R3(BΔ23-27)R/I5 CHIMERIC PEPTIDE, A SELECTIVE ANTAGONIST FOR
GPCR135 AND GPCR142 OVER RELAXIN RECEPTOR LGR7: IN VITRO AND IN
VIVO CHARACTERIZATION
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Running title: Selective antagonist for GPCR135 and GPCR142 over LGR7
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Abstract
Both relaxin-3 and its receptor (GPCR135) are predominantly expressed in brain regions known
to play important roles in processing sensory signals. Recent studies have shown that relaxin-3
is involved in the regulation of stress and feeding behaviors. The mechanisms underlying the involvement
of relaxin-3/GPCR135 in the regulation of stress, feeding, and other potential functions remain to
be studied. Since relaxin-3 also activates the relaxin receptor (LGR7), which is also expressed in the
brain, selective GPCR135 agonists and antagonists are crucial to study the physiological functions
of relaxin-3 and GPCR135 in vivo. Previously, we reported the creation of a selective GPCR135 agonist
(a chimeric relaxin-3/INS15 peptide, designated R3/I5). In this report, we describe the creation of a high
affinity antagonist for GPCR135 and GPCR142 over LGR7. This GPCR135 antagonist, R3(BΔ23-27)R/I5,
consists of the relaxin-3 B-chain with a replacement of Gly23 to Arg, a truncation at the C-terminus
(Gly24-Trp27 deleted) and the A-chain of INS15. In vitro pharmacological studies show that R3(BΔ23-27)R/I5
binds to human GPCR135 (IC50 = 0.67 nM) and GPCR142 (IC50 = 2.29 nM) with high affinity and is a
potent functional GPCR135 antagonist (pA2 = 9.15), but is not a human LGR7 ligand. Furthermore,
R3(BΔ23-27)R/I5 had a similar binding profile at the rat GPCR135 receptor (IC50 = 0.25 nM, pA2 = 9.6) and lacked
affinity for the rat LGR7 receptor. When administered intracerebroventricularly, R3(BΔ23-27)R/I5
blocked food intake induced by the GPCR135 selective agonist R3/I5. Thus, R3(BΔ23-
27)R/I5 should prove a useful tool for the further delineation of the functions of the
relaxin-3/GPCR135 system.

Introduction
Relaxin-3 (1) is the most recently identified member of the insulin-relaxin peptide family.
Both relaxin-3 and its receptor, GPCR135 (2), are predominantly expressed in the brain (2, 3).
GPCR135, an inhibitory receptor, is expressed in many regions of the rodent brain such as the
superior colliculus, sensory cortex, olfactory bulb, amygdala and PVN (4-6), suggesting
potential physiological involvement in neuroendocrine and sensory processing. Recent in vivo studies have further shown that relaxin-3 and GPCR135 are involved in the stress
response and in regulation of feeding. More specifically, water restraint stress or
intracerebroventricular (i.c.v.) corticotrophin releasing factor (CRF) infusion induces relaxin-3
expression in cells of the nucleus incertus, a region where CRF receptor 1 is also expressed
(7) and central administration of relaxin-3 induces feeding in rat (8, 9). These findings
suggest that GPCR135 and relaxin-3 may be involved in multiple physiological processes,
some of which might be as yet unknown.

In vitro, relaxin-3 activates GPCR135 (2), GPCR142 (10) and LGR7 (11) receptors. The
predominant brain expression of both relaxin-3 and GPCR135, coupled with their high affinity interaction, strongly suggests that relaxin-3 is the endogenous ligand for GPCR135 (2). Pharmacological characterization, tissue expression profile, and the evolutionary study of GPCR142 and INSL5 indicate that GPCR142 is the endogenous INSL5 receptor (10, 12-14). The high affinity interaction between relaxin and LGR7 as well as knockout studies demonstrate that relaxin is the endogenous ligand for LGR7 (15-18).

Despite the proposed ligand/receptor pairs mentioned above, in vivo administration of relaxin-3 could potentially activate all three receptors (GPCR135, GPCR142 and LGR7), therefore selective pharmacological tools (agonists and antagonists) are crucial to probe the in vivo function(s) of GPCR135. Since GPCR142 is a pseudogene in the rat (13) and is not detected in the mouse brain (5), activation of GPCR142 by central administration of relaxin-3 is not a great concern in these species. However, potential activation of LGR7 by relaxin-3 remains as potential confounding issue, especially since LGR7 is expressed in the brain and is reported to play a role in drinking (8, 19) and potentially other physiological functions (20-22).

Previous studies showed that the B-chain of relaxin-3 is capable of binding to and activating GPCR135 (2), suggesting that the B-chain of relaxin-3 contains the receptor binding domains for GPCR135. Later studies demonstrated that a chimeric peptide (R3/I5) composed of the relaxin-3 B-chain and the INSL5 A-chain selectively activates GPCR135 over LGR7 (23), which further support our hypothesis. Homology modeling of the relaxin-3 structure (Fig. 1) and solution structure analysis of relaxin-3 (24) show that the middle segment of the relaxin-3 B-chain forms an α-helix. Amino acid residues Arg12, Ile15, Arg16, Ile19, Phe20 are presented on one side of the α-helix that faces away from the A-chain, suggesting that these residues may play a role in the interactions between relaxin-3 and its receptors. In addition, although the N-termini of the B-chains of the members in the insulin/relaxin family have no conservation (Fig. 1), a few different members can activate the same receptor (relaxin-1, 2, and relaxin-3 for LGR7; Relaxin-1, 2, and INSL3 for LGR8; relaxin-3 and INSL5 for GPCR142), suggesting the N-terminus of relaxin-3 may not be important for the interactions between relaxin-3 and its receptors. In this report, using mutagenesis studies, we describe the identification of the GPCR135 binding domain (the alpha helix region of the relaxin-3 B-chain) and the receptor activation domain (the C-terminus of the relaxin-3 B-chain) of relaxin-3. In addition, we report the creation of a selective GPCR135 antagonist (R3(BΔ23-27)R/I5) that consists of the relaxin-3 B-chain with a truncation at the C-terminus (Gly23–Trp27, the GPCR135 activation domain), an addition of an Arg residue in place of Gly23 of the B-chain, and the A-chain of INSL5. This novel, high affinity GPCR135 antagonist (R3(BΔ23-27)R/I5) does not interact with LGR7. In addition, we demonstrate increased feeding in satiated Wistar rats following intracerebroventricular (icv) dosing of R3/I5 (a selective GPCR135 agonist), which is blocked by prior administration of the GPCR135-specific antagonist R3(BΔ23-27)R/I5.

