealth of structural, pharmacological, and anatomical information, the study of central actions of relaxin-3 in intact animal models is confounded by its pharmacological activation of both RXFP3 *and* RXFP1. There is a clear need, therefore, for agonist and antagonist peptides that only bind and activate RXFP3, and in a recent important study, selective RXFP3 ligands were produced that consisted of a series of chimeric peptides comprising a H3 relaxin B chain and the A chain of other members of relaxin-family peptides.[13] Among these chimeric peptides, H3 relaxin B chain linked to INSL5 A chain (R3/I5) displayed good selectivity for RXFP3 over RXFP1, but not full specificity, with RXFP1 activity at >1 μM.[13] Nevertheless, the R3/I5 chimera has been employed in more recent attempts to study the in vivo effects of RXFP3 activation. It enabled a confirmation of the ability of RXFP3 signaling to increase food intake in rats[12] and revealed the ability of relaxin-3/RXFP3 signaling to alter the activity of the septohippocampal pathway and spatial memory.[23] In subsequent studies with the R3/I5 peptide, it was demonstrated that replacement of the GGSRW residues at the B chain C terminus with an Arg led to creation of a high-affinity antagonist, ΔR3/I5.[12] Neither R3/I5 or ΔR3/I5 are wholly selective for RXFP3 over RXFP1, their chemical synthesis has proven to be challenging, and high-scale production for in vivo studies is costly. Therefore, in recent study, we created a high-affinity single chain antagonist (H3 B1−22R) by replacing the GGSRW of the H3 relaxin B chain with Arg and mutating the native cysteine (Cys) to Ser.[24] This peptide has a similar RXFP3 affinity to native H3 relaxin for RXFP3 and does not bind to either RXFP1 or RXFP4. The introduction of Arg into the B chain sequence was thought to create an additional contact point between the receptor and the peptide as the peptide without this Arg has very low affinity for RXFP3.

In the current study, therefore, we aimed to develop small agonistic analogues of H3 relaxin with specificity for RXFP3 over RXFP1 and which would be easier to synthesize chemically. As the B chain of H3 relaxin alone has been demonstrated to be a weak agonist, we first attempted to produce a high-affinity single-chain agonist based on stabilizing the helical structure of the B chain or using an Arg insertion, which was successful for the specific single-chain antagonist. Additionally, we attempted to minimize and simplify the native H3 relaxin peptide to a structure that was easy to synthesize and which retained high affinity and potency at RXFP3 while having little or no activity at RXFP1. Any such selective agonist and related antagonist will not only be valuable pharmacological tools in studies of the role of relaxin-3 in vivo but will also serve as leads for the development of clinically useful compounds.

RESULTS

Synthesis and Chemical Characterization of H3 Relaxin Analogues. Two series of analogues, single B chain and domain minimized analogues of native H3 relaxin, were designed and synthesized. The single-chained analogues (Table 1) were each synthesized in high yields (50−70%), and the overall yields obtained for the syntheses of the two-chained analogues ranged from 10 to 20% relative to the starting B chain, due to the multiple steps required to form the two interchain disulfide bonds. The sequential disulfide bond formation was achieved by first converting the Cys(tBu)[10] in the A chain to Cys(SPy) (yield 60−70%) followed by chain combination between A chain Cys(SPy) and B chain Cys(SH) (yield 30−40%). The second interchain disulfide bond was formed by iodine oxidation, and the final yield obtained at this step was often low (10−20%) as compared to other steps (Scheme 1). The reaction at each step was monitored by RP-HPLC, and the resulting products were purified to single peak purity (>95%). The monoisotopic masses were determined using MALDI-TOF/TOF mass spectrometry (Table 2). The peptide content of each was determined using amino acid analysis and ranged from 30 to 80% of the peptide weight. This information was used to accurately determine the peptide concentration for bioassays.

Determination of Secondary Structure of H3 Relaxin Analogues. The secondary structures of the analogues were determined using circular dichroism (CD) spectroscopy

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Table 1. Primary Structures of H3 Relaxin B Chain and It Analogues with Their Receptor Binding Affinity (pK_i)

peptide	sequence	pK_i
native H3 B chain	H-RAAPYGVRL**C**GREFIRAVIFT**C**GGSRW-OH	
$C^{10,22}$-**S**	H-RAAPYGVRL**S**GREFIRAVIFT**S**GGSRW-OH	5.53 ± 0.07
Y^{5}, T^{21}-**C**	H-RAAP**C**GVRLSGREFIRAVIF**C**SGGSRW-OH	<5
C^{10-14}, C^{18-22}	H-RAAPYGVRL**C**GRE**C**IRA**C**IFT**C**GGSRW-OH	<5
BisMal-C^{15-22}	H-RAAPYGVRLSGREF**C**RAVIFT**C**GGSRW-OH	<5
A^{17}-**K**	H-RAAPYGVRLSGRE**K**IRKVIFTSGGSRW-OH	<5
B chain dimer	H-RAAPYGVRL**C**GREFIRAVIFT**C**GGSRW-OH	5.34 ± 0.03
	H-RAAPYGVRL**C**GREFIRAVIFT**C**GGSRW-OH	
T^{21}-**R**	H-RAAPYGVRLSGREFIRAVIF**R**SGGSRW-OH	6.13 ± 0.09
C^{22}-**R**	H-RAAPYGVRLSGREFIRAVIFT**R**GGSRW-OH	5.47 ± 0.33
G^{23}-**R**	H-RAAPYGVRLSGREFIRAVIFTS**R**GSRW-OH	5.68 ± 0.12
S^{22} **R** G^{23}	H-RAAPYGVRLSGREFIRAVIFTS**R**GGSRW-OH	5.59 ± 0.08

(Figure 2). The percentage of α-helicity was calculated from the theta value (θ). H3 relaxin and analogue **2** displayed similar α-helicity of about 28%, whereas the single B chain analogues, B chain dimer, and analogue **1** did not exhibit any significant secondary structure, and they appeared as random coils.

Determination of Receptor Binding Affinity of H3 Relaxin Analogues. Competition binding assays were used to determine the binding affinity of the analogues at RXFP1, RXFP3, and RXFP4, using europium-labeled H2 relaxin for RXFP1, europium-labeled H3 relaxin B chain/INSL5 A chain chimeric peptide for RXFP3, and europium-labeled mouse INSL5 for RXFP4 (Table 3). The linear single B chain analogues and the B chain dimer (Table 1) displayed very low binding affinity with K_i values ranging from 1 to 10 μM.

In contrast, analogues **2** and **3** demonstrated binding affinities very similar to H3 relaxin (pK_i of 7.87 ± 0.12, 7.6 ± 0.05, and 7.78 ± 0.06, respectively; Figure 3A). Analogues **1** and **4**–**6** each displayed similar binding affinity for RXFP3 *albeit* lower than H3 relaxin and analogues **2** and **3** (Figure 3A). Importantly, none of the analogues had any affinity for RXFP1 in contrast to native H3 relaxin (8.60 ± 0.01) (Figure 3C). Analogue **3** bound to RXFP4 with low affinity, whereas analogue **2** had similar binding affinity to native INSL5 for RXFP4 (Figure 3E).

