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## Novel Chiral diazepines function as specific, selective receptor agonists with variable coupling and species variability in human, mouse and rat BRS-3 receptor cells

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### Abstract

Bombesin receptor subtype-3(BRS-3) is an orphan G-protein coupled receptor which is classified in the bombesin receptor(BnR) family with which it shares high homology. It is present widely in the central nervous system and peripheral tissues and primarily receptor-knockout studies suggest it is involved in metabolic-glucose-insulin homeostasis, feeding and other CNS behaviors, gastrointestinal motility and cancer growth. However, the role of BRS-3 physiologically or in pathologic disorders has been not well defined because the natural ligand is unknown. Until recently, no selective agonists/antagonists were available; however, recently synthetic high-affinity agonists, chiral-diazepines nonpeptide-analogs(3F, 9D, 9F, 9G) with low CNS penetrance, were described, but are not well-categorized pharmacologically or in different laboratory species. The present study attempted to characterize(affinity, potency, selectivity) of the chiral diazepine BRS-3 agonists in human and rodents(mice, rat). In human BRS-3 receptors, the relative affinities of the chiral diazepines was 9G > 9D > 9F > 3F; each was selective for BRS-3. For stimulating PLC activity, in hBRS-3 each of the four chiral diazepine analogs was fully efficacious and their relative potencies were: 9G (EC<sub>50</sub>: 9 nM) > 9D (EC<sub>50</sub>: 9.4 nM) > 9F (EC<sub>50</sub>: 39 nM) > 3F (EC<sub>50</sub>: 48 nM). None of the four chiral diazepine analogs activated r,m,hGRPR. The nonpeptide agonists showed marked differences from each other and a peptide agonist in receptor-coupling-stoichiometry and in affinities/potencies in different species. These results demonstrate that chiral diazepine analogs (9G, 9D, 9F, 3F) have high/affinity/ptoeny for the BRS-3 receptor in human and rodent cells, but different coupling relationships and species differences from a peptide agonist.

### Keywords

BRS-3; chiral diazepine; bombesin receptor; obesity; diabetes; nonpeptide agonist

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### Conflicts of Interest

The authors have no conflicts of interest

## 1. Introduction

Bombesin receptor subtype-3 (BRS-3) is an orphan G-protein coupled orphan receptor present in the central nervous systems and peripheral tissues [10,13,22,30]. Because of its 51% and 47% amino acid homology with the two human Bombesin (Bn) receptors, gastrin-releasing peptide receptor (GRPR) and neuromedin B receptor (NMBR), it is classified in the bombesin receptor (BnR) family [10,22,43].

BRS-3 is receiving increased attention because studies, primarily using BRS-3 receptor knockout mice, suggest it is involved in insulin, glucose, lipid and metabolic homeostasis [13,17,40,52]; appetite and regulation of food intake [13,25,40], behavioral regulation [13,67,68]; gastrointestinal motility [22]; lung development and injury [55,59] and regulation of growth of normal and neoplastic tissues, as well as oncogene expression [13,24,37,57,59,65,66]. This has resulted in BRS-3 receiving considerable attention for a possible therapeutic role in treatment of obesity and/or diabetes mellitus [13,29]. However, the exact role of BRS-3 in physiological and pathologic disorders has not been well defined, because the natural ligand is unknown [22], and, in contrast to the other Bn receptors, until recently, no selective agonists/antagonists were available [13,19,22,29,43,62,64].

In contrast to other Bn receptors, BRS-3 has a low affinity for all natural occurring Bn-related peptides [13,34,48,49]. The only known high-affinity agonist for the hBRS-3 is the synthetic peptide agonist [Tyr<sup>6</sup>, β-Ala<sup>11</sup>, Phe<sup>13</sup>, Nle<sup>14</sup>]Bn-(6–14), however this Bn analog also has high affinity for GRPR and NMBR in different species [22,34,41,50,61] and has low affinity for the rat and mouse BRS-3 (r,mBRS-3) [27]. Recent studies report that the nonpeptide MK-5046 is a potent and selective agonist in different species [18,44,54] and it also has potent effects on glucose/energy homeostasis [13,17,18,54]. However, in animal and human studies, MK-5046 in addition to having the desired effects on body weight, appetite and glucose/metabolic control, had cardiovascular and body temperature/thermogenesis side effects [13,17,26,44,54]. It was proposed that these side effects could be due to a CNS action of MK-5046, because it is CNS permeable and BRS-3 receptors are widely distributed in the CNS [17,21,51,69]. To investigate this possibility further, recently, chiral diazepine nonpeptide analogs with low CNS penetrance (3F, 9D, 9F, 9G) have been reported which are potent agonists for the human BRS-3 [35,36]. However, a full pharmacological characterization of their selectivity and affinities for human and rodent Bn receptors (mouse, rat) has not been done which is needed before detailed *in vivo* studies are performed. This characterization is particularly important with Bn receptor agonists, because previously studies demonstrate this class of receptors has considerable species variations with agonists, as well as antagonists [8,22,28,61,63,64] and because until recently, similar to many other gastrointestinal hormone/neurotransmitter G-protein-coupled receptors [12,20,38], no nonpeptide agonists existed and little is known of their pharmacology at these receptors. Therefore, in the present study, we have investigated the affinity and potency of these novel chiral diazepine analogs for in human, rat and mouse BnR's.

## 2. Materials and methods

### 2.1. Materials

BALB 3T3 cells (mouse fibroblast) and AR42J cells (rat pancreatic acinar cells) were from American Type Culture Collection (ATCC), Rockville, MD; Dulbecco's minimum essential medium (DMEM), phosphate-buffered saline (PBS), G418 sulfate, fetal bovine serum (FBS), penicillin, streptomycin and sodium pyruvate from Gibco Life Technology (Grand Island, NY); bacitracin, soybean trypsin inhibitor, 3-isobutyl-1-methylxanthine (IBMX), formic acid, ammonium formate, disodium tetraborate, and alumina were obtained from Sigma-Aldrich (St. Louis, MO); iodine-125 (100 mCi/ml) and myo-[2-3H]inositol were from Perkin Elmer Life Sciences (Boston, MA); 1,2,4,6-tetrachloro-3,5-diphenylglycouril (Iodo-Gen) from Pierce Chemical Co. (Rockford, IL); AG 1-X8 resin from BIO-RAD (Richmond, CA); human brain cDNA and mouse hypothalamic and brain cDNA library were from Zyagen (San Diego, CA). Standard protected amino acids and other synthetic reagents were obtained from Bachem Bioscience Inc. (King of Prussia, PA). MK-5046, (2S)-1,1,1-trifluoro-2-[4-(1H-pyrazol-1-yl)phenyl]-3-(4-[[1-(trifluoromethyl)cyclopropyl]methyl]-1H-imidazol-2-yl)propan-2-ol[54] was a gift from Merck, Sharp and Dohme (West Point, PA.); 9f, [(5R)-4-((3-[(6-methylpyridin-3-yl)oxy]phenyl)acetyl)-8-(trifluoromethyl)-2,3,4,5-tetrahydro-1H-pyrido[2,3-e][1,4]diazepin-5-yl)acetic acid [36]; 9g, [(5R)-4-([3-(2-methylpropoxy)phenyl]acetyl)-8-(trifluoromethyl)-2,3,4,5-tetrahydro-1H-pyrido[2,3-e][1,4]diazepin-5-yl)acetic acid [36], 3F and 9D were gifts from Daiichi Sankyo Co. (Tokyo, Japan).