**Materials and Methods**

**Generation of relaxin-3 mutant peptides**

Different relaxin-3 mutant peptides with various mutations at the B-chain were created, including truncations at the N-terminus (mutants Δ1-6, Δ1-7, Δ1-8, and Δ1-9), different point mutations at residues Arg^8, Arg^12, Ile^15, Arg^16, Ile^19, Phe^20, Arg^26, and Trp^27 as well as truncations at the C-terminus [R3(BΔ23-27), R3(BΔ24-27), R3(BΔ25-27), R3(BΔ26-27), and R3(BΔ23-27)/I5]. All peptides (except as specified) were generated recombinantly in mammalian cells similar to the production of relaxin-3 as previously described (2). All relaxin-3 mutant coding regions were created by a two-step PCR using primers shown in Sup. Table 1. In the first round PCR, overlapping 5’ end and 3’ end coding regions for each mutant were PCR amplified. The human relaxin-3 cDNA construct (2) was used as the template for the first step PCR reactions for all mutants.
except for the chimeric peptides, R3(B∆23-27)/I5, for which, R3/I5 expression cDNA (23) was used as the templates. The first step PCR products (5' end and 3' end) were then mixed and used as the templates for the respective second round PCR reactions using primers P1 and P2 [P15 for R3(B∆23-27)/I5] listed in Sup. Table 1. All PCRs were run under the conditions of 94°C 20 sec, 65°C 20 sec, 72°C 1 min for 20 cycles. The final PCR products were cloned into a modified pCMV-SPORT1 vector containing a coding region for an alpha signal peptide for secretion, which was followed by a FLAG peptide coding region for affinity purification (2). All mutant peptides (except for R3(B∆23-27)/I5) have the intact A-chain of the wild type relaxin-3 but with different B-chains. R3(B∆23-27)/I5 has an A-chain of human INSL5. The B-chain sequences of the mutant peptides with truncations at the C-terminus of relaxin-3 B-chain are shown in Table 1. B-chain sequences of other mutants are shown in Sup. Table 2. All recombinant peptides were co-expressed with furin protease in COS-7 cells for efficient removal of the C-chain (2, 25). The N-terminal FLAG tagged peptides were first purified using an anti-FLAG affinity column and then the tag was removed by enterokinase (Novagen, Madison, WI) digestion. The peptides, free of the tag, were then further purified by reversed phase HPLC. The purified peptides were analyzed by mass spectrometry as described (2) to verify the peptide identities. R3(B∆23-27)R and R3(B∆23-27)R/I5, which are derivative of R3(B∆23-27) and R3(B∆23-27)/I5 respectively, have an extra Arg residue at the C-terminus of the B-chain due to incomplete processing (Table 1). Mutant peptides R12K, R12K&R16K, W27R were made by solid phase peptide synthesis using methods described previously (1, 26).

Radioligand binding assays

125I-Relaxin-3/INSL5 (125I-R3/I5), a radiolabeled chimeric peptide with the human relaxin-3 B-chain and the human INSL5 A-chain (23) was used at a final concentration of 50 pM as the tracer to characterize the binding properties of GPCR135 and GPCR142 for the mutant relaxin-3 peptides. 125I-H2 relaxin (PerkinElmer Biosciences, Boston) was used at a final concentration of 50 pM to characterize the binding properties of the relaxin receptor LGR7 for the peptides. COS-7 cells in 24-well tissue culture plates that transiently expressed GPCR135, GPCR142, or LGR7 were used in radioligand binding assays as described (13). The results were analyzed by GraphPad Prism 4.0 software (Graphpad, San Diego). The IC₅₀ values, which are the ligand concentrations that inhibited 50% of the maximum binding, were calculated and then converted to Kᵢ values using the Cheng/Prusoff formula (31) using Kₐ values of 0.41 nM, 0.89 nM, and 0.18 nM for binding of 125I-R3/I5 to GPCR135, 125I-R3/I5 to GPCR142, and 125I-H2 relaxin to LGR7, respectively.

Agonist and Antagonist analysis for mutant relaxin-3 peptides

All peptides were tested for their agonist activities against GPCR135, GPCR142, and LGR7 expressed in SK-N-MC/CRE cells as previously described (23). SK-N-MC/CRE-β-gal cells harbor a β-galactosidase (β-gal) gene under the control of a CRE promoter. An increase in cAMP concentration in these cells is associated with increased β-gal expression, which can be measured using Chlorophenol Red-β-D-Galactopyranoside (CRGP) as a substrate and reading the optical absorbance at 570 nm. GPCR135 and GPCR142 are coupled with Gαᵢ proteins, therefore agonists inhibit forskolin-stimulated β-gal expression in GPCR135 or GPCR142 expressing cells. LGR7 is Gs-linked, therefore agonists stimulate β-gal expression in LGR7 expressing cells. R3(B∆23-27)R/I5 was tested for its ability to produce a rightward-shift in relaxin-3’s or R3/I5’s dose-response curve in the presence of 10 nM, 100 nM or 1 μM R3(B∆23-27)R/I5 to demonstrate functional antagonism. Wild type relaxin-3 peptide was used as positive control in all experiments. The results were analyzed using GraphPad Prism 4.0 software (Graphpad, San Diego). The EC₅₀ values, which are the ligand concentrations that stimulate 50% of the maximum responses, were then calculated.
The agonism and antagonism of R3(BΔ23-27)R/I5 for rat GPCR135 was tested in the same way as the human GPCR135 using SK-N-MC/CRE-β-gal cells stably expressing rat GPCR135. The agonism and antagonism of peptides for rat LGR7 was assayed using a cAMP luminescence assay. Briefly, HEK293 cells were transiently transfected with a cDNA construct expressing rat LGR7 (4). Two days post transfection, cells were detached with PBS + 10 mM EDTA and plated at a density of 25,000 cells/well in 96-well white opaque plates (Thermo Electron Corporation, Cat #7571). To test the agonism of R3(BΔ23-27)R/I5, cells expressing rat LGR7 were stimulated with different concentrations of R3(BΔ23-27)R/I5 with relaxin-3 as the positive control. To test the antagonism of R3(BΔ23-27)R/I5 for rat LGR7, different concentrations of relaxin-3 were added to cells expressing rat LGR7 in the presence of 10 nM, 100 nM or 1 µM of R3(BΔ23-27)R/I5. Cells were then incubated at room temperature for 1 hr. The cAMP in the cells was measured with a cAMP detection kit (DiscoveRx HitHunter, Cat. #90-0041) according to the manufacturer’s protocol. The results were analyzed using GraphPad Prism 4.0 software (GraphPad, San Diego).

**Autoradiographic studies**

R3(BΔ23-27)R/I5 peptide was evaluated pharmacologically using endogenous GPCR135 from rat brain slices in autoradiographic studies as previously described (4). Briefly, 125I-R3/I5 was applied in a binding buffer to rat brain slices. Unlabeled human relaxin-3 or R3(BΔ23-27)R/I5 was used at various concentrations as competitors to displace GPCR135 binding of 125I-R3/I5. The specific binding of 125I-R3/I5 to the rat brain slices was quantitated using a Fuji Bio-Imaging Analyzer System (BAS-5000).

**In vivo studies**

Experimentally naïve, male, Wistar rats (Charles River, Wilmington, MA) weighing 200-225 grams at the time of arrival were used. The animals were initially housed at two per cage and given a one-week acclimation period to the vivarium prior to icv cannula implantation. All animals had free access to food and water throughout the experiment. The animal colony was maintained at 22 ± 2 °C during a 12-hr light/12-hr dark illumination cycle with lights on from 06:00 to 18:00 hours. All behavioral testing occurred during the light phase between 08:00 and 16:00 hrs. All studies were carried out in accordance with the US National Institute of Health Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the US National Institutes of Health.

**Surgical preparation**

Following the acclimation period, the animals were anesthetized with 4 % isoflurane and surgically implanted with a 20-gauge guide cannula aimed at the lateral ventricle. Guide cannulae (Plastics One, Roanoke, VA) were unilaterally implanted using a stereotaxic apparatus (David Kopf, Tujunga, CA) using the following coordinates relative to Bregma (flat skull): AP = +1.0 mm, ML = −1.3 mm, DV = −3.8 mm from the top of the skull (25). Three screws were mounted in the skull and covered with dental cement, which served as an anchor for the guide cannula. Animals were then individually housed and given a 7-day recovery period from surgery. During the surgical recovery period, the animals were handled 2-3 times to minimize stress effects that might occur due to handling at the time of behavioral testing.

**Apparatus**

The testing apparatus consisted of a plastic cage (containing no bedding) in which a wire grid was placed on the floor of the cage. A food hopper and drinking spout were located on opposite walls of the cage. The drinking spout was connected to an automated watering system and thereby delivered water to the animal throughout the session(s) on demand.

A predetermined amount of standard rat chow (Formulab Diet, no. 5008) was placed in the food hopper at the start of the 4-hr session(s). The amount of food remaining in the food hopper was determined by subtracting the weight of the food at 1 and 4 hrs from the initial food weight (i.e., weight of the food at the start of the session). Food crumbs detected on the
floor of the apparatus were included in the determination of food weights.