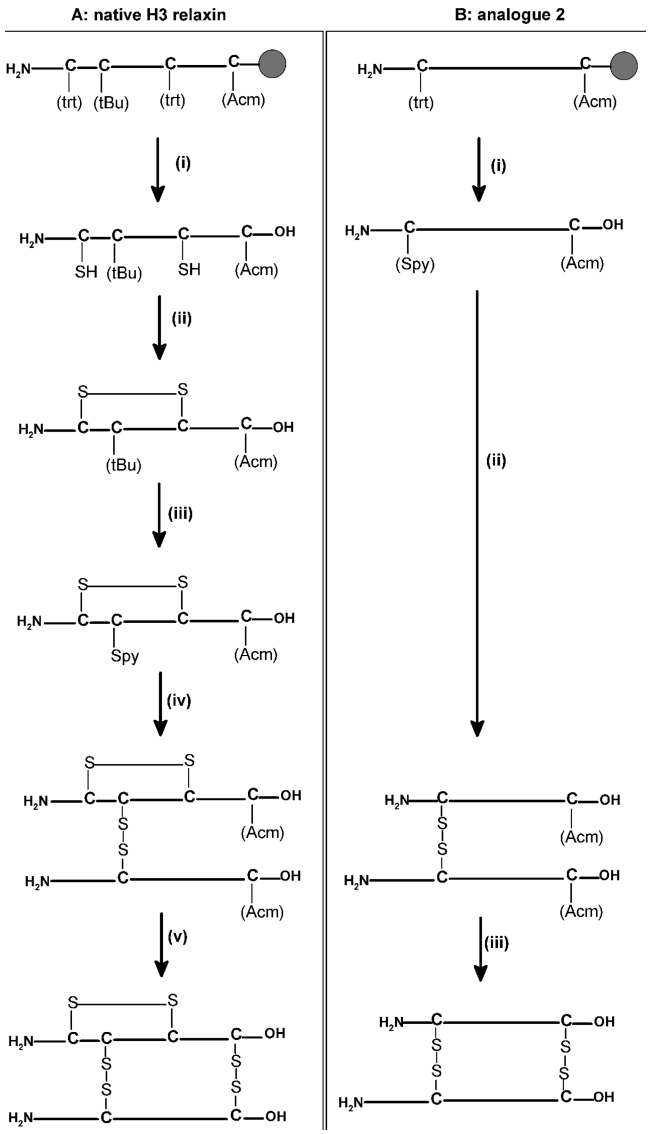
Inhibition of Forskolin-Induced cAMP Accumulation in RXFP3- and RXFP4-Expressing Cells. The potency of the analogues at activating RXFP3 and RXFP4 was determined by measuring their ability to inhibit forskolin-induced cAMP production (pEC_{50}) (Table 3). Importantly, all of the peptides except analogue **3** were able to activate RXFP3 and RXFP4, indicating that they were full agonists at both receptors. H3 relaxin and analogues **2** and **4** had the highest potency at RXFP3 with pEC_{50} values of 9.0 ± 0.07, 8.43 ± 0.09, and 8.37 ± 0.1 ($p > 0.05$), respectively (Figure 3B). Analogues **1**, **5**, and **6** could equally activate the receptor to the same extent (Figure 3B). Analogues **2**, **4**, and **5** displayed high activation potency at RXFP4, and analogue **6** had even higher potency (8.46 ± 0.17) that was similar to native INSL5 (8.51 ± 0.11) and H3 relaxin (8.94 ± 0.13) (Figure 3F).

As anticipated, analogue **3** did not activate RXFP3 or RXFP4, as it failed to inhibit forskolin-induced cAMP production. However, it was able to dose dependently antagonize the activity of H3 relaxin at RXFP3 and INSL5 at RXFP4 (Figure 4A,B).

Stimulation of cAMP Accumulation in RXFP1-Expressing Cells. Analogues **1**, **2**, and **4**–**6** were also tested for their ability to increase cAMP production in human embryonic kidney (HEK)-239 cells expressing RXFP1. H3 relaxin was able to activate RXFP1 (pEC50 = 8.74 ± 0.06) and induce cAMP production (Figure 3D). Analogue **1** activated RXFP1 at a high concentration (1 μM), but the other analogues failed to activate RXFP1 even at high concentrations (Table 3), consistent with their inability to compete with europium-labeled H2 relaxin binding.

Extracellular Signal-Regulated Kinase (ERK)1/2 Phosphorylation in RXFP3-Expressing Cells. The ability of native H3 relaxin and analogue **2** to activate ERK1/2 kinase

Scheme 1. Schematic Diagram of Synthesis of Native H3 Relaxin and Analogue 2[a]



[a](A-i) TFA:DoDt:H_2O:TIPS (94:2.5:2.5:1, v/v) for 90 min. (B-i) TFA:DoDt:H_2O:TIPS (94:2.5:2.5:1, v/v) and 10-fold excess of DPDS cleaved for 90 min. (A-ii) One milligram of peptide dissolved in 100 mM NH_4CO_3/0.2 mL of 1 mM DPDS. (A-iii) Peptide:DPDS (1:4 μmol) dissolved in TFA:thioanisole (9:1) 50 μg/μL, chilled on ice to 0 °C, and then, a similar volume of TFA:TFMSA was added and stirred for 45 min. (A-iv, B-ii) A chain dissolved in 100 mM NH_4CO_3 and equimolar amount of B chain dissolved in H_2O were added and stirred for 30 min. (A-v, B-iii) Peptide dissolved in acetic acid (2 mg/mL) and 60 mM HCl was added (5% total volume) followed by addition of 20 mM I_2 (25 eq/Acm) and stirred for 45 min at room temperature.

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Table 2. Primary Structures of H3 Relaxin and Its Minimized Analogues with Their Calculated and Obtained Monoisotopic Masses ([M + H])

Peptide	Sequence	[M+H]$^+$ Calculated	[M+H]$^+$ Experimental
H3 relaxin	H-RAAPYGVRLCGREFIRAVIFTCGGSRW-OH H-DVLAGLSSSCCKWGCSKSEISSLC-OH	5497	5498
1	H-RAAPYGVRLCGREFIRAVIFTCGGSRW-OH H-DVLAGLSSSACKWGASKSEISSLC-OH	5434.7	5440.7
2	H-RAAPYGVRLCGREFIRAVIFTCGGSRW-OH H-CKWGASKSEISSLC-OH	4533.7	4533.8
3	H-RAAPYGVRLCGREFIRAVIFTCR-OH H-CKWGASKSEISSLC-OH	4149.1	4150
4	H-GVRLCGREFIRAVIFTCGGSRW-OH H-CKWGASKSEISSLC-OH	3977	3977
5	H-RLCGREFIRAVIFTCGGSRW-OH H-CKWGASKSEISSLC-OH	3819	3819.2
6	H-CGREFIRAVIFTCGGSRW-OH H-CKWGASKSEISSLC-OH	3550.7	3550.4

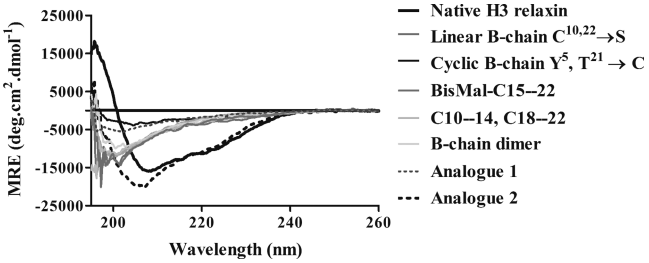

Figure 2. CD spectroscopy profiles of native H3 relaxin and analogues **1** and **2** in PBS (pH 7.4) at room temperature. H3 relaxin and analogue **2** display a measurable amount of helicity at 222 nm, whereas analogue **1** and the single B chain analogues had no secondary structure and were principally random coils.

phosphorylation was determined. Both H3 relaxin and analogue **2** induced ERK1/2 phosphorylation in a concentration-dependent manner with analogue **2** producing a slightly greater effect [pEC_{50} values of 9.83 ± 0.14 (n = 5) and 10.4 ± 0.11 (n = 4), respectively (Figure 5A), $p < 0.05$]. In contrast, analogue **3** did not induce phosphorylation of ERK1/2, but increasing concentrations of analogue **3** antagonized ERK1/2 phosphorylation induced by H3 relaxin (10 nM) (Figure 5B). The ability of analogue **3** to inhibit H3 relaxin-induced p38MAPK phosphorylation was also tested in the same way as ERK1/2 phosphorylation. Analogue **3** antagonized H3 relaxin-induced p38MAPK phosphorylation in a dose-dependent manner (data not shown).

