Both relaxin-3 and its receptor (GPCR135) are expressed predominantly 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. Because relaxin-3 also activates the relaxin receptor (LGR7), which is also expressed in the brain, selective GPCR135 agonists and antagonists are crucial to the study of the physiological functions of relaxin-3 and GPCR135 in vivo. Previously, we reported the creation of a selective GPCR135 agonist (a chimeric relaxin-3/INSL5 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(Δ23–27)/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 INSL5. In vitro pharmacological studies showed that R3(Δ23–27)/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(Δ23–27)/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 to rats intracerebroventicularly, R3(Δ23–27)/R/I5 blocked food intake induced by the GPCR135 selective agonist R3/I5. Thus, R3(Δ23–27)/R/I5 should prove a useful tool for the further delineation of the functions of the relaxin-3/GPCR135 system.

Relaxin-3 (R3)2 (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 paraventricular nucleus (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 corticotropin-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 knock-out 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), and therefore selective pharmacological tools (agonists and antagonists) are crucial to probe the in vivo function(s) of GPCR135. Because 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 a potentially confounding issue, especially because LGR7 is expressed in the brain and is reported to play a role in drinking (8, 19) and potentially in 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...
Arg16, Ile19, and Phe20 are presented on one side of the minal regions of the B-chain.

The homology model differs from the NMR structure in the conformation of the N- and C-termini. 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-termini of the B-chain. R3(I5)/H9251-A chain selectively activates GPCR135 over LGR7 (23), suggesting that the N terminus of relaxin-3 may not be important for interactions between relaxin-3 and its receptors. In addition, we demonstrate increased feeding in satiated Wistar rats following intracerebroventricular 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 Arg8, Arg12, Ile15, Arg16, Ile19, Phe20, Arg26, and Trp27 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 described previously (2). All relaxin-3 mutant coding regions were created by a two-step PCR using primers shown in supplemental 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(BΔ23–27)/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 (PI5 for R3(BΔ23–27)/I5; listed in supplemental Table 1). All PCRs were run under the conditions of 94°C for 20 s, 65°C for 20 s, and 72°C for 1 min for 20 cycles. The final PCR products were cloned into a modified pCMV-SPORT1 vector containing a coding region for an α-signal peptide for secretion, which was followed by a FLAG peptide coding region for affinity purification (2). All mutant peptides (except for R3(BΔ23–27)/I5) have the intact A-chain of the wild type relaxin-3 but different B-chains. R3(BΔ23–27)/I5 has an A-chain of human INS15. 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 supplemental Table 2. All recombinant peptides were co-expressed with furin 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 high pressure liquid chromatography (HPLC). The purified peptides were analyzed by mass spectrometry as described (2) to verify the peptide identities. R3(BΔ23–27)R and R3(BΔ23–27)/I5, which are derivative of R3(BΔ23–27) and R3(BΔ23–27)/I5 respectively, have an extra Arg residue at

for GPCR135. Later studies have demonstrated that a chimeric peptide (R3/I5) composed of the relaxin-3 B-chain and the INS15 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, and 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/rexin family have no conservation (Fig. 1), a few different members can activate the same receptor (relaxin-1 and -2, and relaxin-3 for LGR7; relaxin-1, -2, and INS3 for LGR8; relaxin-3 and INS15 for GPCR142), suggesting that the N terminus of relaxin-3 may not be important for interactions between relaxin-3 and its receptors. In this report, using mutagenesis studies, we describe the identification of the GPCR135 binding domain (the α-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 INS15. 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 dosing of R3/I5 (a selective GPCR135 agonist), which is blocked by prior administration of the GPCR135-specific antagonist R3(BΔ23–27)R/I5.
the C terminus of the B-chain because of incomplete processing (Table 1). Mutant peptides R12K, R12K,R16K, and W27R were made by solid phase peptide synthesis using methods described previously (1, 26).