**Drugs**

The peptides (i.e., R3/I5, R3(BΔ23-27)R/I5) were dissolved in vehicle (sterile physiological saline plus 0.1% bovine serum albumin). All solutions were infused in a 5 µl volume. R3/I5 and R3(BΔ23-27)R/I5 were infused at a concentration of 2 µg/µl.

**Feeding Procedure**

Following the surgical recovery period, the animals were randomly assigned to one of the four treatment conditions (i.e., vehicle (5 µl) + vehicle; vehicle + R3/I5 (10 µg); R3(BΔ23-27)R/I5 (10 µg) + vehicle; R3(BΔ23-27)R/I5 (10 µg) + R3/I5 (10 µg)).

Testing consisted of a two-day protocol. Day 1 served as the baseline session. No injections were administered during this session and it served as a habituation period to the testing apparatus, while also providing a baseline measure of food intake. Day 2 served as the test session. Immediately prior to this session, all animals were removed from their home cage and two infusions were administered directly into the lateral ventricle. Test substances were given via a pre-loaded catheter without removing the catheter between injections. A 0.5 µl air bubble separated each injection to prevent mixing. The animals were first infused with vehicle or R3(BΔ23-27)R/I5, followed by a second infusion that consisted of vehicle or R3/I5. The infusions were separated by 10 minutes and the injection needle remained in the guide cannula for one minute following the termination of the final infusion. Following the second infusion, the animals were placed in the testing apparatus and food intake was measured at 1 and 4 hrs during a 4 hr session. Food intake measured at the end of the session served as a measure of total food intake. All animals were euthanized with carbon dioxide and cannula placements were verified at the end of behavioral testing.

**Results**

Probing the receptor binding and activation domains of relaxin-3

The functions of the N-terminus of the relaxin-3 B-chain were evaluated by removing residues Arg^1^ to Gly^5^ (Δ1-6), or Arg^1^ to Val^7^ (Δ1-7), or Arg^1^ to Arg^8^ (Δ1-8), or Arg^1^ to Leu^9^ (Δ1-9) from the N-terminal region of the relaxin-3 B-chain. The A-chains of those mutant peptides were left unchanged. The RΔ1-6 and Δ1-7 mutants were expressed in mammalian expression system with the similar yield to that of the wild type relaxin-3 peptide (5). Δ1-8 mutant had a significantly reduced expression level (~100 µg/L) while the expression level for Δ1-9 mutant was too low to generate enough peptide for functional testing. Δ1-6 and Δ1-7 mutants retained high binding affinity and agonist potency for GPCR135, GPCR142, and LGR7. Δ1-8 mutant retained low binding affinity and agonist potency for GPCR135 and LGR7 but moderate affinity and agonist potency for GPCR142. The EC₅₀ and Kᵢ values of GPCR135, GPCR142, and LGR7 for these relaxin-3 mutants are listed in Fig. 2 and Sup. Table 3.

Computer modeling (Fig. 1) and NMR structure analysis (24) suggest that residues Arg^12^, Ile^15^, Arg^16^, Ile^19^, and Phe^20^ of the B-chain are presented at one side of the α-helix region facing away from the A-chain, suggesting that these residues may be involved in receptor interactions. The following mutations on the relaxin-3 B-chain were made to assess their function in receptor interactions: Arg^8^ to Ala (R8A) or Ser (R8S); Arg^12^ to Ala (R12A), Leu (R12L), Ser (R12S) or Lys (R12K); Arg^16^ to Ala (R16A) or Ser (R16S); Ile^15^ to Gly (I15G), Ala (I15A), or Val (I15V); Ile^19^ to Ala (I19A) or Glu (I19E); Arg^12^ to Lys and Arg^16^ to Lys (R12K&R16K); Phe^20^ to Ala (F20A), Arg (F20R), Ser (F20S), or Tyr (F20Y); For all mutants described above, the A-chain was kept unchanged. The mutant peptides were tested for their ligand properties against GPCR135, GPCR142, and relaxin receptor LGR7. When Arg^8^ was changed to Ala (R8A) or Ser (R8S), the mutants showed reduced agonist potency and affinity for GPCR135, GPCR142 and LGR7 (Fig. 2 and Sup. Table 3). Arg^12^ to Ala (R12A), Leu (R12L), or Ser (R12S) mutants had no detectable agonist activity or affinity for LGR7
but retained weak agonist activity and low affinity for GPCR135 while possessed high affinity and almost full agonist potency for GPCR142. R12K had high affinity and potency for both GPCR135 and GPCR142 but no activity for LGR7 (Fig. 2). Changing Arg to Ala (R16A) or Ser (R16S) abolishes relaxin-3’s agonist activity and binding affinity for GPCR135 while weakening its agonist activity and binding affinity for GPCR135 and GPCR142 but lost affinity and agonistic activity for LGR7 (Fig. 2). The I15G mutant has very weak activity and low binding affinity for GPCR135 and GPCR142 and no activity for LGR7 (Fig. 2). Changing Ile to Ala (II19A) or Glu (I19E) abolished relaxin-3’s interaction with LGR7 completely. Interestingly, those changes had no effects on relaxin-3 function for LGR7 (Fig. 2). To investigate whether the C-terminal exposure of Trp is necessary for relaxin-3’s ligand activity for GPCR135, GPCR142, and LGR7, a mutant with an extra-Ser residue was placed after Trp (+28S) and the mutant peptide was evaluated. The results showed that this C-terminal addition of Ser reduced the agonist activity of the peptide for both GPCR135 and GPCR142 receptors to marginal levels, however its agonist activity for LGR7 remained as potent as that of the wild type relaxin-3 peptide (Fig. 2). Radioligand binding assays showed that this mutant peptide (+28S) retained high affinity for all three receptors tested (Fig. 2). The detailed description of the EC50 and Ki values of these relaxin-3 mutants for GPCR135, GPCR142, and LGR7 are shown in Sup. Table 3.

Expression and purification of mutant relaxin-3 peptides with a truncation at the C-terminus of the B-chain

Expression constructs encoding mutant relaxin-3 peptides with truncations at the C-terminus of the B-chain were created. The C-termini of various mutant relaxin-3 B-chains were modified so that amino acids Gly23-Trp27 (R3(BΔ23-27)), Gly24-Trp27 (R3(BΔ24-27)), Ser25-Trp27 (R3(BΔ25-27)), or Arg26-Trp27 (R3(BΔ26-27)) were deleted in the mature peptide. The junctions between the B-chain, C-chain, and A-chain contain a furin cleavage site (Arg-Arg-Arg-Arg) for efficient cellular processing when co-transfected with furin (2). Upon cellular processing to the mature peptide, the arginines were removed by furin and endogenous carboxypeptidase-B (26), yielding the mature peptide sequences shown in Table 1. Mass spectrometry analysis showed that R3(BΔ23-27), R3(BΔ24-27) and R3(BΔ26-27) had molecular masses of 5013 Da, 5070 Da and
5157 Da, respectively, which matched the predicted molecular masses. However, the R3(B∆23-27) product had a molecular mass of 5112 Da, which was 156 Da greater than predicted (4956 Da). The 156 Da difference is consistent with a residual C-terminal Arg. This residual C-terminal Arg on the B-chain was probably the result of steric hinderance that prevented carboxypeptidase-B from cleaving the last Arg residue. This peptide was designated R3(B∆23-27)R to reflect the additional Arg at the C-terminus of the B-chain. Peptide R3(B∆23-27)I5 was designed to contain a truncated relaxin-3 B-chain (Gly23-Trp27 removed) and the A-chain from INSL5. However, mass analysis of the resulted peptide indicated that it had a mass of 4851 Da, again 156 Da greater than the predicted mass of R3(B∆23-27)I5 (4695 Da), indicating it also had an additional Arg residue at the C-terminus of its B-chain. This peptide was therefore designated R3(B∆23-27)R/I5.