Inhibition of Transforming Growth Factor (TGF)-β1-Induced Collagen Expression in Human Dermal Fibroblast Cells. The inhibitory effects of H2 relaxin, H3 relaxin, and analogue **2** on TGF-β1-stimulated collagen deposition via RXFP1 activation was assessed by measuring the level of hydroxyproline after 72 h of treatment of BJ3 cells with each peptide. H2 and H3 relaxin comparably lowered the level of collagen by 35−40%, respectively, as compared to TGF-β1 only treatment of the cells. However, as anticipated from RXFP1 binding studies, analogue **2** did not alter the level of TGF-β1-stimulated collagen expression (Figure 6), further demonstrating its selectivity for RXFP3.

Effect of Central Administration on Food Intake in Rats. Central administration of analogue **2** (1.1 nmol, icv) to satiated rats during the early light phase significantly increased food intake within the first hour after injection, as compared to control vehicle or mock injections (Figure 7). The magnitude of the effect on food intake was comparable to that observed after administration of R3/I5 (1 nmol, icv). Central administration of analogue **3** (4.8 nmol, icv) had no significant effect on food intake relative to control but inhibited the effect of a subsequent icv injection of analogue **2** (1.1 nmol, icv). The amount of food consumed after the different treatments was as follows: control, 1.07 ± 0.25 g; analogue **2**, 3.48 ± 0.81 g, $P < 0.01$ vs control; R3/I5, 3.30 ± 0.48 g, $P < 0.01$ vs control; analogue **2** + **3**, 0.32 ± 0.22 g, $P < 0.001$ vs R3/I5 and analogue **2**; and analogue **3** plus vehicle, 0.029 ± 0.017 g, $P < 0.001$ vs R3/I5 and analogue **2** (Figure 7). None of the treatments had a significant effect on the amount of water consumed in conjunction with the stimulation of feeding (data not shown).

DISCUSSION AND CONCLUSIONS

Relaxin-3 is a polypeptide hormone that belongs to the insulin-relaxin superfamily. There are 10 members within this superfamily, each of which is expressed as a single-chain prepro-hormone. They undergo a similar proteolytic processing to produce double-chain mature proteins with the exception of IGF-I and IGF-II, which remain as single chain.[6,25,26] There are six Cys residues that are fully conserved across the members of this superfamily that are responsible for imparting a remarkably similar overall tertiary structure by forming three disulfide bonds (one intra-A chain and two interchain).[6,25,26]

Table 3. Human Relaxin-3 and Its Analogues Assayed for Receptor Binding Affinity (Expressed as pK_i and pIC_{50}) and Activation Potency (pEC_{50}) at RXFP1, RXFP3, and RXFP4[a]

	pK_i		pIC_{50}	pEC_{50}		
peptide	RXFP3	RXFP1	RXFP4	RXFP3	RXFP1	RXFP4
H3 relaxin	7.78 ± 0.06	8.6 ± 0.01	ND	9.0 ± 0.07	8.74 ± 0.06^	8.94 ± 0.13
hINSL5	ND	ND	7.33 ± 0.08	ND	ND	8.51 ± 0.11
1	7.3 ± 0.12**	<6	ND	7.9 ± 0.05#	6.23 ± 0.07^	ND
2	7.87 ± 0.12*	<5	7.1 ± 0.07	8.43 ± 0.09	N/A	7.7 ± 0.07§
3	7.6 ± 0.05*	<5	6.6 ± 0.17″	N/A	ND	N/A
4	6.95 ± 0.03**	<5	ND	8.37 ± 0.10	N/A	7.85 ± 0.16§
5	6.95 ± 0.13**	<5	ND	8.29 ± 0.11#	N/A	8.1 ± 0.21§
6	6.96 ± 0.11**	<5	ND	7.9 ± 0.02#	N/A	8.46 ± 0.17

[a] n = 3−9 independent experiments. N/A, no activity; ND, not determined. **$p < 0.05$ vs H3 relaxin. *$p > 0.05$ vs H3 relaxin. #$p < 0.05$ vs H3 relaxin and analogues **2** and **4**. §$p < 0.05$ vs hINSL5 and analogue **6**. ^$p < 0.05$. ″$p < 0.05$ vs hINSL5 and analogue **2**.