### 2.2. Stable transfections

hBRS-3, h,mGRPR and h,rNMBR stably transfected into BALB cells were made as described previously [2-5,48]. The rat BRS-3 receptor (rBRS-3) was obtained by PCR from a rat brain cDNA library from Zyagen (San Diego, CA) and the mouse BRS-3 receptor (mBRS-3) and NMBR (mNMBR) from a mouse hypothalamic and brain cDNA library Zyagen (San Diego, CA). The PCR product for r,mBRS-3 and mNMBR were inserted in pCMV6-Entry (OriGene, Rockville, MD) and they then subcloned into pcDNA3 at the EcoRI site. BALB cells were stably transfected with r,mBRS-3 or mNMBR as previous described [2-5,48]. Three days after transfection, cells were split in a ratio of 1:3, and the selection antibiotic G418 (Life Technologies, Grand Island, NY) was added to the regular growth medium at a concentration of 800 µg/ml. Single colonies were isolated 2 weeks later and expanded in growth medium containing G418 (300 µg/ml). Colonies containing mNMBR were selected by binding studies using <sup>125</sup>I-[D-Tyr<sup>6</sup>, β-Ala<sup>11</sup>, Phe<sup>13</sup>, Nle<sup>14</sup>]Bn-(6-14). Colonies containing mBRS-3 or rBRS-3 were selected by assessing MK-5046-stimulated charge in [<sup>3</sup>H]Inositol phosphates (IP) performed as described below.

### 2.3. Cell culture

h,r,mBRS-3 stably transfected BALB cells; h,mGRPR stably transfected BALB cells; h,r,mNMBR stably transfected BALB cells were made grown in DMEM media supplemented with 10% FBS, 100 U/ml of penicillin, 100 µg g/ml of streptomycin and 300 µg/ml of G418 sulfate. AR42J cells containing native rGRPR [31,47] were grown in DMEM

media supplemented with 10% FBS, 100 U/ml of penicillin and 100 µg/ml of streptomycin. All cells were incubated at 37°C in a 5% CO<sub>2</sub> atmosphere.

#### 2.4. Preparation of <sup>125</sup>I-Labeled Peptides

<sup>125</sup>I-[D-Tyr<sup>6</sup>, β-Ala<sup>11</sup>, Phe<sup>13</sup>, Nle<sup>14</sup>]Bn-(6–14), with specific activity of 2200 Ci/mmol, was prepared by a modification of methods described elsewhere [33,34]. In brief, 0.8 µg of IODO-GEN (in 0.02 mg/ml chloroform) was transferred to a vial, dried under a stream of nitrogen, and washed with 100 µl of 0.5 M KH<sub>2</sub>PO<sub>4</sub>, pH 7.4. To the reaction vial 20 µl of 0.5 M KH<sub>2</sub>PO<sub>4</sub>, pH 7.4, 8 µg of peptide in 4 µl of water, and 2 mCi (20 µl) Na<sup>125</sup>I were added, mixed gently, and incubated at room temperature for 6 minutes. The incubation was stopped by the addition of 100 µl of distilled water. Radiolabeled peptide was separated using a Sep-Pak (Waters Associates, Milford, MA) and high-performance liquid chromatography as described previously elsewhere [34,50]. The radioligand was stored with 0.5% BSA at 20°C.

#### 2.5. Binding studies

Binding studies with mBRS-3 and rBRS-3 could not be performed because they have low affinity for the universal Bn ligand <sup>125</sup>I-[D-Tyr<sup>6</sup>, β-Ala<sup>11</sup>, Phe<sup>13</sup>, Nle<sup>14</sup>]Bn-(6–14), and no other suitable ligand is available [22,27]. hBRS-3/BALB (0.5 x 10<sup>6</sup> cells/ml), hGRPR/BALB (0.5 x 10<sup>6</sup> cells/ml), rGRPR/AR42J (1 x 10<sup>6</sup> cells/ml), mGRPR/BALB (0.5 x 10<sup>6</sup> cells/ml), hNMBR/BALB (0.05 x 10<sup>6</sup> cells/ml), rNMBR/BALB (0.2 x 10<sup>6</sup> cells/ml) and mNMBR/BALB (0.8 x 10<sup>6</sup> cells/ml) cells were incubated for 60 minutes at 21°C with 50 pM <sup>125</sup>I-[D-Tyr<sup>6</sup>, β-Ala<sup>11</sup>, Phe<sup>13</sup>, Nle<sup>14</sup>]Bn-(6–14) in 300 µl of binding buffer as described previously elsewhere [14,34,38,61]. The standard binding buffer contained 24.5 mM HEPES, pH 7.4, 98 mM NaCl, 6 mM KCl, 2.5 mM KH<sub>2</sub>PO<sub>4</sub>, 5 mM sodium pyruvate, 5 mM sodium fumarate, 5 mM sodium glutamate, 2 mM glutamine, 11.5 mM glucose, 0.5 mM CaCl<sub>2</sub>, 1.0 mM MgCl<sub>2</sub>, 0.01% (w/v) soybean trypsin inhibitor, 0.2% (v/v) amino acid mixture, 0.2% (w/v) BSA, and 0.05% (w/v) bacitracin. After the incubation, 100 µl of each sample were removed and added to 300 µl of incubation buffer in 400-µl Microfuge tubes and centrifuged for 1 minute at 10,000g (Microfuge B; Beckman Coulter, Fullerton, CA) to separate the bound radioligand from unbound radioligand. The supernatant was aspirated, and the pelleted cells were rinsed twice with a washing buffer that contained 1% (w/v) BSA in PBS. The amount of radioactivity bound to the cells was measured in a Cobra II Gamma Counter (Packard Instruments, Meriden, CT). Binding was expressed as the percentage of the total radioactivity that was associated with the cell pellet. Nonsaturable binding was <10% of the total binding in all experiments. Each point was measured in duplicate, and each experiment was replicated at least 3 times. Calculation of affinity was performed by determining the IC<sub>50</sub> using the curve-fitting program Prism GraphPad 4.0 (GraphPad Software, Inc., La Jolla, CA).

#### 2.6. Measurement of [<sup>3</sup>H]Inositol Phosphates

[<sup>3</sup>H]Inositol phosphates (IP) were determined in the different cells as described previously [2,38,46,61]. In brief, h,r,mBRS-3 cells, h,mGRPR/BALB cells, AR42J cells containing native rGRPR and h,r,mNMBR/BALB cells (4.0 x 10<sup>4</sup> cells/ml) were subcultured into 24-well plates in regular propagation media and then were incubated for 24 hours at 37°C in a 5% CO<sub>2</sub> atmosphere. The cells were then incubated with 3 µCi/ml of myo-[2-<sup>3</sup>H]inositol in

growth media supplemented with 2% FBS for an additional 24 hours. After the incubation, the 24-well plates were washed by incubating for 30 minutes at 37°C with 1 ml/well of PBS (pH 7.0) containing 20 mM lithium chloride. The wash buffer was aspirated and replaced with 500 µl of IP assay buffer containing 135 mM sodium chloride, 20 mM HEPES (pH 7.4), 2 mM calcium chloride, 1.2 mM magnesium sulfate, 1 mM EGTA, 20 mM lithium chloride, 11.1 mM glucose, and 0.05% BSA (w/v), and was incubated without (control) or with different concentrations of the peptides studied. After 60 minutes of incubation at 37°C, the experiments were terminated by the addition of 1 ml of ice-cold 1% (v/v) hydrochloric acid in methanol. The total [<sup>3</sup>H]IP was isolated by anion-exchange chromatography as described previously [2,38,46]. Briefly, samples were loaded onto Dowex AG1-X8 anion-exchange resin columns, washed with 5 ml of distilled water to remove free [<sup>3</sup>H]IP and then washed with 2 ml of 5 mM disodium tetraborate/60 mM sodium formate solution to remove [<sup>3</sup>H]glycerophosphorylinositol. Finally, 2 ml of 1 mM ammonium formate/100 mM formic acid solution were added to the columns to elute the total [<sup>3</sup>H]IP. Each eluate was mixed with scintillation cocktail and measured for radioactivity in a scintillation counter.

## 2.7. Statistical Analysis

The results are the mean and S.E.M. from at least three separate experiments. IC<sub>50</sub> and EC<sub>50</sub> were calculated using the curve-fitting program Prism GraphPad 4.0.