**In vitro characterization of C-terminally truncated relaxin-3 peptides as ligands for GPCR135, GPCR142 and LGR7**

Mutant relaxin-3 peptides R3(B∆23-27)R, R3(B∆24-27), R3(B∆25-27), R3(B∆26-27), and R3(B∆23-27)R/I5 were tested in GPCR135, GPCR142, and LGR7 in radioligand binding assay. The results showed that human GPCR135 and GPCR142 had high affinities for R3(B∆23-27)R but human LGR7 had a low affinity for this peptide (Fig. 2). Similarly, human GPCR135 and GPCR142 had high affinities for R3(B∆23-27)R/I5, but human LGR7 showed no affinity for the peptide (Fig. 2). GPCR135 and GPCR142 had moderately high affinities for R3(B∆24-27), R3(B∆25-27) and R3(B∆26-27), while human LGR7 bound these peptides with comparable affinity (Fig. 2). The exact K_i values of these truncated peptides is detailed in Table 2.

Truncated relaxin-3 peptides were tested on SK-N-MC/CRE-β-gal cells stably expressing GPCR135, GPCR142 or LGR7 receptors. Since GPCR135 and GPCR142 are coupled to G_{ai} proteins (2, 10), the agonist activities of the mutant peptides were tested as inhibition of forskolin-induced β-gal expression in SK-N-MC/CRE-β-gal cells expressing GPCR135 or GPCR142 (23). None of the truncated relaxin-3 peptides demonstrated significant agonist activity for human GPCR135 or GPCR142 (Fig. 2, Table 2). LGR7 is linked to G_{α_i} proteins (16), therefore, LGR7 agonism of the truncated relaxin-3 peptides was compared by testing for stimulation of β-gal expression in SK-N-MC/CRE-β-gal cells expressing LGR7 (23). In contrast to GPCR135 and GPCR142, the results showed that R3(B∆24-27), R3(B∆25-27), and R3(B∆26-27) were high potency human LGR7 agonists with E_{C_{50}} values ranging from 3 to 5 nM. R3(B∆23-27)R and R3(B∆23-27)R/I5 showed no LGR7 agonist activity (Fig. 2, Table 2).

R3(B∆23-27)R/I5 binds human GPCR135 and GPCR142 with high affinities but shows no agonist activity to neither receptor. LGR7 has little or no affinity for R3(B∆23-27)R/I5, suggesting that R3(B∆23-27)R/I5 is a selective antagonist for human GPCR135 over LGR7. The antagonism of human GPCR135, GPCR142 and LGR7 by R3(B∆23-27)R/I5 was compared using the functional reporter assay and the results show that R3(B∆23-27)R/I5 dose-dependently shifted relaxin-3’s agonism curves for GPCR135 (pA_2 = 9.1, Fig. 3A) and GPCR142 (pA_2 = 8.2, Fig. 3B) to the right. In contrast, R3(B∆23-27)R/I5 does not affect relaxin-3’s agonism for LGR7 (Fig. 3C) at concentrations up to 1 µM. The pharmacology of R3(B∆23-27)R/I5 was also studied using recombinant rat GPCR135 and LGR7. Recombinant rat GPCR135 binds R3(B∆23-27)R/I5 with high affinity (K_i = 0.25 nM, Fig. 4A) but rat LGR7 lacks affinity for this peptide (Fig. 4B). In a functional reporter assay, R3(B∆23-27)R/I5 potently shifted relaxin-3’s agonism curve for recombinant rat GPCR135 (pA_2 = 9.6, Fig. 4C) to the right, but did not affect relaxin-3’s agonism for recombinant rat LGR7 (Fig. 4D). R3(B∆23-27)R/I5 was further characterized using native GPCR135 in rat brain slices (Fig. 5). Full displacement of ^{125}I-R3/I5 binding sites in rat brain sections by relaxin-3 and R3(B∆23-27)R/I5 was observed at 10 nM. The IC_{50} values for rat brain binding of relaxin-3...
and R3(BA23-27)R/I5 were 0.5 ± 0.1 nM and 0.4 ± 0.1 nM, respectively.

**R3(BA23-27)R/I5 inhibits R3/I5 stimulated food intake in satiated rats**

R3/I5 and R3(BA23-27)R/I5 were tested *in vivo* for their abilities to modulate feeding behaviors in rats. When 10 µg of R3/I5 was administrated i.c.v. to satiated Wistar rats, food intake was stimulated (n = 5-6) for both the first hour ([Fig. 6A](#)) and over 4 hours ([Fig. 6B](#)) after R3/I5 administration. Intracerebroventricular administration of 10 µg R3(BA23-27)R/I5 10 min prior to the R3/I5 dose blocked R3/I5 stimulated food intake. To assure that the effect on food intake could not be attributed to pre-existing group differences in consumption rates, a baseline measure of food intake (i.e., Day 1) was collected prior to introducing any of the treatments. Untreated-satiated animals assigned to the different treatment conditions exhibited similar levels of food intake at 1 hr and 4 hrs on the baseline day (data not shown).

An ANOVA (Treatment x Day) was used to determine the effect of treatment on food consumption for the four treatment conditions shown ([Fig. 6A](#) and [6B](#)). The amount of food consumed at 1 hr and the total amount of food consumed during the 4 hr test session after dosing were used in the analyses. There was a significant effect of Treatment at 1 hr [F(3,19) = 6.103, p = 0.0044] and 4 hrs [F(3,19) = 8.859, p = 0.0007]. A Newman-Keuls’ test revealed that animals infused with vehicle + R3/I5 consumed significantly more food at 1 hr than animals assigned to the other treatment conditions. In addition, the increased food intake exhibited by animals infused with vehicle + R3/I5 was completely blocked when animals were pretreated with R3(BA23-27)R/I5 10 min prior to the R3/I5 injection; with intake amounts for R3(BA23-27)R/I5 + R3/I5 infused animals being comparable to vehicle + vehicle infused animals (p>0.05).

Post hoc analyses on food intake measured at 4 hrs revealed a similar pattern of results as those seen at 1hr. Hence, animals infused with vehicle + R3/I5 consumed significantly more food over the 4 hr test period. Furthermore, the R3/I5-induced increase in food intake was blocked be pretreatment with R3(BA23-27)R/I5. The total amount of food consumed by animals infused with R3(BA23-27)R/I5 did not differ significantly from vehicle-treated controls.

**Discussion**

Relaxin-3/GPCR135 is a new ligand/receptor pair that is believed to play an important role in the central nervous system. Both the ligand and the receptor are highly conserved from the fish to human (13) and are predominantly expressed in brain regions involved in feeding, stress, and sensory perception (2-6). *In vivo* studies of GPCR135 have been hampered by the lack of selective tools for the GPCR135 receptor. Specific or selective ligands (agonists and antagonists) for the GPCR135 receptor will greatly assist detailed studies of the physiological function(s) of relaxin-3 and the GPCR135 receptor. Previously, we reported that by creating a chimeric peptide with the B-chain from relaxin-3 and the A-chain from INSL5, we obtained a very selective agonist for GPCR135 over the relaxin receptor LGR7. This is a valuable tool for selective activation of the GPCR135 receptor in vivo, particularly in the rat, which does not express the GPCR142 receptor. In this report, we performed mutation studies to identify the receptor binding and activation domains of relaxin-3. The resulting information was used to modify the relaxin-3 B-chain, which contains the ligand binding and receptor activation domains for the GPCR135 receptor, to create selective antagonist for GPCR135 over LGR7.