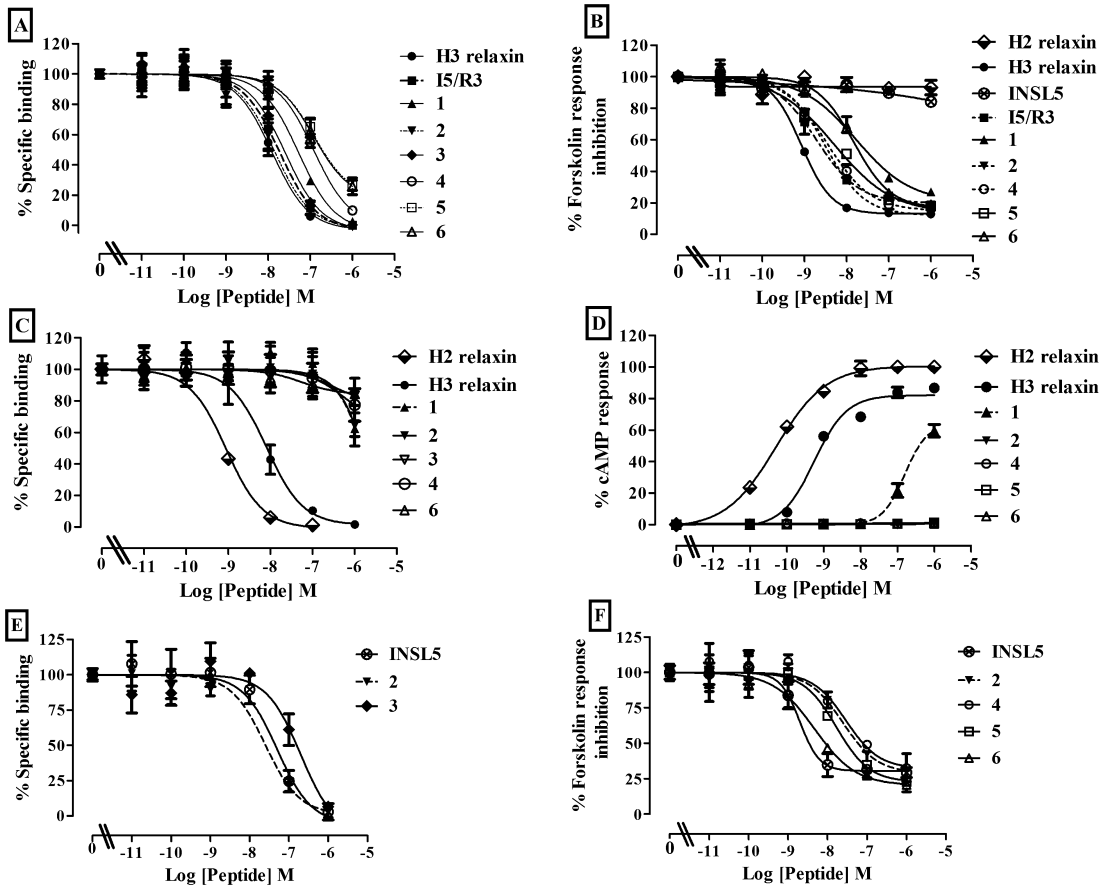

Figure 3. Binding activity (left column) and effects on cAMP production (right column) of H3 relaxin and its analogues at (A, B) RXFP3, (C, D) RXFP1, and (E, F) RXFP4. Europium-labeled H2 relaxin, INSL5/H3 relaxin chimera, and mouse INSL5 were used as labeled ligands in the competition binding assays for RXFP1, RXFP3, and RXFP4, respectively. H3 relaxin and its analogues bind to RXFP3 with an affinity (K_i) of 10−100 nM (A) and activity of 1−50 nM (B). However, H3 relaxin displayed the highest affinity and activation potency in CHO-K1 cells expressing RXFP3. The binding and activation profiles of H3 relaxin and its analogues at RXFP4 (E, F) are similar to those at RXFP3. While H3 relaxin binds RXFP1 with high affinity, analogues **1**−**4** and **6** display little or no binding affinity for RXFP1 (C). H2 relaxin and H3 relaxin induce cAMP accumulation in HEK-293 cells expressing RXFP1 and high concentrations (μM) of analogue **1**-activated RXFP1, whereas analogues **2** and **4**−**6** were inactive (D).

The members of this superfamily activate different types of cell surface receptors, the insulin family (insulin, IGF-I and -II) binds and activates cell surface receptor tyrosine kinase and relaxin-1−3, and INSL3 and INSL5 bind and activate GPCRs. The receptors for INSL4 and INSL6 are currently unknown. Despite the high degree of structural similarity, the peptides in this superfamily have all evolved to assume distinct biological functions through interaction with different receptors.

Relaxin-3, the ancestral member of the relaxin family,[6] has been shown to bind and activate its own receptor, RXFP3, and also receptors for relaxin-2 (RXFP1) and INSL5 (RXFP4).[27,28] RXFP3 and RXFP4 are classic peptide GPCRs, whereas RXFP1 possesses a large extracellular domain containing 10 leucine-rich repeats (LRR). Studies on peptide and receptor mutants have demonstrated that H2 relaxin relies on multiple residues within its A and B chains for high-affinity interaction with both the transmembrane as well as the LRR domains of RXFP1.[29,30] In contrast, similar studies on RXFP3 have demonstrated that H3 relaxin only requires the B chain for binding and activation of RXFP3.[12] The lack of specificity of H3 relaxin has made it difficult to study its physiological roles. However, the B chain of relaxin-3 alone has been shown to selectively bind and activate RXFP3 albeit with much lower affinity and potency as compared to native H3 relaxin.

Therefore, we postulated that we might be able to modify the B chain to increase its affinity and hence develop an RXFP3-selective single chain agonist. The low affinity of the single B chain was thought to be due to the lack of secondary α-helical structure, which is required for the presentation of the key receptor binding residues in a correct spatial orientation to the binding surface on the receptor. Thus, in the first instance, the B chain was constrained by incorporation of disulfide constraint (distanced at position i, i + 4) in the helical region of the B chain as well as between the strand and the helix, as shown by us previously to induce helical structure and increase receptor binding affinity in the B chain of the related peptide INSL3.[31,32] A Glu^-−Lys^+ salt bridge was also placed in the middle of the B chain helix to potentially increase peptide helical stability. A longer constraint was also incorporated along the helix at position i, i + 7 by cross-linking two Cys using 1,6-bismaleimidohexane in an attempt to induce α-helical structure, as previously shown to induce α-helicity in amyloid β peptide.[33] Unfortunately, these modifications could not induce the desired α-helicity and, as a result, failed to increase RXFP3 affinity (Table 1).[34,35] We have also shown that the B chain dimer of INSL3 has a receptor binding affinity about 1000-fold greater than the B chain monomer.[31,32] However, the dimerization of

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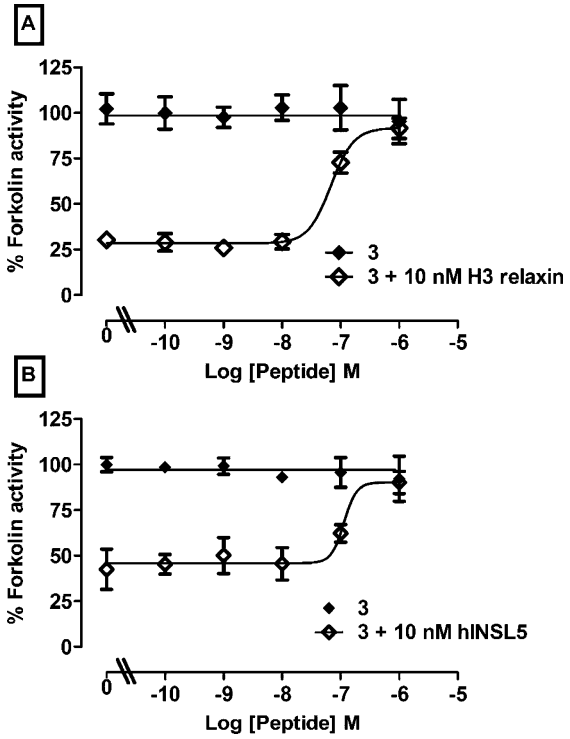

Figure 4. Antagonistic activity of analogue **3** at (A) RXFP3 and (B) RXFP4. Analogue **3** did not inhibit forskolin-induced cAMP production via either receptor. The addition of 10 nM H3 relaxin and INSL5 reduced the level of forskolin-induced cAMP to about 40% of maximal in the presence of their respective receptor. This inhibitory effect of H3 relaxin and INSL5 was antagonized by the addition of increasing concentrations of analogue **3**, which restored the level of cAMP to about 90% control, consistent with its binding to RXFP3 or RXFP4 to prevent their activation by either H3 relaxin or INSL5.

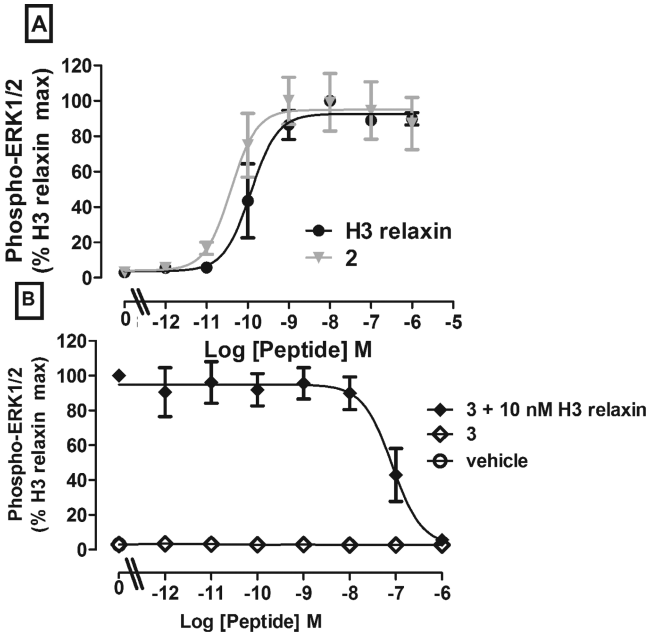

Figure 5. Effect of native H3 relaxin and analogue **2** on ERK1/2 kinase phosphorylation in CHO-K1 cells stably expressing RXFP3. (A) pEC_{50} values of H3 relaxin and analogue **2** were 9.83 ± 0.14 (n = 5) and 10.4 ± 0.11 (n = 4), respectively. Analogue **3** did not induce phosphorylation of ERK1/2. (B) Increasing concentrations of analogue **3** (1 pM−1 μM) antagonized the ERK1/2 phosphorylation produced by H3 relaxin (10 nM). Data are means ± SEMs of 4−6 independent experiments.