Radioligand Binding Assays—

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 nM as the tracer to characterize the binding properties of GPCR135 and GPCR142 for the mutant relaxin-3 peptides. 125I-I-H2 (human gene-2) relaxin (PerkinElmer Life Sciences) 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 previously (13). The results were analyzed by GraphPad Prism 4.0 software (San Diego). The IC50 values, which are the ligand concentrations that inhibited 50% of the maximum binding, were calculated and then converted to Kd values using the Cheng-Prusoff formula (31) with Kd values of 0.41, 0.89, and 0.18 nM for binding of 125I-R3/I5 to GPCR135, 125I-R3/I5 to GPCR142, and 125I-I-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 described previously (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-galactopyranosidase as a substrate and reading the optical absorbance at 570 nm. GPCR135 and GPCR142 are coupled with Gα proteins; therefore agonists inhibit forskolin-stimulated β-gal expression in GPCR135- or GPCR142-expressing cells. LGR7 is Gαi-linked, and therefore agonists stimulate expression in LGR7-expressing cells. R3(BΔ23–27)/R15 was tested for its ability to produce a rightward-shift in the relaxin-3 or R3/I5 dose-response curve in the presence of 10 nM, 100 nM, or 1 μM R3(BΔ23–27)/R15 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. 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)/R15 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 phosphate-buffered saline plus 10 mM EDTA and plated at a density of 25,000 cells/well in 96-well white opaque plates (Thermo Electron Corp., catalog no. 7571). To test the agonism of R3(BΔ23–27)/R15, cells expressing rat LGR7 were stimulated with different concentrations of R3(BΔ23–27)/R15 with relaxin-3 as the positive control. To test the antagonism of R3(BΔ23–27)/R15 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(Δ23–27)/R15. Cells were then incubated at room temperature for 1 h. The cAMP in the cells was measured with a cAMP detection kit (DiscoveRx HitHunter, catalog no. 90–0041) according to the manufacturer’s protocol. The results were analyzed using GraphPad Prism 4.0 software.

Autoradiographic Studies—

In autoradiographic studies as described previously (4). Briefly, 125I-I-R3/I5 was applied in a binding buffer to rat brain slices. Unlabeled human relaxin-3 or R3(BΔ23–27)/R15 was used at various concentrations as competitors to displace GPCR135 binding of 125I-I-R3/I5. The specific binding of 125I-I-R3/I5 to the rat brain slices was quantitated using a Fuji Bio-Imaging analyzer system (BAS-5000).

In Vivo Studies—

Experimentally naive male Wistar rats (Charles River, Wilmington, MA), weighing 200–225 g at the time of arrival, were used. The animals were initially housed at two/cage and given a 1-week acclimation period to the vivarium prior to intracerebroventricular 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-h light/12-h dark illumination cycle with lights on from 6:00 a.m. to 6:00 p.m. All behavioral testing occurred during the light phase between 8:00 a.m. and 4:00 p.m. All studies were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U. S. 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 mounted in the skull and covered with dental cement served as an anchor for the guide cannula. Animals were then housed individually 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 because of being handled 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 on demand throughout the session(s). A predetermined amount of standard rat chow (Formulab Diet no. 5008) was placed in the food hopper at the start of the 4-h session(s). The amount of food remaining in the food hopper was determined by subtracting the weight of the food at 1 and 4 h 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)/R15) 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 g/l. Feeding Procedure—Following the surgical recovery period, the animals were randomly assigned to one of the four treatment conditions: 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 base-line session. No injections were administered during this session, and it served as a habituation period to the testing apparatus, while also providing a base-line 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 preloaded 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 min, and the injection needle remained in the guide cannula for 1 min 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 h during a 4-h 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 Arg1–Gly6, Arg1–Val7, Arg1–Arg8, or Arg1–Leu9 from the N-terminal region of the relaxin-3 B-chain. The A-chains of those mutant peptides were left unchanged. The R1–6 and R1–7 mutants were expressed in mammalian expression system with a similar yield to that of the wild type relaxin-3 peptide (5). R1–8 mutant had a significantly reduced expression level (100 μg/liter), but the expression level for R1–9 mutant was too low to generate enough peptide for functional testing.