**The N-terminus of relaxin-3 B-chain is not important for receptor binding and activation**

Relaxin (relaxin-1 & 2) (27, 28) and relaxin-3 share no conservation at the N-termini (amino acid residues 1 to 6) of their B-chains, but all three peptides are potent LGR7 receptor agonists. Similarly, relaxin (1 & 2) and INSL3 (29) share no homology at their N-termini (amino acid residues 1 to 7) of their B-chains. However all three peptides are potent LGR8 receptor ligands. Moreover, while relaxin-3 and INSL5 share no homology at the N-termini (amino acid residues 1 to 6) of their B-chains, but both peptides are high affinity agonists for
the GPCR142 receptor. These lines of information suggest the N-terminus (amino acid residues 1 to 6) of the relaxin-3 B-chain might not play a critical role in receptor interactions. The results in this study show that deletion of the N-terminal 7 residues of the B-chain do not significantly affect the ligand activity for the GPCR135, GPCR142, or LGR7 receptors, thus confirming that the N-terminus of the relaxin-3 B-chain does not play a critical role either for direct receptor interaction or by serving as a structural component. Arg\(^8\) may play a role for relaxin-3 secretion or maintaining the stability of the peptide. Mutants (\(\Delta 1-8\) and \(\Delta 1-9\)) with N-terminal truncations with Arg\(^8\) in the deleted regions were poorly expressed in mammalian cells. Supporting this hypothesis, we also observed that relaxin-3 mutants with Arg\(^8\) to Ala, or Ser changes have reduced production levels in the mammalian expression system. A homology model of relaxin-3 (Fig. 1) based on the crystal structures of human relaxin (30) and human insulin (31) shows that Arg\(^8\) of the relaxin-3 B-chain is close to the A-chain, suggesting that this Arg residue plays a role in the interaction between the B-chain and the A-chain.

The \(\alpha\)-helical region of relaxin-3 B-chain is important for receptor binding

The B-chain of relaxin-3 has been shown to play key roles in the interaction between relaxin-3 and GPCR135 or GPCR142 (2, 10, 23). The R-X-X-X-R-X-X-I motif in the relaxin B-chain has been demonstrated to be important for relaxin receptor (LGR7) binding (32). In the relaxin-3 B-chain, there is a stretch of amino acid residues (R\(^8\)-L-C-G-R\(^{12}\)-E-F-I\(^{15}\)-R\(^{16}\)-A-V-I\(^{19}\)-P\(^{20}\)), which contains two R-X-X-X-R-X-X-I sequences, suggesting that Arg\(^8\), Arg\(^{12}\), and Arg\(^{16}\) may play a role for ligand/receptor interaction. The homology model (Fig. 1) and the NMR solution structures (24) of relaxin-3 suggest that Arg\(^8\), Ile\(^{15}\), Arg\(^{16}\), Ile\(^{19}\) and Phe\(^{20}\) are presented on one side of the B-chain \(\alpha\)-helix facing away from the A-chain, suggesting that those residues may be involved in the ligand/receptor interactions with GPCR135, GPCR142, and LGR7 receptors. In this study, we first investigated the functions of the Arg residues (Arg\(^{12}\), and Arg\(^{16}\)) on the relaxin-3 B-chain regarding their role in relaxin-3 binding by GPCR135, GPCR142, and LGR7 receptors. Our results showed that Arg\(^{12}\) is very important for GPCR135 and LGR7 receptor binding but not for GPCR142 receptor binding. Changing of Arg\(^{12}\) to a hydrophobic amino acid (Ala, Leu) or a polar amino acid (Ser) significantly reduced its binding affinity at the GPCR135 and LGR7 receptors. These results strongly suggest that Arg\(^{12}\) is involved in GPCR135 and LGR7 receptor binding. In contrast, the Arg\(^{12}\) mutations did not affect relaxin-3’s function at GPCR142. In fact INSL5, which is the purported endogenous ligand for GPCR142 (14), has a Leu at the corresponding position of Arg\(^{12}\) at the relaxin-3 B-chain. The presence of Arg\(^{12}\) on the relaxin-3 B-chain is particularly critical for LGR7. Even a conserved change (Arg\(^{12}\) to Lys), which does not dramatically affect relaxin-3’s activity for GPCR135 and GPCR142, abolishes relaxin-3 activity for LGR7. Similarly, the presence of Arg\(^{16}\) on the B-chain is equally critical for relaxin-3 to interact with LGR7. Mutation studies concerning Arg\(^{12}\) and Arg\(^{16}\) of the relaxin-3 B-chain indicate that Arg\(^{12}\) and Arg\(^{16}\) are involved in receptor binding since the loss of the agonist activity of these mutants for all three receptors correlates with the loss of the binding affinity (Fig. 2).

The two Ile residues (Ile\(^{15}\) and Ile\(^{19}\)) in the \(\alpha\)-helical region of the relaxin-3 B-chain were studied. We changed Ile\(^{15}\) of the relaxin-3 B-chain to Gly, Ala, and Val to investigate whether different lengths in their amino acid residue side chains play a role in receptor interactions. Our results showed that with the increased length of the side chain of the residue, the activity of the mutants for all three receptors increases with the I15V mutant having the same activity as that of relaxin-3, suggesting that Ile\(^{15}\) might interact with GPCR135, GPCR142, or LGR7 through the hydrophobic side chain, with the length of the side chain important for ligand/receptor binding. However, we cannot exclude the possibility that the function of a hydrophobic residue at the position of Ile\(^{15}\) is purely to form the hydrophobic core of the relaxin-3 peptide (31), which hence stabilizes the three dimensional structure of relaxin-3. The presence of a Gly residue at this position will certainly
destabilize the hydrophobic core. This result would predict that INSL4, which has a Gly at the corresponding position of Ile19 of the relaxin-3 B-chain, could not be a ligand for GPCR135, GPCR142, or LGR7. This has been tested and confirmed by our previous studies (5, 6, 39). The mutation studies at Ile19 showed that this residue is very critical for LGR7 binding. An Ile19 to Ala change abolished relaxin-3 activity for LGR7 in terms of both binding and activation but did not affect relaxin-3’s activities for GPCR135 and GPCR142. These results suggest that Ile19 interacts with LGR7 but may not directly interact with GPCR135 or GPCR142. However, an Ile19 to Glu (the corresponding residue at the B-chain of INSL6 (40)) change not only abolished the activity of relaxin-3 for LGR7 but also decreased relaxin-3’s activity for GPCR135 and GPCR142 dramatically, suggesting that INSL6 might not be a ligand for GPCR135, GPCR142 or LGR7. These results further support that R12-E-F-I-R16-A-V-I19 is serving as the R-X-X-X-R-X-X-I motif for LGR7 binding (34).

Mutation studies at the Phe20 position indicate that Phe20 is important for GPCR135 and GPCR142 binding but not important for LGR7. Changing Phe20 to Tyr did not affect relaxin-3 ligand activity significantly for GPCR135 and GPCR142, which have been predicted because the amino acid substitution is conservative and human INSL5, the endogenous ligand for GPCR135 and GPCR142, actually has a Tyr at the position corresponding to Phe20 of relaxin-3. However, changing Phe20 to Ala and Ser lowered the potency of the relaxin-3 while a change to Arg abolished the ligand activity of relaxin-3 for both GPCR135 and GPCR142. All changes made at Phe20 did not affect their activities for LGR7. These results indicate that Phe20 is very important for relaxin-3 to interact with GPCR135 and GPCR142, but is irrelevant to the LGR7 interaction. Since the potency loss of the mutants correlates well with the affinity loss, our results suggest that Phe20 is mainly involved in the ligand/receptor binding between relaxin-3 and GPCR135 or GPCR142.

