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Ala^5-galanin (2–11) is a GAL$_2$R specific galanin analogue

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ABSTRACT

It is over 30 years since the regulatory peptide galanin was discovered by Professor Mutt and co-workers. Galanin exerts its effects by binding to three galanin G-protein coupled receptors, namely GAL$_1$R, GAL$_2$R and GAL$_3$R. Each galanin receptor has a different distribution in the central nervous system and the peripheral nervous system as well as distinctive signaling pathways, which implicates that the receptors are involved in different biological- and pathological effects. The delineation of the galaninergic system is however difficult due to a lack of stable, specific galanin receptor ligands. Herein, a new short GAL$_2$R specific ligand, Ala^5-galanin (2–11), is presented. The galanin (2–11) modified analogue Ala^5-galanin (2–11) was tested in ^{125}I-galanin competitive binding studies for the three galanin receptors and the G-protein coupled receptor signaling properties was tested by the ability to influence second-messenger molecules like inositol phosphate and cyclic adenosine monophosphate. In addition, two different label-free real-time assays, namely EnSpire® based on an optical biosensor and xCELLigence® based on an electric biosensor, were used for evaluating the signaling properties using cell lines with different levels of receptor expression. Ala^5-galanin (2–11) was subsequently found to be a full agonist for GAL$_2$R with more than 375-fold preference for GAL$_2$R compared to both GAL$_1$R and GAL$_3$R. The single amino acid substitution of serine to alanine at position 5 in the short ligand galanin (2–11) resulted in a ligand subsequently unable to bind neither GAL$_3$R nor GAL$_1$R, even at concentrations as high as 0.1 mM.

1. Introduction

It is now over 30 years since the neuropeptide galanin was discovered by Professor Mutt and colleagues (Tatemoto et al., 1983) when isolating C-terminally amidated peptides from porcine intestine. Galanin was found to be widely distributed in the central nervous system (CNS) the peripheral nervous system (PNS) as well as other tissues (for review see Lang et al., 2015; Webling et al., 2012; Bauer et al., 2010; Lang and Kofler, 2011). Over the following years, attention has been drawn to this regulatory peptide due to a wide variety of physiological and pathological conditions where galanin is a part - including cancer, epilepsy, feeding, nociception and pain (for review see Lang et al., 2015; Webling et al., 2012). Galanin exerts its biological effects by binding to three G-protein coupled receptors (GPCRs), namely GAL$_{1-3}$R. GAL$_1$R and GAL$_3$R are mainly signaling through G$_{i/o}$ (Smith et al., 1998) while GAL$_2$R signals through several G proteins and subsequently influence multiple intracellular signaling pathways (for review see Lang et al., 2015). The specific distribution of galanin receptor subtypes in the brain as well as different binding affinities for galanin fragments indicates different biological effects (O'Donnell et al., 1999; Smith et al., 1998). One major limitation to delineate the galaninergic system is the lack of stable, receptor subtype specific ligands. Liu et al. (2001) presented the first selective galanin ligand, a truncated peptide consisting of amino acids 2–11 (also called AR-M1896) of full-length galanin (Table 1) (Liu et al., 2001). The deletion of the first glycine resulted in loss of affinity for GAL$_1$R (Liu et al., 2001), which has been used extensively for designing GAL$_2$R selective ligands ever since (Runesson et al., 2009; Saar et al., 2013a, 2013b; Sollenberg et al., 2006) (Table 1). The finding that galanin (2–11) also had similar affinity to GAL$_3$R (Lu et al., 2005a) was a step back, but galanin (2–11) is still widely used as a non-GAL$_1$R ligand.

Over the years, reports have accumulated that the signaling cascades of GPCRs are more complex than previously anticipated. In addition to the different G protein-mediated signaling, several GPCR interacting

Abbreviations: BBB, blood-brain barrier; CHO cells, Chinese hamster ovary cells; CNS, central nervous system; EDTA, ethylenediaminetetraacetic acid; FBS, fetal bovine serum; Fmoc, 9-fluorenylmethyloxycarbonyl; FSK, forskolin; GAL$_1$R, Galanin receptor 1; GAL$_2$R, Galanin receptor 2; GAL$_3$R, Galanin receptor 3; GIP, GPCR interacting proteins; GPCRs, G-protein coupled receptors; HKR, HEPES buffered Krebs-Ringer solution; HPLC, high performance liquid chromatography; IP, inositol phosphate; MBHA, p-methylbenzhydrylamine; PBS, phosphate-buffered saline; PNS, peripheral nervous system; Sar, sarcosine.