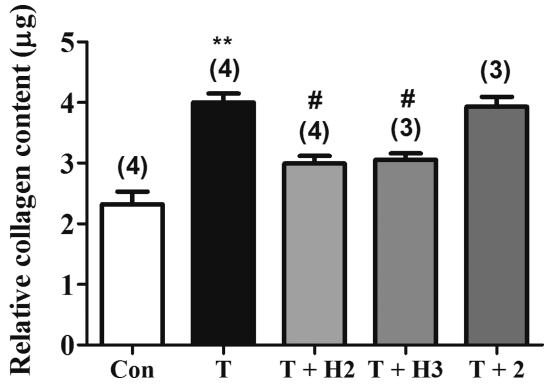

Figure 6. Effect of H2 relaxin (H2), H3 relaxin (H3), and analogue **2** on TGF-β1(T)-induced collagen accumulation in human dermofibroblast cells expressing RXFP1. H2 relaxin and H3 relaxin inhibited the expression of TFG-β1-induced collagen by 35 and 38%, respectively, as compared to TGF-β1 alone. In contrast, analogue **2** did not alter collagen expression (n = 3−4 per group, $^{**}P < 0.01$ vs control, $^{\#}P < 0.05$ vs TGF-β1).

H3 relaxin B chain did not improve the level of RXFP3 binding beyond that of the B chain alone.

Recently, we have shown that truncation of the B chain from the C terminus up to native Cys[21] (that is mutated to Ser) and addition of an Arg at position 22 led to a high-affinity single-chain antagonist, H3 B1−22R.[24] It is clear that the high affinity of this peptide is due to an additional binding contact being created by the addition of Arg at the C terminus. We therefore examined the replacement of residues at positions 21, 22, and 23 with Arg or the addition of an Arg at position 23 in the native B chain in an attempt to create a high-affinity agonist. Unfortunately, this approach was not effective at improving the affinity of the B chain for RXFP3. Clearly, the presence of GGSRW at the C terminus, which is essential for agonist activity, results in the loss of the additional binding site afforded by the Arg at the C terminus of the antagonist peptide. These findings suggest that there are residues within the A chain that are making contacts with residues on the B chain and helping the B chain to maintain its helical structure. As a result, it was decided to restore elements of the A chain to the native H3 relaxin B chain to maintain the structural integrity of the peptide and to improve its binding specifically to RXFP3 but not RXFP1.

In a recent study, we demonstrated that substitution of the intra-A chain disulfide bond with the homologous dicarba bond had no significant effect on the binding and activity of H3 relaxin at RXFP3.[36] In the current study, the intra-A chain disulfide bond was deleted by replacing the Cys residues with Ala to investigate its importance for both the structure and the function of H3 relaxin. The resultant analogue **1** displayed significant receptor binding affinity and activity only slightly less than H3 relaxin. Of particular interest was the observation that analogue **1** had a substantially reduced propensity for binding to RXFP1. Preliminary NMR spectroscopic studies revealed analogue **1** to be highly flexible with a much less-structured insulin core (K. J. Rosengren, personal communication). CD spectroscopic analyses also revealed that removal of the intramolecular disulfide bond resulted in a decrease in α-helicity as compared to the native peptide. We then prepared

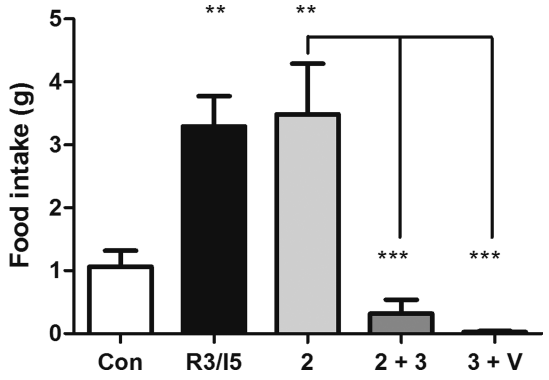

Figure 7. Effect of central administration of analogues **2** and **3** on food intake in satiated, adult male Sprague–Dawley rats. Effect of intracerebroventricular administration of vehicle (aCSF, 5 μL) or mock injection (V/Con), R3/I5 (~1 nmol; 5 μg in 5 μL of aCSF), analogue **2** (~1.1 nmol; 5 μg in 5 μL of aCSF; **2**), analogue **3** (~4.8 nmol; 20 μg in 2.5 μL of aCSF; A3), and analogue **3** (10 min prior) + analogue **2** or aCSF vehicle on food intake, during the first 60 min postinjection. Feeding experiments were performed in different groups of rats over several days with a crossover design, and the results were combined (n = 7–11 per group; $^{**}P < 0.01$ vs control, $^{***}P < 0.001$ vs **2**).

analogue **2**, in which analogue **1** was truncated by 10 residues from the N terminus of the A chain. Analogue **2** possessed α-helical content that was similar to native H3 relaxin and consequently also displayed comparable receptor binding affinity and potency for RXFP3 as compared to native H3 relaxin. Importantly, it had no activity at RXFP1 although still had high affinity for, and potency at, RXFP4. This is in accordance with our previous study in which we demonstrated that native H3 relaxin can be truncated from the N terminus of the A chain without loss of RXFP3 binding and activation while RXFP1 binding and activation are greatly reduced.[27] H3 and H2 relaxin bind to and activate RXFP1 utilizing residues in both the A and the B chains, and loss of any of these receptor binding surfaces results in a dramatic reduction of receptor binding affinity and activity.

The fact that H3 relaxin is able to tolerate removal of its A chain intramolecular disulfide bond without loss of binding or activation potency at RXFP3 is a phenomenon reported for the first time for any member of the relaxin-insulin superfamily. This is perhaps due to the capacity of this peptide to adopt the correct conformation for binding and activation in the absence of this disulfide bond. We have previously demonstrated that removal of the corresponding disulfide bond from human INSL3 resulted in the complete loss of its capacity to activate RXFP2.[39] This is analogous to the situation with insulin and its tyrosine kinase receptor in which the activity of insulin is nearly completely abolished following loss of its intramolecular disulfide bridge.[37,38] It is reasonable to postulate that retention of the highly efficient three disulfide-bonded core insulin-like structure would be mandatory for maintaining the overall structure of insulin, H2 relaxin, and INSL3 and the subsequent presentation of their multiple receptor contact points on both the A and the B chains of the peptides. For H3 relaxin, it would appear that only a short A chain and no intrachain disulfide bond is necessary for maintaining sufficient structure in the B chain for receptor binding and activation.

An emerging paradigm in GPCR pharmacology is that different ligands engender unique conformations of receptors consequently selectively activating diverse signaling pathways. This phenomenon of ligand-directed stimulus-bias was recently demonstrated for RXFP3.[40] Therefore, it is very important to ensure that novel peptides such as analogue **2** are mimicking the activity of the native ligand at multiple signal transduction pathways. Consequently, analogue **2** was further characterized by determining its ability to induce ERK1/2 phosphorylation, and it displayed higher potency than native H3 relaxin in this assay. The selectivity of analogue **2** for RXFP3 over RXFP1 was further tested in a cell line expressing endogenous RXFP1. Both H2 relaxin and H3 relaxin inhibited TGF-β1 induced accumulation of collagen, whereas analogue **2** failed to alter collagen expression, consistent with a lack of activity at RXFP1. Further sequential truncation of analogue **2** from the N terminus of the B chain led to shorter peptides (analogues **4**–**6**) with minimal loss of receptor binding affinity and activity at RXFP3.