The C-terminus of relaxin-3 B-chain is important for GPCR135 and GPCR142 activation but not for LGR7

Arg26 and Trp27 are two conserved amino acid residues at the C-terminus of relaxin-3 and INSL5 B-chains, which are endogenous ligands for GPCR135 and GPCR142, respectively. To determine the contribution of Arg26 or Trp27 of relaxin-3 to GPCR135 or GPCR142 receptor binding and activation, we made specific changes in the region. We changed Arg26 to Ala or Thr and found that both changes abolished relaxin-3’s agonist potency for GPCR135 and GPCR142. However, both mutants retained a moderately high affinity for GPCR135 and GPCR142 (Table 3). Similarly Trp27 might also play a role in GPCR135 and GPCR142 activation. Mutations of Trp27 to Ala, Phe, or Arg reduced relaxin-3 agonist potency for GPCR135 and GPCR142 but retained high affinity for both receptors, indicating that the C-terminal Arg26 and Trp27 are mainly serving as the receptor activation domain for GPCR135 and GPCR142, which is obvious when the pKi and pEC50 values of those mutants are compared side by side (Fig. 2). In addition, we made another mutant by adding an extra Ser residue after Trp27 (+Ser28) and found that this added Ser blocks relaxin-3’s agonist function but not receptor binding by GPCR135 or GPCR142. This suggests the C-terminal exposure of the relaxin-3 B-chain might be necessary for GPCR135 and GPCR142 receptor activation. In contrast to GPCR135 and GPCR142, mutations at Arg26, Trp27, and +Ser28 of the relaxin-3 B-chain do not affect the peptides’ agonist potency and binding affinity for LGR7. Previous studies showed that rat and mouse relaxins (35) and rhesus monkey relaxin (36) do not have a Trp27 equivalent residue but retain high LGR7 activity. Tregear et al., showed that up to seven amino acid residues can be removed from the C-terminus of the B-chain of porcine relaxin and retain full biological activity (37). Our results demonstrate that, similar to relaxin, the C-terminus of the relaxin-3 B-chain is not critical either for LGR7 binding or for LGR7 activation. These results suggest that the interaction mode between relaxin-3 and LGR7 may be different from those between relaxin-3 and GPCR135 or GPCR142. The results from previous studies
(23) and this study demonstrate that both the receptor binding and activation domains for GPCR135 and GPCR142 are likely located at the B-chain of relaxin-3 while for LGR7, both the B-chain and the A-chain of relaxin-3 may be required for receptor binding and activation.

Knowing that the C-terminus of the relaxin-3 B-chain is mainly involved in receptor activation of GPCR135 and GPCR142, we attempted a series of truncations of the C-terminus of the relaxin-3 B-chain, some of which result in high affinity GPCR135 and GPCR142 antagonists. Results of the truncation study further support our hypothesis that the C-terminus of the relaxin-3 B-chain is the activation domain for GPCR135 and GPCR142.

**R3(BΔ23-27)R/I5, a selective GPCR135 antagonist, is active in vivo**

In the process of designing a selective GPCR135 antagonist, we initially created relaxin-3 mutants R3(BΔ23-27)R, R3(BΔ24-27), R3(BΔ25-27), and R3(BΔ26-27). Pharmacological characterization shows that these peptides are GPCR135 antagonists with R3(BΔ23-27)R being the most potent. Since R3(BΔ24-27), R3(BΔ25-27) and R3(BΔ26-27) are also potent LGR7 agonists, R3(BΔ23-27)R was singled out as the best GPCR135 antagonist candidate because it lacks LGR7 agonist activity. However, R3(BΔ23-27)R remains a low affinity LGR7 ligand (Kᵢ ~ 200 nM) and additional selectivity is desirable. To further increase the selectivity of R3(BΔ23-27)R, we replaced the A-chain of relaxin-3 with the A-chain of INSL5, a strategy used in the past to create selective GPCR135/GPCR142 agonist R3/I5 (23). The resulting peptide, R3(BΔ23-27)R/I5, proved to be a selective high affinity GPCR135/GPCR142 antagonist for which LGR7 has essentially no affinity. R3(BΔ23-27)R/I5, was also shown to displace GPCR135 binding sites in native tissue (rat brain tissue sections, Fig. 5).

As an initial in vivo test of these chimeric GPCR135 ligands, we chose to look for feeding changes in satiated rats during the light phase. Previous studies from McGowan et al. or Hidal et al. showed that acute (8) and chronic (9) intracerebroventricular or intraparaventricular administration of relaxin-3 increased food intake. In this study, intracerebroventricular administration of R3/I5, a selective GPCR135 agonist in the rat (23), stimulates food intake in this paradigm (Fig. 6). Prior dosing of R3(BΔ23-27)R/I5 blocks the R3/I5 induced feeding response (Fig. 6). Since the test system described here involves light phase feeding in satiated rats, the lack of significant effect of the antagonist given alone to alter feeding compared to vehicle treated animals is likely due to a lack of feeding drive under these conditions. This result is consistent with earlier reports (8, 9). By using a selective agonist and by blocking its effect with a selective antagonist we clearly demonstrate the involvement of GPCR135 in feeding induced by relaxin-3.

**GPCR135 is abundantly expressed in areas of the rodent brain such as the amygdala, superior colliculus, sensory cortex, and olfactory bulb (4, 38, Fig. 5).** The expression of GPCR135 and GPCR135 binding sites are consistent with demonstrated projections of the nucleus incertus (39), which is the primary source of relaxin-3 in the rat (2, 3). The overall expression patterns of relaxin-3 and its receptor are consistent with roles in spatial memory, emotional, neuroendocrine and sensory processing. In addition to expressing relaxin-3
and GPCR135, the nucleus incertus is a prominent source of CRF-R1 expression in the hindbrain (40). Water restraint stress induces relaxin-3 expression in the nucleus incertus, suggesting the involvement of relaxin-3 in the stress response. Recent visualization of relaxin-3 like immunoreactivity in GABA projection neurons of the nucleus incertus is consistent with prior observations and suggests additional actions of relaxin-3, for instance on arousal and locomotor activity (6). The availability of GPCR135 selective agonist and antagonist provides useful tools to study the role of relaxin-3/GPCR135 in stress and possibly other involvements.

As a summary, in this study, we made a series of mutations of the human relaxin-3 B-chain to probe the receptor binding and activation domains for GPCR135, GPCR142, and LGR7. Our results showed that the N-terminal 1 to 7 residues of the relaxin-3 B-chain are not critical for receptor activation for all three receptors (Fig 7). Arg<sup>8</sup> of the relaxin-3 B-chain might play a role for relaxin-3’s structural stability and might also participate in ligand/receptor binding. Arg<sup>12</sup> and Arg<sup>16</sup> are very important for GPCR135 and LGR7 receptor binding but play a less critical role in the interaction with GPCR142. In contrast, Phe<sup>20</sup>, which is not involved in the LGR7 interaction, plays a pivotal role in GPCR135 and GPCR142 receptor binding. Our results also showed that the C-terminus of the relaxin-3 B-chain is not important for relaxin-3/LGR7 binding but rather serves as the important domain for GPCR135 and GPCR142 activation (Fig. 7). By dissection of the receptor binding and activation domains of relaxin-3 for different receptors, our studies provide useful information for designing receptor specific agonists and antagonists, which has led to the creation of a potent GPCR135 antagonist (R3(BΔ23-27)R/I5) which is selective with respect to LGR7 activity. Specific stimulation (with R3/I5) or inhibition (with R3(BΔ23-27)R/I5) of GPCR135 is now possible in the rat. As an initial test of the in vivo effects of these chimeric GPCR135 ligands, we have confirmed and extended the finding that GPCR135 is involved in feeding induced by relaxin-3. Upcoming experiments will further assess the physiological role(s) of GPCR135 in the central nervous system using these selective pharmacological tools.

REFERENCES


FIGURE LEGENDS

Figure 1. A. Amino acid sequence and disulfide bond formation of the human relaxin-3 peptide. B. Homology model of relaxin-3, constructed using the crystal structures of human relaxin (41) and human insulin (42). The B-chain is in front (cyan; A-chain is behind in gold), and the eight residues examined with single-point mutations are shown. After this work was completed, an NMR structure of relaxin-3 was published (24). The homology model differs from the NMR structure in the conformation of the N- and C-terminal regions of the B-chain. C. B-chain sequence comparison among different members in insulin/relaxin peptide family.