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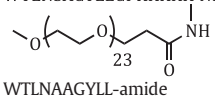
Table 1
The amino acid sequences of peptide ligands discussed.

Name	Sequence	Reference
Rat galanin	GWTLNSAGYLLGPHAIDNHRSFSDKHGLT-amide	Vrontakis et al. (1987)
Human galanin	GWTLNSAGYLLGPHAVGNHRSFSDKNGLTS	Schmidt et al. (1991)
Tuna galanin	GWTLNAAGYLLGPHGIDGHRTLGDKPGLA-amide	Habu et al. (1994)
Human GALP	APAHPGRGGWTLNSAGYLLGPVLHLPQMGDQDG KRETALEILDLWKAIDGLPYSHPPQPS	Ohtaki et al. (1999)
Galanin (2–11)	WTLNSAGYLL-amide	Liu et al. (2001)
AR-M1896		
Human spexin	NWTPQAMLYLKGAQ-amide	Mirabeau et al. (2007)
Gal-B2	(Sar)WTLNSAGYLLGPKKK$_{Palmitoyl}$ K-amide	Bulaj et al. (2008)
NAX 5055		
M1145	RGRGNWTLNSAGYLLGPVLPPPALALA-amide	Runesson et al. (2009)
M1153	RGRGNWTLNSAGYLLGPK(ε-NH-C(O)X)-amide	Saar et al. (2011)
M1160	RGRGNWLNSAGYLLGPVLPPPALALA-amide	Saar et al. (2013b)
NAX 409-9	WTLNSAGYLLGPKKKKK-NH_2 	Metcalf et al. (2015)
Ala^5-galanin (2–11)	WTLNAAGYLL-amide	

Sar = sarcosine; X = —CH(NH_2)—CH_2—CH_2—COOH.

proteins (GIP) have been described (Xiao et al., 2010) (for review see Jacobson, 2015; Shukla et al., 2014; Khoury et al., 2014; Luttrell et al., 2015; Walther and Ferguson, 2015). Activated β-arrestins have been shown to trigger a second wave of cell signaling (Ferrie et al., 2013) alongside a third wave of GPCR signaling from internalized receptors in endosomes (Irannejad et al., 2013; Irannejad and von Zastrow, 2014; Tsvetanova et al., 2015), which adds to the complexity of GPCR signaling.

These findings highlight that measuring a single second messenger to evaluate the signaling property for GPCRs that signal through multiple pathways as inadequate to show the true biological response (for review see Hall and Lefkowitz, 2002; Kenakin, 2009; Zhang and Xie, 2012).

However, to evaluate second-messengers from all known signaling pathways of certain GPCRs would be time-consuming and difficult due to biased ligands.

Since GPCR activation leads to cytoskeletal-guided transport of molecules and subsequent changes in cell adhesion and morphological re-arrangements, new real-time label free techniques have been developed to evaluate the sum of cellular events of activated GPCRs (for review see Grundmann and Kostenis, 2015; Ke et al., 2015; Lee, 2009; Nayler et al., 2010; Peters and Scott, 2009; Scott and Peters, 2010; Zhang and Xie, 2012). The sum of these cellular events gives a combined biological response measured in real time by these label-free techniques as either changes in wavelength due to mass redistribution of the cells (optical biosensor) or measuring changes in impedance due to cell adhesion changes on electrodes (an electric biosensor).

In this study, two real-time label free techniques, one based on optical techniques and one based on impedance were used alongside classical signaling measurements, i.e. cyclic adenosine monophosphate (cAMP) measurements and inositol phosphate (IP) turnover, to evaluate the signaling property of ligands and receptors.

Here we present a new short galanin receptor 2 subtype specific ligand, Ala^5-galanin (2–11), with no detectable binding to neither human GAL_1R nor human GAL_3R (for sequence see Table 1). Two classical signaling methods, IP turnover and cAMP assay, are also compared to two different real-time label-free assays for GPCR signaling, for both Ala^5-galanin (2–11) and full-length galanin.

2. Materials and methods

2.1. Peptide design

Galanin (1–15) is highly conserved among species (for review see Langel and Bartfai, 1998) which implicates the N-terminus to be important for receptor binding. The removal of the first glycine along with omission of galanin 12–29/30 of galanin has been shown to produce a non-GAL_1R-ligand (Liu et al., 2001; Lu et al., 2005b).