## 3. Results

In this study, we compared the ability of four new chiral diazepamines [35,36] with that of the naturally Bn-related peptides GRP and NMB, the universal Bn-receptor agonist peptide #1 [34,41] and the nonpeptide BRS-3 agonist MK-5046 [13,38,54] to interact and activate the three Bn-receptor subtypes –GRPR, NMBR and BRS-3– in human, rat and mouse.

In hBRS-3/BALB cells, the affinity of chiral diazepamine 9G was 1.5-fold greater than MK-5046 (9G; IC<sub>50</sub>: 70 nM and MK-5046; IC<sub>50</sub>: 102 nM, Table 1, Fig. 1). The remaining three chiral diazepamines had a 1.7-13-fold lower affinity compared with 9G (chiral diazepamines 9D, 9F and 3F; IC<sub>50</sub>: 121–909 nM, Table 1, Fig. 1). As described previously [13,32,34,61] for hBRS-3, GRP and NMB had very low affinity (IC<sub>50</sub>: >3000 nM, Table 1, Fig. 1); peptide #1 had a high affinity (IC<sub>50</sub>: 2.89 nM, Table 1, Fig. 1) and it was 24-fold higher than 9G (IC<sub>50</sub>: 70 nM, Table 1, Fig. 1). None of the four chiral diazepamines interact with hGRPR or hNMBR at concentration up to 3,000 nM (Table 1, Fig. 2–3). In contrast, GRP and peptide #1 or NMB and peptide #1 interacted with high affinity with GRPR or NMBR, respectively (GRPR; GRP; IC<sub>50</sub>: 0.48 nM; peptide #1; IC<sub>50</sub>: 0.71 nM, Table 1, Fig. 2A and NMBR; NMB; IC<sub>50</sub>: 0.60 nM; peptide #1; IC<sub>50</sub>: 0.21 nM, Table 1, Fig. 3A). Because of a lack of a radioligand for rat and mouse BRS-3, the affinities of various ligands the could not be assessed by binding studies, however their potency for cell activation could be assessed.

In binding studies with rGRPR/AR42J cells and rNMBR/BALB cells, the four chiral diazepamines and MK-5046 did not interact with rGRPR or rNMBR, even at concentrations of 3,000 nM (Table 1, Fig. 2B, 3B). However, peptide #1 showed the highest affinity for rGRPR or rNMBR (IC<sub>50</sub>: 0.03–0.53 nM, Table 1, Fig. 2B, 3B). GRP showed a high affinity for rGRPR (IC<sub>50</sub>: 0.41 nM, Table 1, Fig. 2B) and it was >1,000-fold greater than GRP for

rNMBR (IC<sub>50</sub>: 440 nM, Table 1, Fig. 3B). Similarly, NMB had a high affinity for rNMBR (IC<sub>50</sub>: 1.21 nM, Table 1, Fig. 3B) and 66-fold lower in affinity for rGRPR (IC<sub>50</sub>: 80 nM, Table 1, Fig. 2B).

In binding studies with mGRPR/BALB and mNMBR/BALB cells, the four chiral diazepamines and MK-5046 did not interact with mGRPR or mNMBR at concentrations up to 3,000 nM (Table 1, Fig. 2C, 3C). In contrast, peptide #1 had a high affinity for mGRPR and mNMBR (IC<sub>50</sub>: 0.42 and 5.76 nM, Table 1, Fig. 2C, 3C); and GRP and NMB showed a high affinity for mGRPR and mNMBR, respectively (IC<sub>50</sub>: 0.60 and 5.76 nM, Table 1, Fig. 2C, 3C). GRP had a low affinity for mNMBR and NMB had low affinity for mGRPR (IC<sub>50</sub>: 206 and 170 nM, Table 1, Fig. 2C, 3C).

To assess the potencies of the various BRS-3 selective agonists, we utilized the fact that each of the Bn-receptor subtypes is coupled to PLC activation [15,22,43], and assessed their abilities to stimulate [<sup>3</sup>H]IP generation in each of the BRS-3, GRPR, and NMBR containing cells. We compared the results with the natural ligands (GRP, NMB), the universal Bn-receptor agonist peptide #1, the nonpeptide BRS-3 agonist MK-5046 and the four chiral diazepamines (3F, 9D, 9F, 9G) (Fig. 4–6).

In human, rat and mouse BRS-3 containing cells, each of the four chiral diazepamines, as well as MK-5046, were full efficacious for activating PLC causing a 3.8-, 5.8- and 3.9-fold increase, respectively (Table 2, Fig. 4). In human BRS-3/BALB cells, of the four chiral diazepamines, 9G and 9D were the most potent (IC<sub>50</sub>: 9.2 and 9.4 nM, Table 2, Fig. 4A); which was equal in potency to MK-5046 (EC<sub>50</sub>: 8.70 nM, Table 2, Fig. 4A); with 9F and 3F having, respectively a 4.3- and 5.2-fold lower potency than 9G or 9D (3F; EC<sub>50</sub>: 47.7 nM, and 9F; EC<sub>50</sub>: 39.6 nM, Table 2, Fig. 4A). For rat and mouse BRS-3, the relative and absolute potencies for the chiral agonists, MK-5046 and peptide #1 differed from that seen in hBRS-3/BALB cells. Specifically, in rBRS-3/BALB cells, the chiral diazepamines 9G and 3F were equally potent (3F; EC<sub>50</sub>: 34.6 nM and 9G; EC<sub>50</sub>: 37.9 nM, Table 2, Fig. 4B), but were 165-fold less potent than MK-5046 (EC<sub>50</sub>: 0.23 nM, Table 2, Fig. 4B); whereas 9D and 9F had 2- and 7.8-fold lower potency for rBRS-3/BALB (9D; EC<sub>50</sub>: 70.4 nM and 9F; EC<sub>50</sub>: 295 nM, Table 2, Fig. 4B). In mBRS-3/BALB cells, of the four chiral agonists, 9D and 9G were the most potent (9D; EC<sub>50</sub>: 18.4 nM and 9G; EC<sub>50</sub>: 20.1 nM, Table 2, Fig. 4C), but were 3.5–3.8-fold less potent than MK-5046 (EC<sub>50</sub>: 5.24 nM, Table 2, Fig. 4C). In contrast, the chiral diazepamines 3F and 9F, compared to 9D or 9G, showed a 1.5- and 4.3-fold lower for mBRS-3/BALB cells (3F; EC<sub>50</sub>: 31 nM and 9F; EC<sub>50</sub>: 86.8 nM, Table 2, Fig. 4C). In hBRS-3/BALB cells, GRP or NMB, and in r,mBRS-3/BALB cells, GRP or NMB, did not interact with BRS-3 at concentrations up to 3,000 nM (Table 2, Fig. 4A–C). Similarly, peptide #1 did not activate r,mBRS-3 at concentrations up to 3,000 nM (Table 2, Fig. 4B, 4C).

In h,mGRPR/BALB cells, rGRPR/AR42J cells and h,r,mNMBR/BALB cells, none of the four chiral diazepamines or MK-5046 activated GRPR or NMBR at concentrations up to 3,000 nM (Table 2, Fig. 5A–C, 6A–C). However, peptide #1 had a high potency for activating GRPR and NMBR in human, rat or mouse (EC<sub>50</sub>: 0.12–0.58 nM, Table 2, Fig. 5A–C, 6A–C); which was 8.4-, 0.3- and 0.2-fold greater than GRP, respectively (EC<sub>50</sub>: 4.79, 0.30 and 1



nM, Table 2, Fig. 5A–C, 6A–C). Similarly, peptide #1 showed a 3.4-, 3.5- and 5.2-fold greater potency than NMB for human, rat or mouse NMBR ( $EC_{50}$ : 2.5, 1.6, 4.7 nM, Table 2, Fig. 5A–C, 6A–6C). In contrast, peptide #1 had a >250-fold higher potency than GRP for h,r,mNMBR ( $EC_{50}$ : 1,300, 125 and 499 nM, Table 2, Fig. 5A–C, 6A–C) and 40-930-fold greater potency than NMB for h,r,mGRPR ( $EC_{50}$ : 530, 17 and 23 nM, Table 2, Fig. 5A–C, 6A–C).