Figure 2. pKi and pEC50 value comparison of different relaxin-3 mutants to GPCR135, GPCR142, and LGR7. All mutants contain the intact A-chain of relaxin-3. The pKi and pEC50 values are converted from Ki and EC50 values (Sup. Table 3). Mutants with pKi or pEC50 smaller than 6 show no bar values in the graph.

Figure 3. Characterization of R3(B∆23-27)R/I5 as an antagonist for human GPCR135 and GPCR142. In a functional assay using SK-N-MC/CRE-β-gal cells expressing human GPCR135 (A), GPCR142 (B) or LGR7 (C), ascending concentrations of relaxin-3 (R3) were used to generate concentration response curves either in the absence or presence of 10 nM, 100 nM or 1 µM R3(B∆23-27)R/I5. For Gαi linked GPCR135 and GPCR142, the assay was performed as inhibition of forskolin-induced β-gal expression. LGR7 is linked to Gαs, therefore addition of forskolin was not necessary. The antagonism of R3(B∆23-27)R/I5 is indicated by the rightward-shift of the relaxin-3’s dose-response curve. R3(B∆23-27)R/I5 does not affect relaxin-3’s stimulation of LGR7. β-gal expression was measured by colorimetric assay using CPRG as the substrate and reading the absorbance at 570nm. Relaxin-3/INSL5 chimeric peptide (R3/I5) was used as agonist in comparison to R3 for all three receptors. The antagonism of R3(B∆23-27)R/I5 to R3/I5 for GPCR135 and GPCR142 has also studied and the results is almost identical to that when R3 is used as agonist.

Figure 4. Pharmacological characterization of R3(B∆23-27)R/I5 as a selective antagonist for rat GPCR135 over rat LGR7. COS-7 cells transiently expressing recombinant rat GPCR135 or LGR7 were used to characterize the binding affinity of rat GPCR135 (A) and LGR7 for (B) R3(B∆23-27)R/I5. 125I-R3/I5 was used as the tracer for GPCR135 and 125I-relaxin-2 was used as the tracer for LGR7 binding. Various concentrations of Relaxin-3 (R3), R3/I5, and R3(B∆23-27)R/I5 were used as the competitor and unlabeled human relaxin-3 was used as the positive control in the binding assay. SK-N-MC/CRE-β-gal cells stably expressing rat GPCR135 (C) or HEK293 cells transiently expressing rat LGR7 (D) were used to characterize the antagonism of R3(B∆23-27)R/I5 for rat GPCR135 and rat LGR7. For rat GPCR135, which is Gαi coupled, the assay was performed as inhibition of forskolin-induced β-gal expression. β-gal expression was measured by colorimetric assay using CPRG as the substrate and reading the absorbance at 570nm. The antagonism of R3(B∆23-27)R/I5 to the rat GPCR135 is indicated by the rightward-shift of the relaxin-3’s dose-response curve. The antagonism of R3(B∆23-27)R/I5 to R3/I5 for rat GPCR135 has also studied and the results is almost identical to that when R3 is used as agonist. For rat LGR7, which is Gαs coupled, the activation of receptor was monitored by measuring the agonist-induced cAMP production using a cAMP luminescent assay kit (DiscoveryRx Hithunter). R3/I5 did not show significant agonist activity for rat LGR7 even at 1 µM. R3(B∆23-27)R/I5 (1 µM) did not demonstrate any detectable antagonism for rat LGR7.
Figure 5. High affinity binding of R3(Δ23-27)R/I5 to rat brain sections. Autoradiograms of $^{125}$I-R3/I5 binding sites in the rat brain are shown (A) with competition by relaxin-3 (10 nM, B) or R3(Δ23-27)/I5 (10 nM, C). D. Dose-response curve for the inhibition of $^{125}$I-R3/I5 by relaxin-3 and R3(Δ23-27)R/I5 in rat brain slices. Nonspecific binding was determined using 1 µM unlabeled relaxin-3. Values shown are mean ± SEM (n = 3/data points).

Figure 6. A. Food consumption in satiated Wistar rats during the first hour of the test session (i.e., Day 2) following the i.c.v. administration (5 µl) of vehicle + vehicle, vehicle + R3/I5 (10 µg), R3(Δ23-27)R/I5 (10 µg) + vehicle, and R3(Δ23-27)R/I5 (10 µg) + R3/I5 (10 µg) (mean ± SEM; n = 5-6 per group). B. Total food consumption over 4 hours in the same paradigm.

Figure 7. Amino acid residues in the relaxin-3 B-chain that affects relaxin-3’s ligand activity for GPCR135, GPCR142, and LGR7.
### Table 1. Amino acid sequence of relaxin-3 and mutant peptides

<table>
<thead>
<tr>
<th>Peptides</th>
<th>B-chain</th>
<th>A-chain</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>23 27</td>
<td></td>
</tr>
<tr>
<td>Relaxin-3</td>
<td>RAAPYGVRGREFIRAVIFTCGGSRW</td>
<td>DVLAGLSSCCKWGCSKSEISSLC</td>
</tr>
<tr>
<td>R3(B∆23-27)</td>
<td>RAAPYGVRGREFIRAVIFTC</td>
<td>DVLAGLSSCCKWGCSKSEISSLC</td>
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<tr>
<td>R3(B∆23-27)R</td>
<td>RAAPYGVRGREFIRAVIFTCR</td>
<td>DVLAGLSSCCKWGCSKSEISSLC</td>
</tr>
<tr>
<td>R3(B∆24-27)</td>
<td>RAAPYGVRGREFIRAVIFTCG</td>
<td>DVLAGLSSCCKWGCSKSEISSLC</td>
</tr>
<tr>
<td>R3(B∆25-27)</td>
<td>RAAPYGVRGREFIRAVIFTCGG</td>
<td>DVLAGLSSCCKWGCSKSEISSLC</td>
</tr>
<tr>
<td>R3(B∆26-27)</td>
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<td>DVLAGLSSCCKWGCSKSEISSLC</td>
</tr>
<tr>
<td>R3/I5</td>
<td>RAAPYGVRGREFIRAVIFTCGGSRW</td>
<td>&lt;EDLQTLCCTDGCSMTDLSALC</td>
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<tr>
<td>R3(B∆23-27)/I5</td>
<td>RAAPYGVRGREFIRAVIFTC</td>
<td>&lt;EDLQTLCCTDGCSMTDLSALC</td>
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<tr>
<td>R3(B∆23-27)R/I5</td>
<td>RAAPYGVRGREFIRAVIFTCR</td>
<td>&lt;EDLQTLCCTDGCSMTDLSALC</td>
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All truncated relaxin-3 peptides, except for R3(B∆23-27)R/I5, have truncations at the C-terminus of the B-chain and contain the intact relaxin-3 A-chain. Truncated chimeric peptide R3(B∆23-27)R/I5 possesses a truncated relaxin-3 B-chain and an intact INSL5 A-chain. Wild type relaxin-3 and chimeric R3/I5 peptide sequences are shown for comparison. A Gln to pyro-Glu (<E) conversion at the N-terminus of the INSL5 A-chain occurs in R3/I5 and mutant peptides with INSL5 A-chain.