The design of Ala^5-galanin (2–11), with the amino acid sequence WTLNAAGYLL-amide (see Table 1), was based on the short galanin fragment galanin (2–11) but with a single amino acid substitution where serine at position five was changed to alanine. Alanine instead of serine at position six in full-length galanin is found in yellowfin tuna (Habu et al., 1994).

2.2. Peptide synthesis

The peptides were synthesized in a stepwise manner using small scale (0.1 mmol) 9-fluorenylmethyloxycarbonyl (Fmoc) solid-phase peptide synthesis strategy on an automated Syro multipeptide synthesizer (MultiSynTech, GmbH, Witten, Germany) using p-methylbenzhydrylamine (MBHA) resin (Iris Biotech GmbH, Marktredwitz, Germany), which generated a C-terminally amidated peptide after final cleavage. Fmoc amino acids (Iris Biotech GmbH, Marktredwitz, Germany) were coupled as hydroxybenzotriazole (HOBt) esters. The peptides were cleaved from the resin using a solution of 95% trifluoroacetic acid (TFA), 2.5% triisopropylsilane (TIS) and 2.5% H_2O for 3 h. The peptides were subsequently purified by reverse-phase HPLC (Nucleosil 120-3 C-18 column), freeze-dried and the correct molecular weight was determined by matrix assisted laser desorption ionization time-of-flight mass spectrometry (Voyager-DE STR, Applied Biosystems, Foster City, CA, USA). The freeze-dried peptides were stored at −20 °C until used.

2.3. Cell culture

Bowes human melanoma cells (American type Culture Collection CRL-9607) stably expressing human GAL_1R (PMID: 7524088) were cultured in Eagle's minimum essential medium with GlutaMAX^{-1} supplemented with 10% fetal bovine serum (FBS), 1% sodium pyruvate, 1% non-essential amino acids, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin.

Chinese Hamster Ovary (CHO) K1 cells stably expressing human GAL_2R (a kind gift from Kathryn A. Jones and Tiina P. Iismaa, Sydney, Australia) were cultured in Dulbecco's modified essential medium F12 with GlutaMAX^{-1} supplemented with 10% FBS, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin.

SH-SY5Y cells stably transfected with an tetracycline inducible overexpression (see Table 2) of either GAL_1R or GAL_2R (PMID: 14592962) were cultivated in Dulbecco's modified eagle medium supplemented with 10% FBS, 2 mM L-glutamine, 1% non-essential amino acids, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin.

Table 2
The cell lines used in this study as well as the concentration of the receptors expressed.

Cell line and receptor expressed	Concentration membrane bound receptor in pmol/mg protein	References
Bowes melanoma cells expressing human GAL_1R	0.54 ± 0.185	Runesson et al. (2009)
Chinese hamster ovary cells expressing human GAL_2R	0.675 ± 0.152	Runesson et al. (2009)
Flp-In T-Rex 293 cells expressing human GAL_3R	0.696 ± 0.293	Runesson et al. (2009)
SHSY5Y stably expressing human GAL_1R	0.4 ± 0.1	Berger et al. (2004)
SHSY5Y with tetracycline induced human GAL_1R overexpression	10.2 ± 1.2	Berger et al. (2004)
SHSY5Y stably expressing human GAL_2R	0.05 ± 0.016	Berger et al. (2004)
SHSY5Y with tetracycline induced human GAL_2R overexpression	3.2 ± 0.3	Berger et al. (2004)

Flp-In T-REx 293 GAL_3R cell line (Runesson et al., 2010) was cultured in Dulbecco's modified essential medium supplemented with 10% FBS, 2 mM L-glutamine, 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, 15 μg ml^{-1} blasticidin S and 150 μg ml^{-1} Hygromycin B (Roche Diagnostics). Tetracycline (1 μg/ml) (Sigma Aldrich, St. Louis, MO, USA) to induce the expression of GAL_3R were added 24 h prior to cell harvesting for the ^{125}I galanin competitive study, and after 24 h of cell seeding in the real-time impedance based 96-well E-plate VIEW.

Cell cultures were grown at 37 °C in a 5% CO_2 incubator. Cell culture reagents were purchased from Invitrogen (Carlsbad, CA, USA) and cell plastics from Corning (Corning, NY, USA).