Comparison of affinities for occupying the hBRS-3 receptor determined from binding studies ( $IC_{50}$ ) and its potencies for activating hBRS-3 ( $EC_{50}$ ) in [ $^3H$ ]IP demonstrated the different chiral diazepamines, peptide #1 and MK-5046 had markedly different degrees of receptor sparseness. Specifically, for the four chiral agonists, 9G had the lowest  $IC_{50}/EC_{50}$  ratio of 7.6; whereas with 9D, 9F, 3F and the  $IC_{50}/EC_{50}$  ratios were 12.5, 12.8 and 19, respectively. With MK-5046 the  $IC_{50}/EC_{50}$  ratio was 11.7, whereas with peptide #1 the  $IC_{50}/EC_{50}$  ratio was 0.74, demonstrating no receptor sparseness (Table 3).

#### 4. Discussion

The propose of this study was to assess the abilities of four new nonpeptide chiral diazepamines with low CNS penetrance [35,36] to interact with and activate Bn receptors. This study was performed because of the increasing interest in BRS-3 both in physiological and pathological conditions, as well as in it therapeutic potential in diseases such as obesity/diabetes [13,17,18,29,30,36,40,43,44]. Until recently, little was known of the role of BRS-3 in physiological, or pathological conditions, because it is an orphan receptor with an unknown ligand, ever though it is included in the Bn receptor family, because of its high homology to other receptors in this family [10,13,22]. The report that BRS-3 knockout animals develop, hypertension, diabetes and obesity, resulted in increased interest in this receptor [13,40]. This resulted in different pharmacological companies developing, nonpeptide agonists and antagonists [11,13,17,18,35,36,38,44,54], aimed at exploring BRS-3's role in physiology/pathophysiology and possible role in treatment of obesity/diabetes [13,17,23,29,30,44]. Initial human and animal studies with the benzodiazepine analogue, MK-5046, showed promise with weight loss, stimulation of insulin release and correction of hyperglycemia/diabetes [13,18,54]. However, an area of prominent concern was that MK-5046, caused cardiovascular side-effects in both human and mice [18,26,44], which could possibly be due to a CNS effect of MK-5046, because it is a CNS penetrance compound and BRS-3 receptors are widely present in the CNS [13,17,69]. To circumvent this possibility, peripherally acting BRS-3 agonists which are nonpeptide chiral diazepamine analogs were recently reported [35,36]. However, little is known of the pharmacology of these new nonpeptide chiral diazepamines, including their affinities for the different human BnRs, their receptor selectivity, specifically in comparison to the widely used BRS-3 agonist MK-5046, and their potency and efficacy for activating the various BnR's. Because these compounds will be widely used to investigate the physiological/pathological role of BRS-3 and because they are the first peripheral-acting BRS-3 potentially, selective agonists described, the assessment of their pharmacology is important. This is particularly important for BnRs because numerous studies have reported with this class of receptors that various peptide agonists and antagonists show marked species difference in affinity, potency and efficacy, such that in different species a given analogue can function as an antagonist,

agonist or partial agonist [14,22,28,61,63,64]. Furthermore, little information exists for nonpeptide agonists of Bn receptors, similar to case for many other G-protein-coupled receptors (GPCRs) for gastrointestinal (GI) hormones/neurotransmitters. This has occurred because for many GPCR for GI hormones/neurotransmitters such as those for VIP, secretin, substance P, PACAP or gastrin, as well as for the BnR receptors, NMBR and GRPR, no nonpeptide agonists have been described. With other GI hormone/neurotransmitter GPCRs receptors such as for CCK, BRS-3, neurotensin and motilin, only recently have nonpeptide agonists been described [6,58,60], with the result that their pharmacology is largely unknown, including whether they also demonstrated species variability for affinity, potency and efficacy.

Furthermore, the pharmacology, as well as the ability of given Bn receptor ligand to function as agonist/antagonist, varies markedly in different species. Because of this, in the present study a comparison for the ability of the different chiral diazepamines to activate BnRs of mice, rat and human was performed.

A new number of our results support the conclusion that each of four new chiral diazepamines and nonpeptide MK-5046 are highly selective and fully efficacious agonists for the BRS-3 receptor in human, rat and mouse cells. First, in hBRS-3 containing cells, the two chiral diazepamines (9D and 9G) had a similar affinity for the hBRS-3 receptor (69.69–121.1 nM) to that seen with MK-5046 (102 nM). Second, each of the four new chiral diazepamines were selective for hBRS-3 and did not bind to either GRPR or NMBR in human, rat or mouse at concentrations as high as 3,000 nM. In contrast, the agonist, peptide #1, as was reported previously [32–34,41,61], showed a high affinity for all human, rat or mouse Bn receptors ( $IC_{50}$ : 0.03–5.76 nM), except rat, mouse BRS-3. Third, our results demonstrated that the new four chiral diazepamines are fully efficacious BRS-3 agonists for activating phospholipase C in cells containing BRS-3 from human, rat or mouse; that they were selective BRS-3 in each of these cells, not activating activate GRPR or NMBR in human, rat or mouse cells, even at concentrations up to 3,000 nM.

Previous studies report wide variation in the affinity of peptide agonists and peptide antagonists for GRPR and NMBR in different species [8,9,14,22,28,34,61,63,64], as well as their potency/efficiency for activating GRPR or NMBR and functioning as an agonist, partial agonist or antagonist in different species [8,9,22,63,64]. Much less information is available for the BRS-3 receptor because the endogenous ligand is unknown [13,30]. Furthermore, only recently has nonpeptide agonists been described for BRS-3, similar to numerous other gastrointestinal hormone/neurotransmitter GPCRs [12], so there is very little information of whether this variability in potency of a given receptor in different species extends also to a nonpeptide agonist, and exists for BRS-3. This study for the first time demonstrates that the nonpeptide BRS-3 receptor agonists examined in the present study (MK-5046, 3F, 9D, 9F and 9G) demonstrate wide variation in their potencies for receptor activation between human, rat and mouse BRS-3, but not in their efficacy for activating the receptor and stimulating phospholipase C activation. This conclusion is supported by a number of findings. First, MK-5046 in rBRS-3 cell was 22-fold and 38-fold more potent than in mBRS-3 or hBRS-3, whereas the chiral diazepamine 3F was approximately equally potent in the BRS-3 receptors of all these species and the related chiral diazepamines 9G, 9F were more



potent in hBRS-3 over r,mBRS-3. Second, the relative potencies of the different nonpeptide agonists varied markedly between the different species. For example, MK-5046, 9D and 9G were approximately equally potent at activating hBRS-3; whereas for rBRS-3, 9F and 9G were 8-fold and 165-fold less potent than MK-5046 and with mouse BRS-3, and they were 5-fold and 4-fold less potent. Third, in h,r,mBRS-3 cells, each of the the nonpeptide demonstrated similar efficacy in activating phospholipase C, when sufficiently high correlations were used, demonstrate none were partial agonists. These results are consistent with studies of nonpeptide agonists for serotonin 2A [7], histamine (H<sub>1</sub>, H<sub>2</sub>, H<sub>3</sub>, H<sub>4</sub>) [56], endothelium [45] and Kinin-B2 receptors, which are report to demonstrate species variation in their affinities/potencies. These results suggested that if physiological/pathological functions of BRS-3 are explored in different laboratory animals, it will be important to consider potency of the selected agonists in the studied species.