Compared to R3(B∆23-27) and R3(B∆23-27)/I5, R3(B∆23-27)R and R3(B∆23-27)R/I5 peptides have an extra Arg residue at the C-terminus of the B-chain, which is due to incomplete processing of the peptide by carboxypeptidase-B during peptide maturation. The extra Arg residue is highlighted in bold and underlined.
Table 2. The $K_i$ and $EC_{50}$ values of relaxin-3 and mutant peptides with truncations at the C-terminus of the B-chain for human GPCR135, GPCR142, and LGR7

<table>
<thead>
<tr>
<th>Peptides</th>
<th>GPCR135</th>
<th>GPCR142</th>
<th>LGR7</th>
<th>GPCR135</th>
<th>GPCR142</th>
<th>LGR7</th>
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<tbody>
<tr>
<td>Relaxin-3</td>
<td>0.53 ± 0.09</td>
<td>1.49 ± 0.12</td>
<td>2.41 ± 0.23</td>
<td>0.35 ± 0.06</td>
<td>0.95 ± 0.14</td>
<td>1.3 ± 0.15</td>
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<tr>
<td>R3/I5</td>
<td>0.49 ± 0.17</td>
<td>1.32 ± 0.12</td>
<td>583 ± 64</td>
<td>0.47 ± 0.12</td>
<td>1.15 ± 0.14</td>
<td>453 ± 96</td>
</tr>
<tr>
<td>R3(Δ23-27)R</td>
<td>0.95 ± 0.14</td>
<td>3.91 ± 0.56</td>
<td>~200</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>R3(Δ23-27)R/I5</td>
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<td>2.29 ± 0.23</td>
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<tr>
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<td>13.5 ± 1.84</td>
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<td>8.9 ± 1.21</td>
<td>NA</td>
<td>NA</td>
<td>4.3 ± 0.57</td>
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<tr>
<td>R3(Δ25-27)</td>
<td>9.5 ± 1.64</td>
<td>11.9 ± 1.34</td>
<td>7.9 ± 0.72</td>
<td>NA</td>
<td>NA</td>
<td>4.1 ± 0.61</td>
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<tr>
<td>R3(Δ26-27)</td>
<td>11.5 ± 1.96</td>
<td>9.5 ± 1.28</td>
<td>6.3 ± 0.45</td>
<td>NA</td>
<td>NA</td>
<td>3.3 ± 0.42</td>
</tr>
</tbody>
</table>

$^a$ $IC_{50}$ values (mean ± SEM) are the concentrations of ligands that displaced 50% of the specific binding in the competition binding assay. The $K_i$ values are converted from $EC_{50}$ values using Cheng/Prusoff formula ($K_i = IC_{50} / (1 + [L]/K_d)$, where [L] stands for radioligand concentration used in the binding assay. The $K_d$ values of $^{125}$I-R3/I5 for GPCR135 (0.41 nM), GPCR142 (0.89 nM) and the $K_d$ value of $^{125}$I-relaxin for LGR7 (0.18 nM) were determined by saturation binding assay using cells recombinantly expressing human GPCR135, GPCR142 or LGR7 as previously described (23).

$^b$ $EC_{50}$ values (mean ± SEM) are the concentrations of ligands that stimulated agonistic responses by 50%.

$^c$ NA represents no affinity was observed in radioligand binding assay or no agonist activity was observed in the functional assay.
**FIGURES**

**Figure 1**

A

A-Chain: `DVLAGLSSSECKWGCSEISSLC`

B-Chain: `RAAPYGVRGREFIRAVIFTCGGSRW`

B

![Diagram of Relaxin-1, Relaxin-2, Relaxin-3, INSL3, INSL4, INSL5, INSL6, Insulin, and IGF1 and IGF2](image)

C

**B-chain**

<table>
<thead>
<tr>
<th>Relaxin-1:</th>
<th>KWKDDVIKLCRELRAQIA1CG5STWS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Relaxin-2:</td>
<td>DSWMEVIKLCHELRAQIA1CG5STWS</td>
</tr>
<tr>
<td>Relaxin-3:</td>
<td>RAAPYGVRGLCREIRAVIFTCGGSRW</td>
</tr>
<tr>
<td>INSL3:</td>
<td>PTPEMKLCHELHVRALVRVCGEPWSTEAG</td>
</tr>
<tr>
<td>INSL4:</td>
<td>ELSAEELCGPFRGKHHLSYCPPEK</td>
</tr>
<tr>
<td>INSL5:</td>
<td>KESVRLCHELRTVIYICASSRWS</td>
</tr>
<tr>
<td>INSL6:</td>
<td>ISSARKLCHELRYLKEIEKLCRANSQF</td>
</tr>
<tr>
<td>Insulin:</td>
<td>FVMQHLCHELVEALVLCGERGFYYTPKT</td>
</tr>
<tr>
<td>IGF1:</td>
<td>GPETLCHELVVALQFVCGGERGFYFKPTGYG6---</td>
</tr>
<tr>
<td>IGF2:</td>
<td>ARPSETLCHELVDLQFVCGGGERGFYFSRP6---</td>
</tr>
</tbody>
</table>
Figure 2

### GPCR135

- Relxin-3
- R3/I5
- Δ1-4
- Δ1-7
- Δ1-8
- R8A
- R8S
- R11A
- R11L
- R11S
- R12K
- H15A
- H15G
- H15V
- R16A
- R16S
- R13K&R16K
- I19A
- I19E
- F20A
- F20R
- F20S
- F20Y
- R26T
- W27A
- W27F
- W27R
- +28S
- R3(B23-27)R
- R3(B23-27)R15
- R3(B24-27)
- R3(B25-27)
- R3(B26-27)

### GPCR142

- Relxin-3
- R3/I5
- Δ1-4
- Δ1-7
- Δ1-8
- R8A
- R8S
- R11A
- R11L
- R11S
- R12K
- H15A
- H15G
- H15V
- R16A
- R16S
- R13K&R16K
- I19A
- I19E
- F20A
- F20R
- F20S
- F20Y
- R26T
- W27A
- W27F
- W27R
- +28S
- R3(B23-27)R
- R3(B23-27)R15
- R3(B24-27)
- R3(B25-27)
- R3(B26-27)

### LGR7

- Relxin-3
- R3/I5
- Δ1-4
- Δ1-7
- Δ1-8
- R8A
- R8S
- R11A
- R11L
- R11S
- R12K
- H15A
- H15G
- H15V
- R16A
- R16S
- R13K&R16K
- I19A
- I19E
- F20A
- F20R
- F20S
- F20Y
- R26T
- W27A
- W27F
- W27R
- +28S
- R3(B23-27)R
- R3(B23-27)R15
- R3(B24-27)
- R3(B25-27)
- R3(B26-27)
Figure 3

A

GPCR135

Absorbance (570 nm)

Log Concentration of Ligand (M)

B

GPCR142

Absorbance (570 nm)

Log Concentration of Ligand (M)

C

LGR7

Absorbance (570 nm)

Log Concentration of Ligand (M)
Figure 4

A  Rat GPCR135

B  Rat LGR7

C  Rat GPCR135

D  Rat LGR7

Log Concentration of Ligand (M)

Percent of specific 125I-R3/I5 bound

Log Concentration of Ligand (M)

Log Concentration of Ligand (M)

Absorbance (570nm)

RLU

log Concentration of Ligand (M)
Figure 5

$^{125}$I R3/I5

A
+ 10 nM R3(Δ23-27)R/I5

B
+ 10 nM Relaxin-3

C

D

Percent Specific Binding

Log Concentration of Ligand (M)

-12 -11 -10 -9 -8 -7 -6

0 20 40 60 80 100 120

● Relaxin-3

△ R3(Δ23-27)R/I5
Figure 6

A

B

Vehicle + Vehicle
Vehicle + R3/I5
R3(BΔ23-27)R/I5 + Vehicle
R3(BΔ23-27)R/I5 + R3/I5

Food Consumed (g)

0 2 4 6 8 10 12

**

#
Figure 7

Affinity
GPCR135
RAAPYGVRLCGREFIRAVIFTCGGSRW
Activation
△ △ △ △ △

Affinity
GPCR142
RAAPYGVRLCGREFIRAVIFTCGGSRW
Activation
△ △ △ △ △

Affinity
LGR7
RAAPYGVRLCGREFIRAVIFTCGGSRW
Activation
△ △ △ △ △
R3(Bù23-27)R/I5 chimeric peptide, a selective antagonist for GPCR135 and GPCR142 over relaxin receptor LGR7: In vitro and in vivo characterization

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