Analogue **2**, which had the highest receptor binding affinity, was selected for additional modification in an attempt to produce a high-affinity and selective antagonist. Therefore, it was postulated that the GGSRW residues at the C terminus of the B chain should be able to be replaced with Arg to obtain a high-affinity antagonist in a similar fashion to the previously identified single B chain antagonist.[24] As a result, similar changes were made to analogue **2** to produce a selective antagonist of RXFP3, analogue **3**. This antagonist peptide bound RXFP3 and RXFP4 with high affinity but did not activate signaling associated with these receptors. It did, however, antagonize the ability of H3 relaxin and INSL5 to inhibit forskolin-induced cAMP production via their respective receptors. Moreover, to test for ligand-directed stimulus-bias of the novel antagonist analogue **3** at RXFP3, we tested its effect on diverse signaling pathways including ERK1/2 and p38MAPK phosphorylation. In both cases, analogue **3** was able to antagonize H3 relaxin-induced responses and had no effect on either ERK1/2 or p38MAPK phosphorylation on its own. Therefore, analogue **3** is acting as a full antagonist in multiple signaling pathways. Additionally, analogue **2** is behaving as a full agonist and mimicking the effects observed with native H3 relaxin. After this thorough characterization of the analogues in vitro, their in vivo activity was determined by examining their effect on feeding in rats.

Prior studies have shown that central administration of H3 relaxin and the previously identified RXFP3-selective, chimeric analogue, R3/I5, can increase food intake in rats[12,17,20] and that the antagonist, ΔR3/I5, blocked the effect of R3/I5.[12] Therefore, analogues **2** and **3** were administered icv to satiated rats. Analogue **2** increased food intake to the same extent as R3/I5, whereas analogue **3** did not alter food intake but had a strong inhibitory effect on food intake induced by analogue **2**. It is important to note that while H3 relaxin binds and activates RXFP4 in vitro, RXFP4 is a pseudogene in the rat,[41] so any biological activity produced by RXFP3 targeted peptides is not via the related receptor in this species.

In summary, we have demonstrated that the intra-A chain disulfide bond of H3 relaxin does not play a significant role in binding and activation of RXFP3, allowing for its deletion from synthetic peptide analogues, which, in turn, significantly simplifies their chemical assembly. However, this bond must be present in the H3 relaxin sequence for interaction with and activation of RXFP1. Deletion of both the intra-A chain disulfide bond together with 10 residues from the N terminus of the A chain produced a peptide, analogue **2**, which binds and activates RXFP3 with the same potency as native H3 relaxin but has no activity at RXFP1. Further truncation of this analogue by

five residues from the C terminus of the B chain and incorporation of an Arg residue in their place resulted in a selective high-affinity antagonist (analogue **3**). These selective analogues displayed significant activity at RXFP3 in vivo, with analogue **2** stimulating feeding in satiated rats, similar to R3/I5 and analogue **3**, blocking feeding induced by analogue **2**, in a similar fashion to ΔR3/I5. These RXFP3-selective analogues displayed similar effects to the native peptide and represent important lead compounds for the development of drugs to treat a range of metabolic, affective, and cognitive disorders. They are also one-third smaller than existing peptides of this type (e.g., R3/I5) and can be more easily synthesized in high quantity and purity as compared to native H3 relaxin. These new analogues are currently being employed in studies of the pharmacological effects of RXFP3 signaling aimed at further determining the precise physiological roles of relaxin-3 in the brain.

EXPERIMENTAL SECTION

Materials. 9-Fluoroenylmethoxycarbonyl (Fmoc)-protected L-α-amino acids and 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) were obtained from GL Biochem (Shanghai, China). Fmoc-Arg(Pbf)-PEG-PS (0.17 mmol/g) and Fmoc-PAL-PEG-PS (0.19 mmol/g) resins were obtained from Applied Biosystems (Melbourne, Vic, Australia). Fmoc-Cys(Acm)-Ac-S-TentaGel (0.22 mmol/g) was obtained from Rapp Polymere GmbH (Tübingen, Germany). Fmoc-Trp(Boc)-NovaSynTGA (0.19 mmol/g), dimethylformamide (DMF), methanol, diethyl ether, dichloromethane, acetonitrile, and piperidine were obtained from Merck (Kilsyth, Vic, Australia). Trifluoroacetic acid (TFA) was obtained from Auspep (West Melbourne, Vic, Australia). 3,6-Dioxa-1,8-octanedithiol (DODT), triisopropylsilane (TIPS), diisopropylethylamine (DIPEA), and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were obtained from Sigma-Aldrich (Sydney, NSW, Australia). 1,6-Bismaleimidohexane was purchased from LOMB (Taren point, NSW, Australia). 2,2′-Dipyridyl disulfide (DPDS) was from Fluka (Bucha, Switzerland); NH_4HCO_3 and $(NH_4)_2CO_3$ were from BDH Laboratory Supplies (Poole, United Kingdom), and trifluoromethanesulfonic acid (TFMSA) was from MP Biomedicals (Seven Hills, NSW, Australia). Isoflurane was obtained from Delvet (Seven Hills, NSW, Australia). Bovine serum albumin (BSA), Dulbecco's modified Eagle's medium (DMEM), RPMI 1640 medium, 2 mM L-glutamine, fetal calf serum, and penicillin/streptomycin were all obtained from Trace Biosciences (Castle Hill, NSW, Australia). Sodium chloride (NaCl), calcium chloride ($CaCl_2$), disodium hydrogen phosphate (Na_2HPO_4), potassium phosphate (KH_2PO_4), and sodium azide, glucose, ethylenediaminetetraacetic acid (EDTA), magnesium sulfate ($MgSO_4$), and sodium acetate (NaOAc) were obtained from AJAX Chemicals (Sydney, NSW, Australia). Chlorophenolred-β-D-galactopyranoside (CPRG) was obtained from Roche (Indianapolis, IN). Synthetic INSL5 and R3/I5 peptides were produced by S.Z. and MAH (Florey Neuroscience Institutes). TGF-β was obtained from R&D Systems (Minneapolis, MN).

H3 Relaxin and Its Sequential Minimization. Human H3 relaxin, in which the C termini of both chains are conventional carboxylates, was prepared as described.[11] Analogue **1** (Table 2) is similar to native H3 relaxin except that the Cys^{A10} and Cys^{A15} residues that comprise the A chain intramolecular disulfide bond were replaced with Ala. Analogue **2** is an A chain truncated version of analogue **1**. Analogue **3** is a B chain truncated version of analogue **2** in which residues 23−27 inclusive (Gly-Gly-Ser-Arg-Trp) at the C terminus were substituted by a single Arg residue. Analogues **4**−**6** are further B chain N terminally truncated versions of analogue **2**.