Recently with peptide and nonpeptide agonists as well as antagonists, marked different in receptor coupling efficiency for various G-protein coupled receptors have been reported [1,6,16,38,39,42,53,61]. Until recently, no nonpeptide agonists existed for BnRs, so that it was unclear whether a similar relationship frequently occurred with these receptors for nonpeptide agonists. Our results support the conclusion that it frequently occurs with BRS-3 receptors in human cells. This conclusion was supported by the fact that with the peptide agonist (peptide #1), the IC<sub>50</sub>/ EC<sub>50</sub> ratio, a measure of receptor sparseness, was almost unity, showing no receptor sparseness. In contrast, with the nonpeptide BRS-3 agonists the coupling ratio varied from 7.61 to 19.06 demonstrating receptor sparseness in each case, as well as demonstrating this variation occurred even with closely chemically related agonists such as the four chiral diazepines (IC<sub>50</sub>/ EC<sub>50</sub>: 7.61–19.06). These results demonstrated the receptor coupling efficiency both of peptide agonists and nonpeptide agonists can vary considerably for a given receptor of the BnR family. This result is, in contrast, to the findings recently reported with a nonpeptide agonist's ability to activate and occupy CCK [53], vasopressin V2 [39], or bradykinin B2R [1] receptors. With each of these receptors there was no receptor sparseness for the nonpeptide agonist and it resembles the ability of a peptide agonist in its receptor occupation-coupling stoichiometry. This marked variation in receptor coupling activation is important to consider in the use of the BRS-3 compounds, because it can lead to different conclusions on their selectivity for a given BnR [38].

## Acknowledgments

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## Abbreviations

<b>AR42J cells</b>	rat pancreatic acinar cells
<b>BALB 3T3</b>	mouse embryonic fibroblast cells
<b>Bn</b>	Bombesin
<b>BnR</b>	Bombesin receptors
<b>BRS-3</b>	Bombesin receptor subtype-3

<b>BSA</b>	bovine serum albumin fraction V; chiral diazepam analogs (3F, 9G, 9F, 9G)
<b>CNS</b>	central nervous system
<b>DMEM</b>	Dulbecco's minimum essential medium
<b>h</b>	human
<b>EC<sub>50</sub></b>	concentration causing half-stimulation
<b>FBS</b>	fetal bovine serum
<b>GPCR</b>	G-protein-coupled receptor
<b>GRP</b>	gastrin-releasing peptide
<b>GRPR</b>	gastrin-releasing peptide receptor
<b>IC<sub>50</sub></b>	half maximal inhibitor concentration
<b>IBMX</b>	3-isobutyl-1-methylxanthine
<b>IP</b>	inositol phosphate
<b>m</b>	mouse
<b>MK-5046</b>	nonpeptide BRS3 agonist
<b>NMB</b>	neuromedin B
<b>NMBR</b>	neuromedin B receptor
<b>peptide #1</b>	[D-Tyr <sup>6</sup> , β-Ala <sup>11</sup> , Phe <sup>13</sup> , Nle <sup>14</sup> ]Bn-(6–14)
<b>PBS</b>	phosphate buffered saline
<b>PLC</b>	phospholipase C
<b>r</b>	rat

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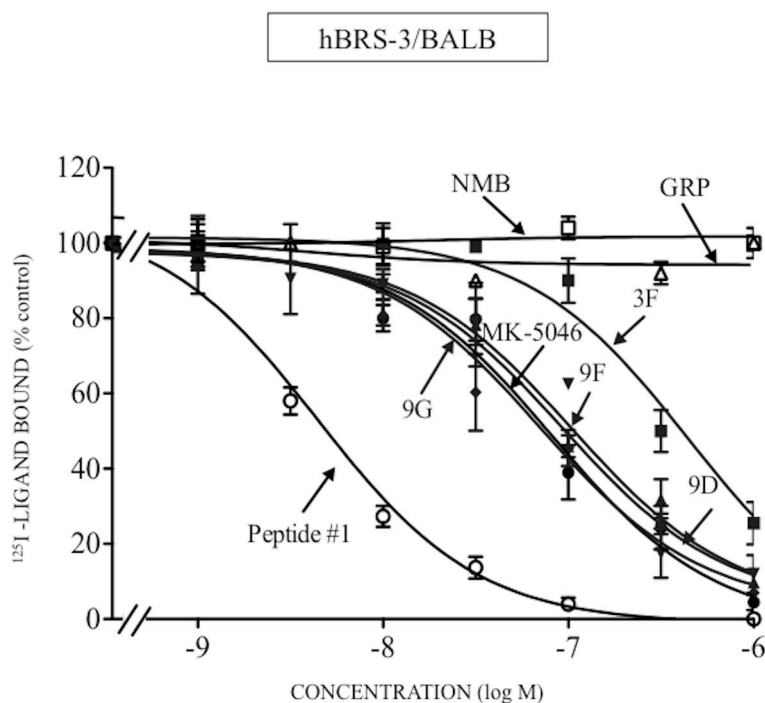
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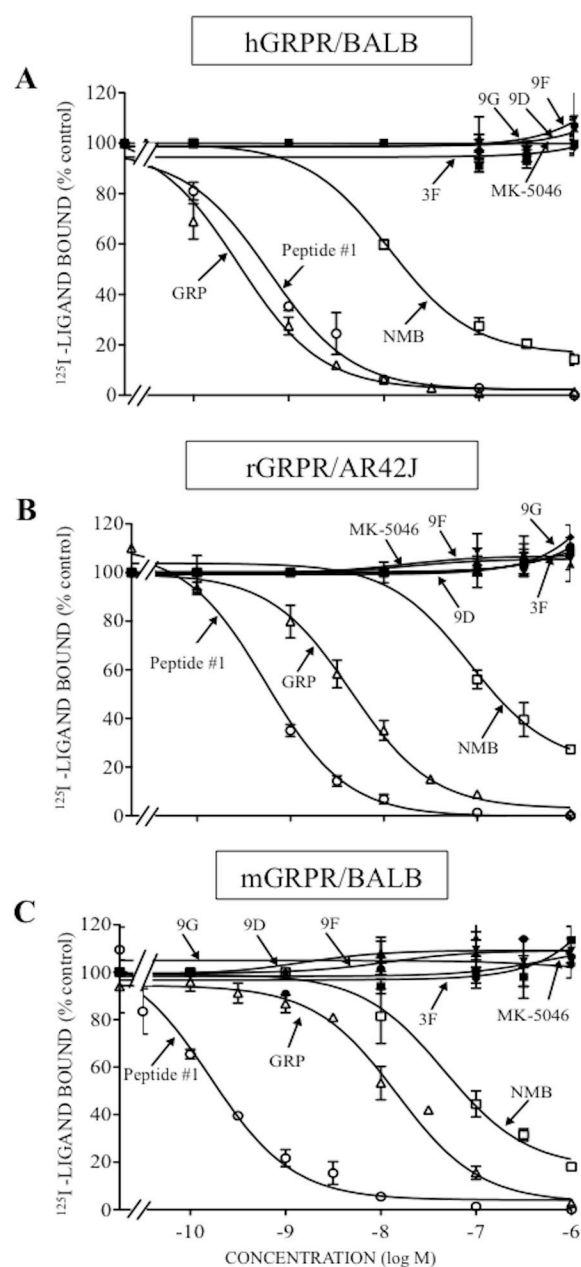


### Highlights

- Recently reported nonpeptide-chiral-diazepine BRS-3 agonists are characterized pharmacologically.
- The chiral-dizapeines have high affinity and selectivity for human, rat, mouse hBRS-3.
- Each analog has high potency, selectivity and functions as full agonist at human, rat, mouse BRS-3.
- Compared to peptide agonists, the chiral diazepines had variable receptor spareness in human BRS-3
- The chiral diazepines nonpeptides analogs had variable potencies in BRS-3 from different species



**Figure 1.** Comparison of the ability of four chiral diazepine analogs (3F, 9D, 9F, 9G); a nonselective Bn analog agonist (Peptide #1); a nonpeptide agonist MK-5046; GRP and NMB to inhibit binding to cells containing human BRS-3. The peptides were incubated with 50 pM  $^{125}\text{I}$ -[D-Tyr<sup>6</sup>,  $\beta$ -Ala<sup>11</sup>, Phe<sup>13</sup>, Nle<sup>14</sup>]Bn-(6–14) for 60 minutes at 21°C in 300  $\mu\text{l}$  of binding buffer with hBRS-3/BALB cells ( $0.5 \times 10^6$  cells/ml), and the saturable binding was determined as described in Materials and Methods. The results are expressed as the percentage of saturable binding without unlabeled peptide added (percentage control). The results are the mean and S.E.M. from at least three separate experiments and in each experiment the data points were determined in duplicated. Abbreviations: See in table 1 legend.