2.4. Galanin receptor binding studies

Cells for ^{125}I-galanin receptor displacement studies were seeded in 150 mm dishes and cultured in complete cell media for 2–3 days until approximately 90% confluent. Complete cell media were changed every other day. The GAL_3R Flp-In T-REx cell line had an addition of tetracycline (1 μg/ml) 24 h prior to harvest for inducing GAL_3R expression. Cells were washed thrice and scraped into phosphate-buffered saline (PBS) and centrifuged twice at 4 °C, 3000 × *g* for 5 min.

The pellet was resuspended in assay buffer (20 mM HEPES, 5 mM $MgCl_2$, pH 7.4) supplemented with EDTA (5 mM EDTA) and incubated on ice for 45 min before centrifugation at 4 °C, 8500*g* for 15 min. After washing the pellet in assay buffer and repeated centrifugation the obtained pellet was resuspended in assay buffer supplemented with 1% protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA) and stored at −20 °C. Protein concentration was determined according to Lowry (BioRad, Stockholm, Sweden). Displacement studies on cell membranes were performed in a final volume of 200 μl, containing 0.1 nM porcine-[^{125}I]-galanin (2200 Ci/mmol, Perkin-Elmer Life Science, Boston, MA, USA), 30 μg cell membrane, and various concentrations of peptide (10^{-9} to 10^{-4} M). Peptide solutions were prepared in assay buffer supplemented with 0.3% bovine serum albumin (BSA) using silanized (dichlorodimethylsilane, Sigma-Aldrich, St. Louis, MO, USA) tubes and pipette tips to prevent unspecific binding. Samples were incubated at 37 °C for 30 min while gently shaking after which the samples were transferred and filtered through a MultiScreen-FB filter plate (Millipore, Billerica, MA, USA) pre-soaked in 0.3% polyethylenimine solution (Sigma-Aldrich, St. Louis, MO, USA) using vacuum. The filters were washed thrice with assay buffer and the retained radioactivity was determined in a β-counter (Tri-Carb Liquid Scintillation Analyser, model 2500 TR, Packard Instrument Company, Meriden, CT, USA) using OptiPhase Supermix Cocktail (Perkin-Elmer Life Science, Boston, MA, USA) as scintillation fluid. IC_{50} values for the peptides were calculated using Prism 6.0 (GraphPAD Software Inc., San Diego, CA, USA) and converted into K_i values using the equation of Cheng-Prusoff (Cheng and Prusoff, 1973).

2.5. Cyclic adenosine-monophosphate (cAMP) measurements

SH-SY5Y cells stably expressing either GAL_1R or GAL_2R were seeded into 48-well plates and grown to confluency. Tetracycline was not added since the experiment aimed to examine a lower receptor expression level (see Table 2). Cells were incubated for 2 h with DMEM and [^{3}H]adenine (3 μCi/ml) (Perkin Elmer, Waltham, MA, USA) and then washed thrice with Hank's balanced salt solution (Gibco) before stimulated with 20 μM forskolin and/or peptide ligand Ala^5-galanin (2–11) (10^{-5} to 10^{-7}) for 30 min at 37 °C in the presence of inhibitors (1 mM 3-isobutylmethylxanthine (IMBX) (Sigma, Vienna, Austria), 1 μM BAY 60-7550 (Santa Cruz, Heidelberg, Germany) and 10 μM rolipram (Sigma, Vienna, Austria)). The cells were subsequently lysed using of 5% (w/v) trichloroacetic acid followed by the addition of 250 μl of 5% (w/v) trichloroacetic acid supplemented with 0.1 mM cAMP and 0.1 mM adenosine triphosphate (ATP) (Sigma, Vienna, Austria) for at least 20 min at 4 °C.

The lysates were then added to Dowex 50W-X4 columns (200–400 mesh) (Bio-Rad, Hercules, CA, USA) and washed twice with deionized water before placed over alumina columns and eluted with 0.1 M imidazole (AppliChem, Darmstadt, Germany). Radioactivity of the eluate was determined in a liquid scintillation counter (Tri-Carb model 1600 TR; Packard Instrument Company) using Ultima Gold XR (Perkin Elmer, Waltham, MA, USA) as scintillation fluid.