Peptide Synthesis and Purification. Appropriately S-protected A and B chains with C-terminal acids were separately synthesized using preloaded Fmoc-Trp(Boc)-NovaSynTGA, Fmoc-Arg(Pbf)-PEG-PS resin, and Fmoc-Cys(Acm)-Ac-S-TentaGel. All peptides were assembled on a CEM Liberty microwave peptide synthesizer (AI Scientific, Scarborough, QLD, Australia). The side chain-protecting groups of trifunctional amino acids were TFA-labile, except for acetamidomethyl (Acm)-protected Cys in positions A24 and B22 and *tert*-butyl (tBu)-protected Cys in position A11. All peptides were synthesized on a 0.1 mmol scale using a 5-fold molar excess of Fmoc-protected amino acids (0.5 mmol) that were activated by using 5-fold excess of HBTU in the presence of DIPEA.[42] N^{α}-Fmoc protecting groups were removed by treating the resin-attached peptide with piperidine (20% v/v) in DMF. The coupling and deprotection were conducted at 75 °C using 25 W microwave power for 5 min and 60 W microwave power for 3 min. The resin-bound peptides were cleaved using a mixture of TFA:DoDt:H_2O:TIPS (94:2.5:2.5:1, v/v) for 90 min. The cleaved peptide was precipitated in ice-cold diethyl ether and centrifuged at 3000 rpm for 3 min. The pellet was washed by resuspending it in ice-cold diethyl ether and recentrifugation. This washing process was repeated at least three times. The intrachain disulfide bond within the single B chain, between the B chain in the B chain dimer and intra-A chain were formed using 2-DPDS.[43] The chain combination between A and B chain was carried out by first converting the A chain $Cys(tBu)^{10}$ to Cys(SPy) using TFMSA to deprotect the tBu group and DPDS to convert it to 2-pyridylsulfenyl derivative.[44,45] The second interchain disulfide bond was formed by iodolysis using 20 mM iodine in acetic acid. The B chain analogue with free thiols at positions 15 and 22 were cross-linked with 1,6-bismaleimidohexane (1.2-fold excess over peptide) in 50% TFE in phosphate buffer saline (pH 7.4). All crude peptides were analyzed and purified by RP-HPLC using, Waters XBridge columns (4.6 mm × 250 mm, C18, 5 μm, and 19 mm × 150 mm, C18, 5 μm, respectively). Each peptide was characterized by MALDI-TOF/TOF mass spectrometry (Bruker Daltonics, Bremen, Germany). Matrices used were sinapinic acid, α-cyano-4-hydroxy-cinnamic acid, and 2,5-dihydroxy benzoic acid (Bruker Daltonics) depending on the molecular size of peptides. The matrices were made up in 50% acetonitrile containing 0.5% TFA. The laser frequency was set at 25 Hz with 300 ns of pulsed ion extraction at 20 kV. The analysis of the spectra was conducted using FlexAnalysis (Version 2.4).

CD Spectroscopy. The CD spectral measurements were carried as previously described.[31] The concentration of peptides used was 0.15 μg/μL, except for H3 relaxin, which was at 0.10 μg/μL.

Whole Cell Receptor Binding Assay for RXFP1, RXFP3, and RXFP4. HEK-293T cells stably expressing RXFP1 and Chinese hamster ovary (CHO)-K1 cells stably expressing RXFP3 and RXFP4 were plated into a 96-well plate (Viewplate; opaque white wall and clear bottom, PerkinElmer, Glen Waverly, Vic, Australia) at a density of 5×10^4 cells/well and grown overnight to reach ∼90% confluence before experimentation. Binding assays were conducted as described.[45] Briefly, the competition binding assay was done using a single concentration of Eu-labeled INSL5A/H3 relaxin B (0.5 nM),[46] Eu-labeled H2 relaxin (1 nM), and Eu-labeled mouse INSL5 (2.5 nM) in the presence of an increasing concentration of H3 relaxin analogues in comparison to the native receptor ligands. Each concentration point was performed in triplicate, and the data were expressed as the mean ± SEM of three independent experiments.

cAMP Signaling Assay. The potency of the various synthetic H3 relaxin analogues on RXFP1, RXFP3, and RXFP4 was assessed by measuring their influence on cAMP signaling using a cAMP reporter gene assay. The RXFP1 activity was assessed in HEK-293T cells stably expressing RXFP1 and pCRE-β-galactosidase, and RXFP3/RXFP4 activity was determined in CHO-K1 cells stably expressing pCRE-β-galactosidase and either RXFP3 or RXFP4. Cells expressing RXFP1 were treated with an increasing concentration of H3 relaxin analogues, while forskolin was used to stimulate RXFP3 (5 μM) and RXFP4 (1 μM) together with increasing concentrations of H3 relaxin analogues. Activities were compared to concentration−response curves for native H2 relaxin, H3 relaxin, and INSL5 for RXFP1, RXFP3, and RXFP4, respectively. The stimulation was carried out for 6 h after which the media were aspirated and the cells were frozen at −80 °C overnight. The cells were thawed to room temperature, and the amount of cAMP-induced β-galactosidase expression was measured by adding 25 μL of buffer A (100 mM Na_2HPO_4, pH 8.0, 2 mM $MgSO_4$, and 0.1 mM $MnCl_2$) and shaking it at room temperature for 10 min to

hypotonically lyse the cells. Buffer B (100 μL; similar to buffer A with additional 0.5% Triton X-100 and 40 mM β-mercaptoethanol) was added to each well with shaking for 10 min at room temperature. Finally, 25 μL of substrate for β-galactosidase (chlorophenol red β-D galactopyranoside) was added to each well with shaking until the color change was observed. The absorbance was measured at 570 nm on a Victor^3 plate reader (PerkinElmer). To test the antagonistic activity of analogue **3**, 10 nM H3 relaxin was used to inhibit the forskolin-induced cAMP response to approximately 50%, and then, an increasing concentration of analogue **3** was added to compete for the inhibitory effect of H3 relaxin. Each concentration point was performed in triplicate, and the data were expressed as the mean ± SEM of three independent experiments.

ERK1/2 Phosphorylation (pERK1/2) Surefire Assay. Stable CHO-RXFP3 cells were plated into 96-well plates (5×10^4 cells/well) and grown overnight in DMEM/Ham's F-12 medium at 37 °C, 5% CO_2. Cells were then washed twice with PBS and serum-starved for 8 h before their stimulation with different concentrations of peptides, serum-free DMEM/Ham's F-12 medium (vehicle control), or 10% FBS (positive control) for 5 min. If antagonists were tested, cells were pretreated with antagonists (analogue **3**) for 1 h prior to stimulation with H3 relaxin or analogue **2** for 5 min. Following treatment, cells were lysed according to the manufacturer's instructions using 100 μL of lysis buffer and frozen at −20 °C. For detection of ERK1/2 kinase phosphorylation, 4 μL of the thawed sample (cell lysate) was transferred to a white 384-well microplates (Proxiplates, PerkinElmer), and 5 μL of the combination buffer with AlphaScreen donor beads (40 parts reaction buffer, 10 part activation buffer, and 1 part acceptor beads) was added. Plates were incubated for 2 h at 23 °C in the dark on an oscillating platform. Subsequently, 2 μL of the dilution buffer with AlphaScreen acceptor beads (20 parts dilution buffer and 1 part donor beads) was added. Plates were again incubated for 2 h at 23 °C in the dark on the oscillating platform. The AlphaScreen signal (counts per second) was measured in 384-well microplates (Proxiplates) on an EnVision Multilabel Plate Reader (PerkinElmer) with excitation at 680 nm and emission at 520−620 nm.

Hydroxyproline Analysis of Collagen Deposition from Human Dermal Fibroblasts. On the basis of our recent observation that H2 and H3 relaxin inhibited TGF-β1-stimulated collagen deposition from rat ventricular fibroblasts in vitro, which only express RXFP1 but not RXFP3,[47] the effects of analogue **2** vs H2 and H3 relaxin were evaluated in TGF-β1-stimulated human dermal fibroblasts (BJ3 cells; kindly provided by Associate Professor William Hahn; Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA), which also express RXFP1. BJ3 cells were plated at a density of 1×10^6/well in six-well plates and either untreated or treated with TGF-β1 (2 ng/mL) alone, TGF-β1 (2 ng/mL) plus H2 relaxin (100 ng/mL; 16.8 μM), TGF-β1 (2 ng/mL) plus H3 relaxin (100 ng/mL; 18.2 μM), or TGF-β1 (2 ng/mL) plus analogue **2** (100 ng/mL; 22 μM) for 72 h. The deposited collagen into the cell layer was hydrolyzed and analyzed for hydroxyproline content, as described.[48] Hydroxyproline values were then converted to total collagen content by multiplying by a factor of 6.94 (based on hydroxyproline representing approximately 14.4% of the amino acid composition of collagen in most mammalian tissues).[49] These experiments were performed 3−4 separate times in duplicate.