**Figure 2.**

Comparison of the ability of four chiral diazepine analogs (3F, 9D, 9F, 9G); a nonselective Bn analog agonist (Peptide #1); a nonpeptide agonist MK-5046; GRP and NMB to inhibit binding to cells containing human, rat or mouse GRPR. The peptides were incubated with 50 pM  $^{125}\text{I}$ -[D-Tyr<sup>6</sup>,  $\beta$ -Ala<sup>11</sup>, Phe<sup>13</sup>, Nle<sup>14</sup>]Bn-(6-14) for 60 minutes at 21°C in 300  $\mu\text{l}$  of binding buffer with hGRPR/BALB cells ( $0.5 \times 10^6$  cells/ml) (A), rGRPR/AR42J cells ( $1 \times 10^6$  cells/ml) (B) or mGRPR/BALB cells ( $0.5 \times 10^6$  cells/ml) (C), and the saturable binding was determined as described under Materials and Methods. The results are expressed as the percentage of saturable binding without unlabeled peptide added (percentage control). The results are the mean and S.E.M. from at least three separate experiments and in each

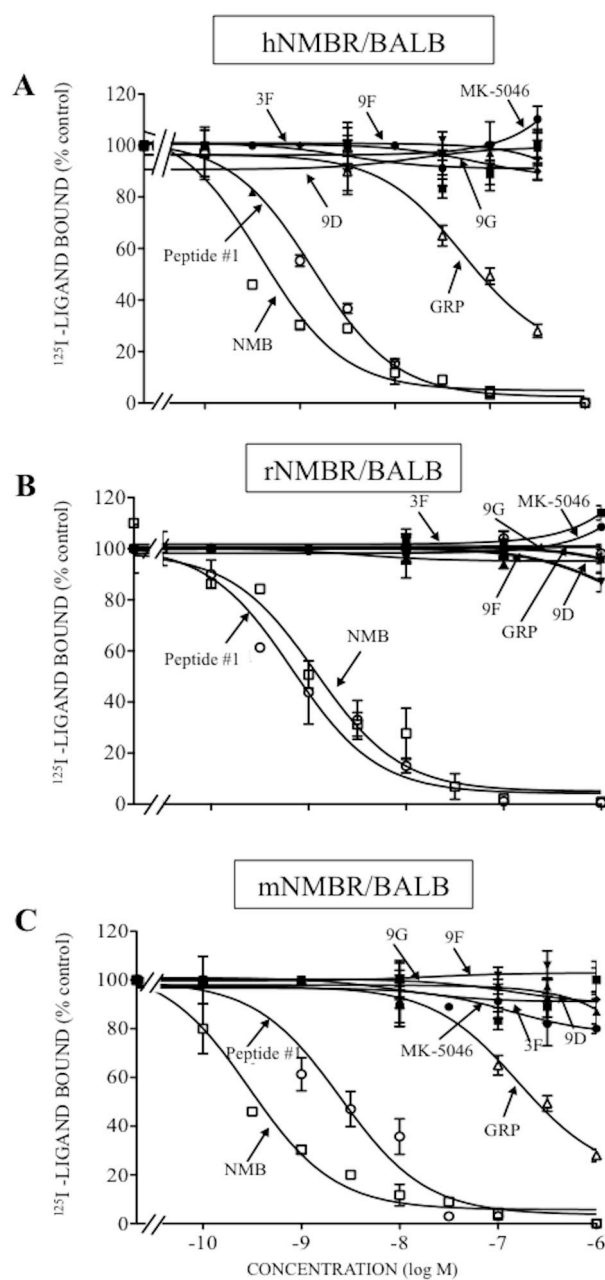
experiment the data points were determined in duplicated. Abbreviations: See in table 1 legend.

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**Figure 3.**

Comparison of the ability of four chiral diazepine analogs (3F, 9D, 9F, 9G); a nonselective Bn analog agonist (Peptide #1); a nonpeptide agonist MK-5046; GRP and NMB to inhibit binding of  $^{125}\text{I}$ -labeled peptide in cells containing human, rat or mouse NMBR. The peptides were incubated with 50 pM  $^{125}\text{I}$ -[D-Tyr<sup>6</sup>,  $\beta$ -Ala<sup>11</sup>, Phe<sup>13</sup>, Nle<sup>14</sup>]Bn-(6–14) for 60 minutes at 21°C in 300  $\mu\text{l}$  of binding buffer with hNMBR/BALB cells ( $0.05 \times 10^6$  cells/ml) (A), rNMBR/BALB cells ( $0.2 \times 10^6$  cells/ml) (B) or mNMBR/BALB cells ( $0.8 \times 10^6$  cells/ml) (C), and the saturable binding was determined as described under Materials and Methods. The results are expressed as the percentage of saturable binding without unlabeled peptide added (percentage control). The results are the mean and S.E.M. from at least three

separate experiments and in each experiment the data points were determined in duplicated.  
Abbreviations: See in table 1 legend.

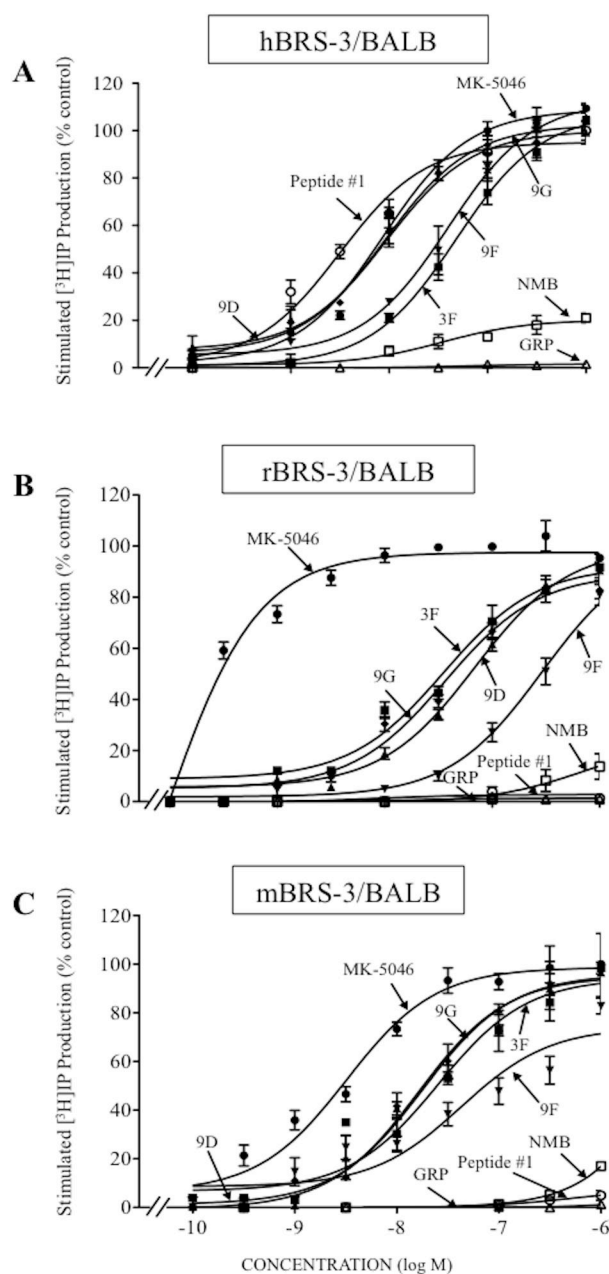
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**Figure 4.**