2.6. Inositol phosphate accumulation

CHO K1 cells stably expressing human GAL_2R were seeded in 12-well plates and cultured in complete cell culturing media for 48 h before incubation with 1 μCi [^{3}H]-myo-inositol (Perkin Elmer) in M-199 medium (Invitrogen, Carlsbad, CA, USA) containing 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin for 24 h. Prior to experiments, cells were incubated with fresh M-199 medium for 10 min at 37 °C and washed twice with HEPES buffered Krebs-Ringer solution (HKR) buffer (5 mM HEPES, 137 mM NaCl, 2.68 mM KCl, 2.05 mM $MgCl_2$, 1.8 mM $CaCl_2$, 1 g l^{-1} glucose, pH 7.4) followed by 10 min pre-incubation in 800 μl HKR buffer with 10 mM LiCl at 37 °C. Cells were then stimulated with peptide in various concentrations (10^{-9} to 10^{-4} M) generating a total volume of 1000 μl. After addition of peptide the samples were incubated for 1 h at 37 °C. The reaction was terminated by addition of 200 μl ice cold 20% perchloric acid followed by incubation on ice for 30 min. The samples were neutralized to pH 7 by addition of 1.5 M KOH/75 mM HEPES. Anion exchange chromatography of the samples was performed over 1.5 cm 50:50 Dowex (AG 1-X8 Resin, 200–400 mesh formate, BioRad, Hercules, CA, USA). The columns were washed with 5 ml distilled water before the inositol phosphates (IP) were eluted with 5 ml of 0.1 M formic acid/1.2 M ammonium formate. Radioactivity of the eluate was determined in a β-counter (Tri-Carb Liquid Scintillation Analyzer, model 2500 TR, Packard Instrument Company, Meriden, CT, USA) using Utima Flo AF (Perkin-Elmer Life Science, Boston, MA, USA) as scintillation fluid. Each sample was normalized against the total count obtained before anion exchange chromatography.

2.7. Label-free assay for signaling using EnSpire®

SH-SY5Y cells stably expressing either GAL_1R or GAL_2R were seeded in EnSpire LFC-348 well plates (Perkin Elmer, Austria) at a concentration of 12,000 cells per well and cultured overnight at 37 °C and 5% CO_2. Tetracycline was not added to be able to examine the signaling in cells expressing the receptor at lower concentrations. The media was then aspired and the cells were washed four times with Hank's balanced salt solution (HBSS) buffer supplemented with HEPES (20 mM) and the

final volume was adjusted to 30 μl per well. The plate was allowed to equilibrate to assay temperature for 1 h in the Enspire multimode reader (Perkin Elmer, Austria). HBSS buffer supplemented with HEPES (20 mM) alone or dilution series of Ala^5-galanin (2–11) dissolved in the same buffer were prepared and placed on a compound plate which were allowed to equilibrate to reach the same temperature as the Enspire plate holding the cells. A baseline reading was first generated by 4 measurements before ligands were added. The plate was measured between 30 and 70 repeats and each experiment was performed in triplicates. The mean was used to generate the dose response curve and EC_{50} values were calculated using Prism 6.0 (GraphPad Software Inc., San Diego, CA, USA).

2.8. Label-free assay for signaling using xCELLigence

One day prior to the experiment, 100 μl of complete cell culture media was added to each well and background recorded. Following background measurement, 100 μl of complete cell media containing the cell suspension were seeded on the 96-well E-plate VIEW™ (ACEA, San Diego, USA), incubated at room temperature for 30 min and then placed onto the device station (Roche Applied Science and ACEA Biosciences) hosted inside an incubator at 37 °C with 5% CO_2. The optimal cell density for the assay was determined to be 40,000 cells per well for Bowes human melanoma cells stably expressing GAL_1R, 25,000 cells per well for CHO K1 cells stably expressing GAL_2R and 20,000 cells per well for Flp-In T-REx 293 cells with inducible GAL_3R expression. The cells were allowed to equilibrate for 24 h (48 h for the GAL_3R cell line where tetracycline (1 μg/ml) was added 24 h after seeding to induce the GAL_3R expression) and impedance was constantly monitored every minute throughout the whole experiment. Prior to galanin ligand addition, complete cell media were replaced with 180 μl low-FBS (1%) containing cell media, according to the manufacturer's instructions, and incubated for 1 h. The ligands were dissolved in PBS at concentrations 10× the wanted final concentration and 20 μl were added gently to each well.