Computer modeling (Fig. 1) and NMR structure analysis (24) predict that residues Arg12, Ile15, Arg16, Ile19, and Phe20 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: Arg8 to Ala (R8A) or Ser (R8S); Arg12 to Ala (R12A), Leu (R12L), Ser (R12S), or Lys (R12K); Arg16 to Ala (R16A) or Ser (R16S); Ile15 to Gly (I15G), Ala (I15A), or Val (I15V); Ile19 to Ala (I19A) or Glu (I19E); Arg12 to Lys and Arg16 to Lys (R12K,R16K); Phe20 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 Arg8 was changed to Ala (R8A) or Ser (R8S), the mutants showed reduced agonist potency and affinity for GPCR135, GPCR142, and LGR7 (Fig. 2 and supplemental Table 3). Arg12 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 retaining 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 Arg16 to Ala (R16A) or Ser (R16S) abolishes relaxin-3 agonist activity and binding affinity for LGR7 while weakening its agonist activity and binding affinity for GPCR135 and GPCR142. R12K,R16K mutant remained a moderately high affinity agonist for GPCR135 and GPCR142 but lost affinity and agonistic activity for LGR7 (Fig. 2). The I15G mutant had very weak activity and low binding affinity for GPCR135 and GPCR142 and no activity or affinity for LGR7 (Fig. 2). Compared with I15G, the I15A mutant had improved potency and affinity to all three receptors (Fig. 2). In contrast, the I15V mutant had nearly the same pharmacological properties as the relaxin-3 wild type peptide for all three receptors (Fig. 2). Changing Ile19 to Ala (I19A) or G1u (I19E) abolished the interaction of relaxin-3 with LGR7 completely. However, the I19A mutant retained full GPCR135 and GPCR142 activity (Fig. 2). The I19E mutant retained weak activity for GPCR135 and GPCR142 (Fig. 2). The pharmacological properties of the modified relaxin-3 peptides with mutations at Phe20 were also evaluated. The results showed that changing Phe20 to Tyr (F20Y) reduced the binding affinity or agonist potency for GPCR135 and GPCR142 but not for LGR7 (Fig. 2). Changing Phe20 to Ala (F20A) dramatically reduced the agonist potency and binding affinity for GPCR135 and GPCR142 but still retained full activity for LGR7. A Phe20 to Ser (F20S) change also dramatically decreased relaxin-3 ligand activity for GPCR135 and GPCR142. Changing Phe20 to Arg (F20R) abolished relaxin-3 ligand activity for both GPCR135 and GPCR142. However, both F20S and F20R retained full ligand activity for LGR7. The EC50 and Kᵢ values of these mutants for GPCR135, GPCR142 and LGR7 are summarized in Fig. 2. The exact EC50 and Kᵢ values of the mutant peptides for GPCR135, GPCR142, and LGR7 are listed in supplemental Table 3.

The functions of residues Arg26 and Trp27 at the C terminus of the relaxin-3 B-chain were also evaluated. Changing Arg26 to Ala (R26A) or Thr (R26T) dramatically reduced relaxin-3 agonist activity but not its binding affinity for GPCR135 and GPCR142. Similar results were observed when Trp27 was replaced with Ala (W27A), Phe (W27F), or Arg (W27R). Interestingly, those changes had no effects on the relaxin-3 function for LGR7 (Fig. 2). To investigate whether the C-terminal exposure of Trp27 is necessary for relaxin-3 ligand activity for GPCR135, GPCR142, and LGR7, a mutant with an extra Ser residue was placed after Trp27 (R3(B26–27)R) 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 supplemental 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(BΔ24–27), R3(BΔ25–27), and R3(BΔ26–27) had molecular masses of 5013, 5070, and 5157.
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The affinity (Fig. 2). The exact where human LGR7 bound these peptides with comparable peptide (Fig. 2). GPCR135 and GPCR142 had moderately high GPCR135 and GPCR142 had high affinities for R3(B23–27)R but that human LGR7 showed no affinity for this peptide (Fig. 2). Similarly, human GPCR135 and GPCR142 had high affinities for R3(B23–27)R/I5, but human LGR7 showed no affinity for the peptide (Fig. 2). GPCR135 and GPCR142 had moderately high affinities for R3(B24–27), R3(B25–27), and R3(B26–27), whereas human LGR7 bound these peptides with comparable affinity (Fig. 2). The exact Ki 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, and LGR7 receptors. Because GPCR135 and GPCR142 are coupled to Gαi 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 Gαi 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(B24–27), R3(B25–27), and R3(B26–27) were high potency human LGR7 agonists, with Ki values ranging from 3 to 5 nM. R3(B23–27)R and R3(B23–27)R/I5 showed no LGR7 agonist activity (Fig. 2, Table 2).

R3(B23–27)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(B23–27)R/I5, suggesting that R3(B23–27)R/I5 is a selective antagonist for human GPCR135 over LGR7. The antagonism of human GPCR135, GPCR142, and LGR7 by R3(B23–27)R/I5 was compared using the functional reporter assay, and the results showed that R3(B23–27)R/I5 dose-dependently shifted the relaxin-3 agonism curves for GPCR135 (pA2 = 9.1, Fig. 3A) and GPCR142 (pA2 = 8.2, Fig. 3B) to the right. In contrast, R3(B23–27)R/I5 does not affect relaxin-3 agonism for LGR7 (Fig. 3C) at concentrations up to 1 μM. The pharmacology of R3(B23–27)R/I5 was also studied using recombinant rat GPCR135 and LGR7. Recombinant rat GPCR135 binds R3(B23–27)R/I5 with high affinity (Ki = 0.25 nm, Fig. 4A), but rat LGR7 lacks affinity for this peptide (Fig. 4B). In a functional reporter assay, R3(B23–27)R/I5 potently shifted the relaxin-3 agonism curve for recombinant rat GPCR135 (pA2 = 9.6, Fig. 4C) to the right but did not affect relaxin-3 agonism for recombinant rat LGR7 (Fig. 4D). R3(B23–27)R/I5 was further characterized using native GPCR135 in rat brain slices (Fig. 5). Full displacement of 125I-R3/I5 binding sites in rat brain sections by R3(B23–27)R/I5 was observed at 10 nM. The IC50 values for rat brain binding of relaxin-3 and R3(B23–27)R/I5 were 0.5 ± 0.1 and 0.4 ± 0.1 nm, respectively.