Animals. Male Sprague−Dawley rats (n = 28; 250−300 g) supplied by Animal Resources Centre (Perth, WA, Australia) were housed under ambient conditions (21 °C) and maintained on a 12 h light:dark cycle (lights on 0700−1900 h) with access to laboratory chow and water ad libitum. Rats were acclimatized to the animal facility for a week before further treatment. Experiments were conducted with the approval of the Howard Florey Institute Animal Welfare Committee and according to the ethical guidelines issued by the National Health and Medical Research Council of Australia.

Stereotaxic Implantation of Cannula into Lateral Ventricle. Rats were anaesthetized with isoflurane. Deep anesthesia was induced with 4% isoflurane in oxygen, 2 L/min, and maintained with 2−3% isoflurane in oxygen, 200 mL/min. Each rat was positioned in a stereotaxic frame (David Kopf Instruments, Tujunga, CA), and a stainless steel guide cannula (22 gauge, cut 5 mm below pedestal; Plastics One, Roanoke, VA) was implanted with the cannula tip inserted into the lateral ventricle (coordinates: anteroposterior, −0.8 mm; mediolateral, −1.4 mm; and dorsolateral, −3.6 mm)[50] and affixed to the skull using surgical screws and dental cement. Rats were then placed under a heat lamp in a clean cage until regaining consciousness. Meloxicam (3 mg/kg, ip; Troy Laboratories, Smithfield, NSW, Australia) and 0.5 mg/mL paracetamol in 5% sucrose/water (for 3 days) were administered to provide acute and ongoing postoperative analgesia, respectively. Rats were single-housed and allowed to recover for 7 days, during which time they were handled and weighed daily to habituate them to the experimenter. A stylet of stainless steel wire (30-gauge) was inserted into each cannula to maintain patency. The stylet extended no further than the base of the cannula.

Infusion Procedure and Verification of Cannulation Using Angiotensin II. Lateral ventricle infusions were made using 30-gauge hypodermic tubing (Small Parts Inc., Miramar, FL) connected to a 10 μL Hamilton microsyringe (Hamilton Instruments, Reno, NV) by polyethylene tubing (0.80 mm outer and 0.40 mm internal diameter; Microtube Extrusions, North Rocks, NSW). Correct positioning of the cannula was verified in each rat by injecting 5 μL of a 4 ng/μL solution of human angiotensin II (Auspep, Parkville, Vic, Australia) in artificial cerebrospinal fluid (aCSF; made from stock of 10 × aCSF: 1470 mM NaCl, 40 mM KCl, 8.5 mM $MgCl_2$, and 23 mM $CaCl_2$) and observing if this produced a positive dipsogenic response, defined as repeated drinking episodes of ≥5 s that commenced within 1 min of angiotensin II administration. Injectors that extended 0.5 or 1 mm below the base of the cannula were tested as required on consecutive days, and the length of the effective injector was recorded for each rat and used for all subsequent experiments.

Peptide Treatments. Previous studies have observed significant increases in food intake following central administration of H3 relaxin[20] and R3/I5, a selective RXFP3 agonist.[12] The latter effect was inhibited by prior injection of R3(BΔ23−27)R/I5, an RXFP3 antagonist.[12] Therefore, we examined the effect of R3/I5 as a positive control. Feeding experiments were performed in different groups of rats over several days with a crossover design, and the results were combined (n = 7−11 per group). Peptides were dissolved in aCSF vehicle. Group 1 (control) received an injection of 5 μL of vehicle aCSF; group 2 (agonist I) received icv infusions of 5 μg (∼1 nmol) of R3/I5 (5 μL of 1 μg/μL solution); group 3 (agonist II) received icv infusions of 5 μg (∼1.1 nmol) of analogue **2** (5 μL of 1 μg/μL solution); group 4 (antagonist and agonist) received an icv infusions of 20 μg (∼4.8 nmol) of analogue **3** (2.5 μL of 8 μg/μL solution) followed 10 min later by 2.5 μg (∼1.1 nmol) of analogue **2** (2.5 μL of 2 μg/μL solution); group 5 (antagonist and vehicle) received an icv infusion of 20 μg (∼ 4.8 nmol) of analogue **3** (2.5 μL of 8 μg/μL solution) followed 10 min later by 2.5 μL of aCSF. The higher dose of analogue **3** used was based on the in vitro relative potency of analogue **3** and antagonist, R3(BΔ23−27)R/I5.

Food and Water Intake. Rats were habituated for a minimum of 7 days to the holding room, and behavioral studies were performed during the light phase beginning at 1030−1100 h. Groups of rats were injected using a crossover design, whereby each rat received peptide and vehicle, with ≥3 days between tests. Cannulated rats that had no drinking response were given "mock" injections by inserting a stylet into their cannula and treated as controls (see analysis). Following the infusion, each rat was placed back into their home cage, where a preweighed amount of rat chow (11−14 g) was located in the food compartment of the wire cage lid. A preweighed water bottle was also placed in its usual compartment. Food and water were weighed at hourly intervals for up to 4 h postinjection, with minimal disturbance to the rat. For rats that received two injections 10 min apart, the preweighed rat chow was placed into the cage lid after the *first* injection, but the weighing of food and water was done at hourly intervals after the *second* injection.

Data Analysis. All data were analyzed using GraphPad PRISM 5 (GraphPad Inc., San Diego, CA). The europium displacement curves were fitted to a one site competition curve, and the inhibition of forskolin-induced cAMP accumulation and ERK1/2 phosphorylation

curves were fitted to a single-site sigmoidal dose−response curve with variable slope. The hydroxyproline assay data were analyzed as relative collagen content in μg. All of the data were expressed as mean ± SEM from at least three independent experiments. Statistical analysis was conducted using one-way ANOVA with Bonferroni's multiple comparison test.[S1] A P value of less than 0.05 was considered as statistically significant. The in vivo data were also expressed as mean ± SEM. Statistical significance was evaluated using a Student's t test or a one-way ANOVA with Bonferroni's post-test: $P < 0.05$, significant (*); $P < 0.01$, highly significant (**); and $P < 0.001$, very highly significant (***). Food and water intake levels of rats that were "mock injected" and were combined with data from the vehicle control group, as there was no significant difference between them ($p = 0.8$).

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

Acm, acetamidomethyl; Arg, arginine; BSA, bovine serum albumin; CD, circular dichroism; CHO, Chinese hamster ovary; Cys, cysteine; DMF, dimethylformamide; DIPEA, diisopropylethylamine; DMEM, Dulbecco's modified Eagle's medium; DoDt, 3,6-dioxa-1,8-octanedithiol; DPDS, 2,2′-dipyridyl disulfide; ERK, extracellular-signal-regulated kinase; Fmoc, 9-fluoroenylmethoxycarbonyl; GPCR, G-protein-coupled receptor; HBTU, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HEK, human embryonic kidney; HEPES, 1,2,4,5-benzene-tetracarboxylic dianhydride, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; INSLs, insulin-like peptides; LRR, leucine-rich repeat; RXFP, relaxin-family peptide; SPy, thiopyridyl; tBu, *tert*-butyl; TIPS, triisopropylsilane; TFA, trifluoroacetic acid; TFMSA, trifluoromethanesulfonic acid; TGF, transforming growth factor

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