Ability of four chiral diazepine analogs (3F, 9D, 9F, 9G); a nonselective Bn analog agonist (Peptide #1); a nonpeptide agonist MK-5046; GRP and NMB to stimulate  $[^3\text{H}]$ IP generation in cells containing human, rat or mouse BRS-3: (A) hBRS-3/BALB, (B) rBRS-3/BALB and (C) mBRS-3/BALB cells.  $[^3\text{H}]$ IP was determined after a 60 minutes incubation at  $37^\circ\text{C}$  after loading the cells for 48 hours with  $3\ \mu\text{Ci/ml}$  myo- $[2\text{-}^3\text{H}]$ inositol as described in Materials and Methods. The results are the mean and S.E.M. from at least three separate experiments and in each experiment the data points were determined in duplicated. With hBRS-3/BALB cells, the maximal stimulated  $[^3\text{H}]$ IP value by  $1\ \mu\text{M}$  MK-5046 was  $70,268 \pm 4,912$  dpm, and the control value was  $18,251 \pm 1,632$  dpm ( $n=13$ ). With rBRS-3/BALB cells, the maximal

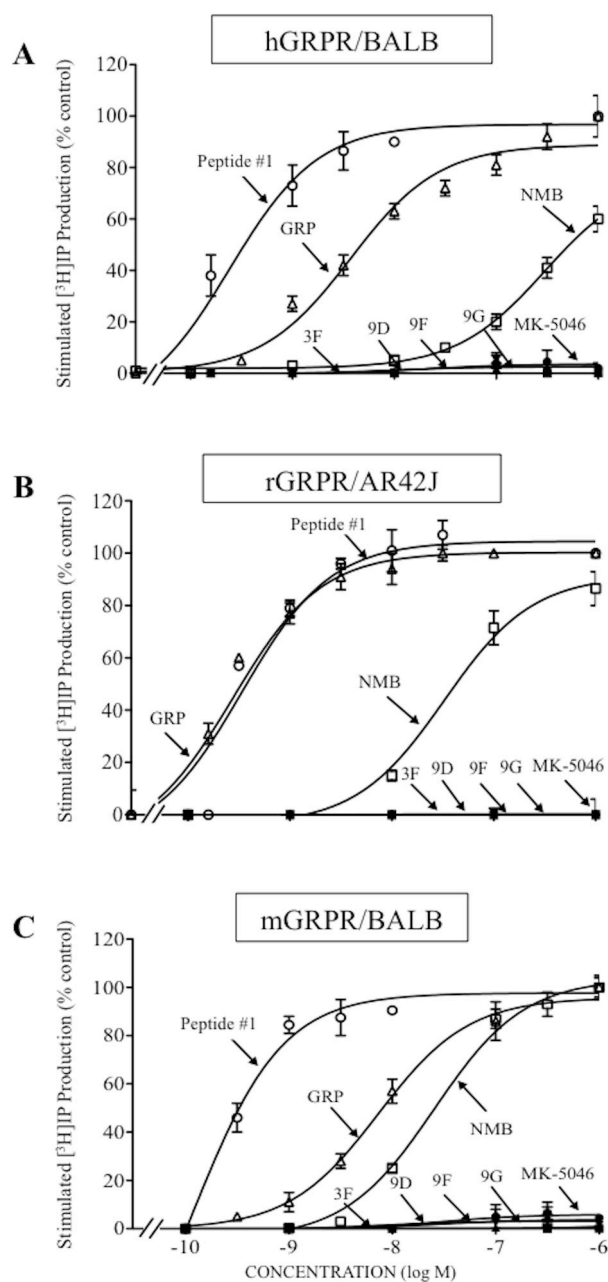
stimulation of a 1  $\mu\text{M}$  concentration of MK-5046 was  $21,812 \pm 1,721$  dpm, and the control value was  $3,749 \pm 315$  dpm (n=7). With mBRS-3/BALB cells, the maximal stimulation of a 1  $\mu\text{M}$  concentration of MK-5046 was  $62,992 \pm 9,298$  dpm, and the control value was  $16,160 \pm 2,396$  dpm (n=3). Abbreviations: See in table 1 and 2 legends.

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**Figure 5.**

Comparison of abilities of four chiral diazepine analogs (3F, 9D, 9F, 9G); a nonselective Bn analog agonist (Peptide #1); a nonpeptide agonist MK-5046; GRP and NMB to stimulate  $[^3\text{H}]$ IP generation in cells containing human, rat or mouse GRPR: (A) hGRPR/BALB, (B) rGRPR/AR42J and (C) mGRPR/BALB cells. The experimental conditions were the same as described in Fig. 4 legend. The results are the mean and S.E.M. from at least three experiments, and in each experiment the data points were determined in duplicate. The results are expressed as the percentage of stimulation causing by maximal effective concentration of peptide #1 ( $1\ \mu\text{M}$ ). The control and the maximal stimulated  $[^3\text{H}]$ IP with peptide #1 ( $1\ \mu\text{M}$ ): hGRPR/BALB cells,  $2,567 \pm 211$  and  $20,974 \pm 2,520$  dpm, respectively

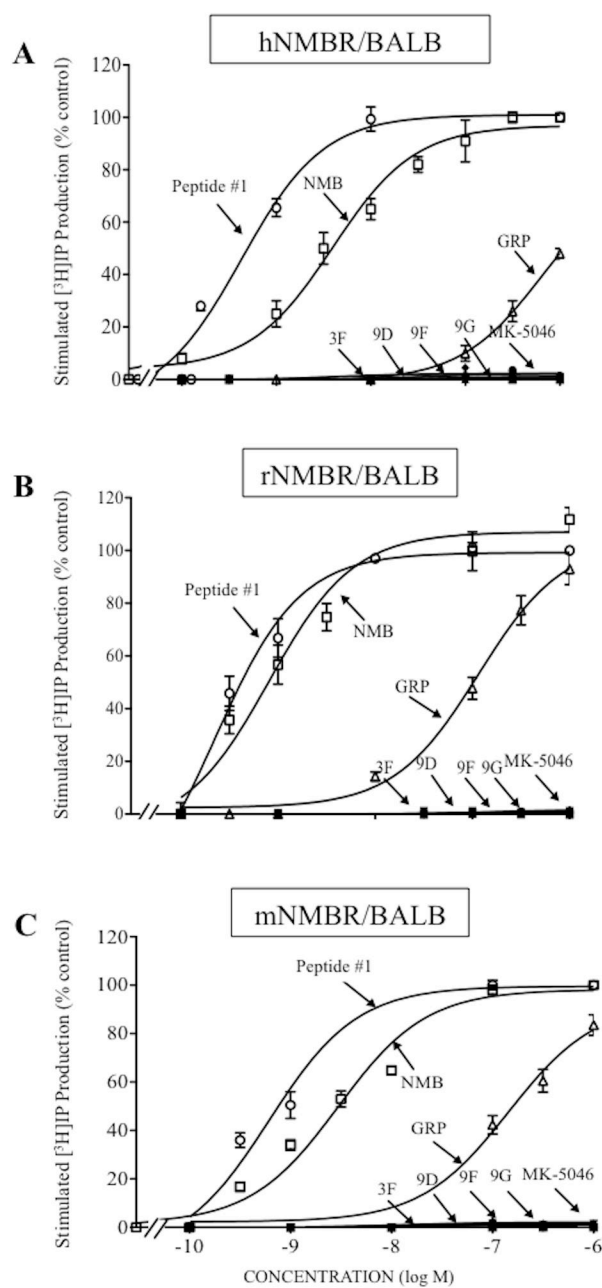
(n=6). rGRPR/AR42J cells,  $577 \pm 6$  and  $9,887 \pm 465$  dpm, respectively (n=3). mGRPR/BALB cells,  $1,490 \pm 113$  and  $12,527 \pm 2087$  dpm, respectively (n=7). Abbreviations: See in table 1 and 2 legends.