R3(B23–27)R/I5 Inhibits R3/I5-stimulated Food Intake in Satiated Rats—R3/I5 and R3(B23–27)R/I5 were tested in vivo for their abilities to modulate feeding behaviors in rats. When 10 μg of R3/I5 was administrated intracerebroventricularly to satiated Wistar rats, food intake was stimulated (n = 5–6) for both the first hour (Fig. 6A) and over 4 h (Fig. 6B) after R3/I5 administration. Intracerebroventricular administration of 10 μg of R3(B23–27)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 preexisting group differences in consumption rates, a baseline measure of food intake (i.e.
Selective Antagonist for GPCR135 and GPCR142 over LGR7

Selective Antagonist for GPCR135 and GPCR142 over LGR7

FIGURE 3. Characterization of R3(B23–27)/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 R3 were used to generate concentration response curves either in the absence or presence of 10 nM, 100 nM, or 1 μM R3(B23–27)/I5. For Gαi-linked GPCR135 and GPCR142, the assay was performed as inhibition of forskolin-induced β-gal expression. LGR7 is linked to Gαs, and therefore the addition of forskolin was not necessary. The antagonism of R3(B23–27)/I5 is indicated by the rightward shift of the relaxin-3 dose-response curve. R3(B23–27)/I5 does not affect relaxin-3 stimulation of LGR7. β-Gal expression was measured by colorimetric assay using CPRG as the substrate and reading the absorbance at 570 nm. Relaxin-3/INSL5 chimeric peptide (R3/I5) was used as agonist in comparison with R3 for all three receptors. The antagonism of R3(B23–27)/I5 to R3/I5 for GPCR135 and GPCR142 was also studied, and the results are almost identical to those when R3 was used as agonist.

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 and -2) (27, 28) and relaxin-3 share no conservation at the N termini (amino acid residues 1–6) of their B-chains, but all three peptides are potent LGR7 receptor agonists. Similarly, relaxin (1 and 2) and INSL3 (29) share no homology at the N termini (amino acid residues 1–7) of their B-chains. However all three

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 and 4 h on the base-line day (data not shown).

An analysis of variance (treatment × day) was used to determine the effect of treatment on food consumption for the four treatment conditions shown (Fig. 6). The amount of food consumed at 1 h and the total amount of food consumed during the 4-h test session after dosing were used in the analyses. There was a significant effect of Treatment at 1 h (F(3,19) = 6.103, p = 0.0044) and 4 h (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 h 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(B23–27)/I5 at 10 min prior to the R3/I5 injection; intake amounts for R3(B23–27)/I5 + R3/I5-infused animals were comparable with vehicle + vehicle-infused animals (p > 0.05).

Post hoc analyses on food intake measured at 4 h revealed a similar pattern of results as those seen at 1 h. Hence, animals infused with vehicle + R3/I5 consumed significantly more food over the 4-h test period. Furthermore, the R3/I5-induced increase in food intake was blocked by pretreatment with R3(B23–27)/I5. The total amount of food consumed by animals infused with R3(B23–27)/I5 did not differ significantly from vehicle-treated controls.

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FIGURE 4. Pharmacological characterization of R3(B23-27)/I5 as a selective antagonist for rat GPCRs 135 and 142 over rat LGR7. COS-7 cells transiently expressing recombinant rat GPCRs 135 or 142 were used to characterize the binding affinity of R3(B23-27)/I5 and LGR7 for R3(B23-27)/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 R3, R3/I5, and R3(B23-27)/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 cells transiently expressing rat LGR7 (D) were used to characterize the antagonist of R3(B23-27)/I5 for rat GPCR135 and rat LGR7. For rat GPCR135, which is Gs-coupled, the assay was performed as inhibition of forskolin-induced cAMP production using a cAMP luminescent assay kit (DiscoveryRx Hithunter). R3/I5 did not show agonist activity for rat LGR7, which is Gi-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(B23-27)/I5 (1 μM) did not demonstrate any detectable antagonist for rat LGR7.