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**Figure 6.**

Comparison of abilities of four chiral diazepine analogs (3F, 9D, 9F, 9G); a nonselective Bn analog agonist (Peptide #1); a nonpeptide agonist MK-5046; GRP and NMB to stimulate [<sup>3</sup>H]IP generation in cells containing human, rat or mouse NMBR: (A) hNMBR/BALB, (B) rNMBR/BALB and (C) mNMBR/BALB cells. The experimental conditions were the same as described in Fig. 4 legend. The results are the mean and S.E.M. from at least three experiments, and in each experiment the data points were determined in duplicate. The results are expressed as the percentage of stimulation causing by maximal effective concentration of peptide #1 (1  $\mu$ M). The control and the maximal stimulated [<sup>3</sup>H]IP with peptide #1 (1  $\mu$ M): hNMBR/BALB cells, 2,046  $\pm$  171 and 56,260  $\pm$  916 dpm, respectively

(n=8); rNMBR/BALB cells,  $2,501 \pm 519$  and  $69,250 \pm 13,261$  dpm, respectively (n=6) and mNMBR/BALB cells,  $3,692 \pm 413$  and  $125,207 \pm 4,108$  dpm, respectively (n=3).  
Abbreviations: See in table 1 and 2 legends.

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TABLE 1

Affinities of different ligands for human, rat and mouse BRS-3, GRP, and NMB receptors in various cells.

Peptides	IC <sub>50</sub> (nM)									
	Human			Rat			Mouse			
	BRS-3	GRP	NMBR	GRP	NMBR	GRP	NMBR	GRP	NMBR	
	hBRS-3/BALB	hGRP/BALB	hNMBR/BALB	rGRP/AR42J	rNMBR/BALB	mGRP/BALB	mNMBR/BALB			
Peptide #1	2.89 ± 0.70	0.71 ± 0.03	0.21 ± 0.01	0.03 ± 0.00	0.53 ± 0.08	0.42 ± 0.04	5.76 ± 1.02			
MK-5046	102 ± 2	>3,000	>3,000	>3,000	>3,000	>3,000	>3,000			
3 F	909 ± 2	>3,000	>3,000	>3,000	>3,000	>3,000	>3,000			
9 D	121 ± 1	>3,000	>3,000	>3,000	>3,000	>3,000	>3,000			
9 F	495 ± 1	>3,000	>3,000	>3,000	>3,000	>3,000	>3,000			
9 G	70 ± 1	>3,000	>3,000	>3,000	>3,000	>3,000	>3,000			
GRP	>3,000	0.48 ± 0.05	65 ± 17	0.41 ± 0.00	440 ± 70	2.70 ± 0.20	206 ± 13			
NMB	>3,000	31.5 ± 2.4	0.60 ± 0.02	80 ± 5	1.21 ± 0.15	170 ± 5	0.60 ± 0.02			

The indicated cell type was incubated with 50 pM <sup>125</sup>I-[D-Tyr<sup>6</sup>, β-Ala<sup>11</sup>, Phe<sup>13</sup>, Nle<sup>14</sup>]Bn-(6–14) for 60 minutes at 21°C and binding was determined as described in Materials and Methods. The IC<sub>50</sub> was the concentration causing half-maximal inhibition of the saturable binding, calculated using a nonlinear regression curve-fitting program (Prism). In each experiment each value was determined in duplicate, and values given are means and S.E.M. from at least three separate experiments. Abbreviations: BALB 3T3, mouse fibroblast cells; BRS-3, bombesin receptor subtype 3; GRPR, gastrin-related peptide receptor; NMBR, neuromedin B receptor; h, human; r, rat; m, mouse; 3F, 9D, 9F, 9G, nonpeptide chiral diazepam analogs; peptide #1, [D-Tyr<sup>6</sup>, β-Ala<sup>11</sup>, Phe<sup>13</sup>, Nle<sup>14</sup>]Bn-(6–14); MK-5046, (2S)-1,1-trifluoro-2-[4-(1H-pyrazol-1-yl)phenyl]-3-(4-[[1-(trifluoromethyl)cyclopropyl]methyl]-1H-imidazol-2-yl)propan-2-yl]propan-2-ol; IC<sub>50</sub>, half-maximal inhibitory concentration; h,r,mBRS-3 stably transfected into BALB cells; h,mGRPGR stably transfected into BALB cells; h,r,mNMBR stably transfected into BALB cells and AR42J cells containing native rGRPGR. Binding studies could not be performed with rat or mouse BRS-3 because no radioligand is available. Data are calculated from dose-inhibition curves shown in Fig. 1–3.

TABLE 2

Ability of different ligands to activate phospholipase C and stimulate [<sup>3</sup>H]IP production in BRS-3, GRP, and NMB receptor containing cells.

Peptides	EC <sub>50</sub> (nM)											
	Human				Rat				Mouse			
	BRS-3	GRPR	NMBR	BRS-3	GRPR	NMBR	BRS-3	GRPR	NMBR	BRS-3	GRPR	NMBR
	hBRS-3/BALB	hGRPR/BALB	hNMBR/BALB	rBRS-3/BALB	rGRPR/AR42J	rNMBR/BALB	mBRS-3/BALB	mGRPR/BALB	mNMBR/BALB			
<b>Peptide #1</b>	3.92 ± 0.26	0.57 ± 0.08	0.71 ± 0.04	>3,000	0.12 ± 0.01	0.46 ± 0.06	>3,000	0.58 ± 0.04	0.91 ± 0.09			
<b>MK-5046</b>	8.70 ± 0.47	>3,000	>3,000	0.23 ± 0.08	>3,000	>3,000	5.24 ± 0.36	>3,000	>3,000			
<b>3 F</b>	47.7 ± 0.7	>3,000	>3,000	34.55 ± 0.56	>3,000	>3,000	31.08 ± 1.02	>3,000	>3,000			
<b>9 D</b>	9.40 ± 0.46	>3,000	>3,000	70.40 ± 0.37	>3,000	>3,000	18.4 ± 0.5	>3,000	>3,000			
<b>9 F</b>	39.6 ± 1.0	>3,000	>3,000	295 ± 0.5	>3,000	>3,000	86.8 ± 0.8	>3,000	>3,000			
<b>9 G</b>	9.20 ± 0.39	>3,000	>3,000	37.9 ± 0.8	>3,000	>3,000	20.1 ± 0.9	>3,000	>3,000			
<b>GRP</b>	>3,000	4.79 ± 0.19	1,300 ± 170	>3,000	0.30 ± 0.10	125 ± 10	>3,000	1.00 ± 0.20	499 ± 32			
<b>NMB</b>	>3,000	530 ± 23	2.52 ± 0.10	>3,000	17.01 ± 1.90	1.59 ± 0.16	>3,000	23.0 ± 8.0	4.73 ± 0.21			

Cells were incubated with 3 µCi/ml myo-[2-<sup>3</sup>H]inositol for 24 hours, then washed and incubated with unlabeled ligand for 60 minutes at 37°C. [<sup>3</sup>H]-IP production was determined as described in Materials and Methods. The EC<sub>50</sub> for each ligand was the concentration causing half-maximal increase in [<sup>3</sup>H]-IP production with the maximal response seen with MK-5046 or peptide #1 at 1 µM. Data were calculated from the experiments shown in Fig. 4–6. In each experiment each value was determined in duplicate, and values given are means and S.E.M. from at least three separate experiments. Abbreviations: EC<sub>50</sub>: concentration causing half-maximal stimulation; for others see, Table 1 legend.

**TABLE 3**

Comparison of affinities/potencies of different ligands for human BRS-3/BALB cells.

	<b>IC<sub>50</sub> (nM)</b>	<b>EC<sub>50</sub> (nM)</b>	<b>IC<sub>50</sub>/ EC<sub>50</sub></b>
<b>Peptides</b>	<b>hBRS-3/BALB</b>	<b>hBRS-3/BALB</b>	<b>hBRS-3/BALB</b>
<b>Peptide #1</b>	2.89 ± 0.70	3.92 ± 0.26	0.74
<b>MK-5046</b>	102 ± 2	8.70 ± 0.47	11.72
<b>3 F</b>	909 ± 2	47.7 ± 0.7	19.06
<b>9 D</b>	121 ± 1	9.40 ± 0.46	12.87
<b>9 F</b>	495 ± 1	39.6 ± 1.0	12.50
<b>9 G</b>	70 ± 1	9.20 ± 0.39	7.61

Abbreviations: See in Table 1 and 2 legends.