FIGURE 5. High affinity binding of R3(B23-27)/I5 to rat brain sections. Autoradiograms are shown of 125I-R3/I5 binding sites in the rat brain (A) and rat brain slices. Nonspecific binding was determined using 1 μM unlabeled relaxin-3. Values shown are mean ± S.E. (n = 3/data points).

peptides are potent LGR8 receptor ligands. Moreover, although relaxin-3 and INSL5 share no homology at the N terminus (amino acid residues 1–6) of their B-chains, both peptides are high affinity agonists for the GPCR142 receptor. These lines of information suggest the N terminus (amino acid residues 1–6) of the relaxin-3 B-chain might not play a critical role in receptor interactions. The results from this study show that deletion of the N-terminal seven 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. Arg⁸ may play a role in relaxin-3 secretion or in maintaining the stability of the peptide. Mutants (Δ11–8 and Δ11–9) with N-terminal truncations with Arg⁸ in the deleted regions were poorly expressed in mammalian cells. Supporting this hypothesis, we also observed that relaxin-3 mutants with Arg⁸ to Ala or Ser changes had 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 Arg⁸ 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 α-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 RXXXRXXI 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 (R¹⁰LCGR¹²EFI¹⁵R¹⁶AVI¹⁹F²⁰), which contains two RXXXRXXI sequences, suggesting that Arg⁸, Arg²⁰, and Arg⁸ may play a role for ligand/receptor interaction. The homology model (Fig. 1) and the NMR solution structures (24) of relaxin-3 suggest that Arg¹², Ile¹⁵, Arg¹⁶, Ile¹⁹, and Phe²⁰ are presented on one side of the B-chain α-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 (Arg¹², and Arg¹⁶) on the relaxin-3 B-chain regarding their role in relaxin-3 binding by GPCR135, GPCR142, and
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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 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 its high affinity for both receptors, indicating that the C-terminal Arg26 and Trp27 serve mainly as the receptor activation domain for GPCR135 and GPCR142, which is obvious when the pH, pEC_{50} values of those mutants are

FIGURE 6. A, food consumption in satiated Wistar rats during the first hour of the test session (i.e. day 2) following intracerebroventricular administration (5 µl) of vehicle + vehicle, vehicle + R3/I5 (10 µg), R3(BA23-27)/I5 (10 µg) + vehicle, and R3(BA23-27)/I5 (10 µg) + R3/I5 (10 µg) (mean ± S.E.; n = 5–6 per group). B, total food consumption over 4 h in the same paradigm.
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compared side by side (Fig. 2). In addition, we made another mutant by adding an extra Ser residue after Trp27 (+28S) and found that this added Ser blocks the relaxin-3 agonist function but not receptor binding by GPCR135 or GPCR142. This suggests that 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 agonist potency and binding affinity of the peptide 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. (37) showed that up to seven amino acid residues can be removed from the C terminus of the B-chain of porcine relaxin and it would still retain full biological activity. 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, whereas 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 involved mainly 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. Mutants R3(BΔ23–27)R and R3(BΔ23–27)R/I5 have an extra Arg at the C terminus of the B-chain because of the incomplete removal of the Arg between the B-chain and C-chain junction that is designed to be removed by endogenous carboxypeptidase B. This proves to be fortuitous because in vitro pharmacology studies show that GPCR135 has a higher affinity for R3(BΔ23–27)R and R3(BΔ23–27)R/I5 than R3(BΔ24–27), R3(BΔ25–27) and R3(BΔ26–27), suggesting the extra Arg present in R3(BΔ23–27)R and R3(BΔ23–27)R/I5 plays a role in the improved affinity. The results from this current report suggest that maintaining positive charges on the B-chain is one of the essential elements for high affinity binding of relaxin-3 by GPCR135. These results suggest that the domains of GPCR135 involved in relaxin-3 binding may contain a negatively charged region.

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. Given that 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. and 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). Because 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 with 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 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 γ-aminobutyric acid 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 involvement.

To summarize, 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 1–7 residues of the relaxin-3 B-chain are not critical for receptor activation for all three receptors (Fig. 7). Arg⁸ of the relaxin-3 B-chain might play a role in relaxin-3 structural stability and might also participate in ligand/receptor binding. Arg¹⁵ and Arg¹⁶ are very important for GPCR135 and LGR7 receptor binding but play a less critical role in the interaction with GPCR142. In contrast, Phe²⁰, 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/15) that is selective with respect to LGR7 activity. Specific stimulation (with R3/I5) or inhibition (with R3(BΔ23–27)R/15) 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.

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