Data were analyzed using the integrated software package expressing changes in cell electrode impedance as changes in cellular index, CI, (Yu et al., 2006) and normalized to the cell index at the time of ligand addition. Concentration effect curves were generated by calculation of the area under curve (AUC) between 0 and 3600 s after peptide addition, and plotted against ligand concentration. The subsequent calculation of EC_{50} values was performed by non-linear regression using Prism 6.0 (GraphPad Software Inc., San Diego, CA, USA).

2.9. Data analysis

For all experiments are data reported as mean ± SEM of at least three individual experiments performed in at least duplicates. Statistical significance were graded after *p-value ≤ 0.05; **p-value ≤ 0.01 and ***p-value ≤ 0.001 using ANOVA Bonferroni's multiple comparison test using Prism 6.0 (GraphPad Software Inc., San Diego, CA, USA).

3. Results

3.1. Galanin receptor binding studies

^{125}I-galanin-receptor displacement studies were performed using full-length rat galanin, galanin (2–11) and Ala^5-galanin (2–11) on membrane preparations of Bowe's human melanoma cells stably expressing human GAL_1R, CHO K1 cells stably expressing human GAL_2R and Flp-In T-REx 293 with inducible expression of human GAL_3R. The high degree of sequence homology between rat and human galanin receptors and the reported similar binding affinity for full-length rat, human and porcine galanin to those two receptors (Wang et al., 1997; Borowsky et al., 1998; Lu et al., 2005a, 2005b; reviewed in Webling et al., 2012) allows full-length galanin from either of these three species to be used as controls.

The displacement of both full-length rat galanin as well as galanin (2–11) revealed for galanin a K_i of 1.70 ± 1.2 nM for GAL_1R, 2.71 ± 0.9 nM for GAL_2R and 4.33 ± 0.6 nM for GAL_3R. Galanin (2–11) had no detectable binding to GAL_1R, and the K_i for GAL_2R was 14.6 ± 2.2 nM and for GAL_3R 186 ± 74 nM (Fig. 1, Table 3). Ala^5-galanin (2–11) had a lower affinity for GAL_2R compared to galanin (2–11) with a K_i of 258 ± 68 and 16.6 ± 3.5, respectively, but Ala^5-galanin (2–11) had no detectable binding to neither GAL_1R nor GAL_3R even at doses as high as 0.1 mM (Table 3, Fig. 1). In Table 3, the highest experimentally tested concentration is presented when no K_i could be calculated and indicated by a > meaning that the true K_i value would be higher. Consequently, Ala^5-galanin (2–11) has more than 375-fold preference for GAL_2R over both GAL_1R and GAL_3R.

3.2. Cyclic adenosine-monophosphate stimulation assay

The ability of Ala^5-galanin (2–11) to initiate GPCR signaling were examined by its ability to change the concentration of cyclic adenosine-monophosphate, cAMP, in SH-SY5Y cells expressing GAL_2R in a 10-fold lower concentration than the CHO K1 cells. Signaling through $G_{i/o}$ would decrease the cAMP concentration and forskolin was therefore used to stimulate cAMP production of the cells. Three different ligand concentrations (0.1, 1 and 10 μM) were tested together with 20 μM forskolin, using forskolin (20 μM) together with 0.1 μM full-length human galanin as control. No significant change in cAMP concentration after addition of Ala^5-galanin (2–11) could be seen even at 10 μM concentration using ANOVA Bonferroni's multiple comparison test whereas forskolin together with full-length galanin caused a statistical significant increase in cAMP concentration (**p-value ≤ 0.01) (Fig. 2).

3.3. Inositol phosphate accumulation

The ability of Ala^5-galanin (2–11) and full-length rat galanin to induce GPCR signaling was examined by the ability of the peptides to stimulate inositol phosphate (IP) production in CHO cells stably expressing human GAL_2R. The ability to induce maximum IP accumulation is similar to that of full-length rat galanin and also the previously published full agonist M1145 (Runesson et al., 2009). Ala^5-galanin (2–11) has relatively low affinity compared with the previously published GAL_2R selective ligands with an EC_{50} value of 1.01 ± 0.18 μM but considered a full agonist (Fig. 3) (Runesson et al., 2009; Saar et al., 2013a, 2013b; Sollenberg et al., 2006). The EC_{50} value of full-length rat galanin in the present study was 66.4 ± 13 nM for GAL_2R, a value in concordance with previous studies were full-length rat galanin had a EC_{50}-value of 75 nM (Runesson et al., 2009). Using ANOVA Bonferroni's multiple comparison significance were graded according *p-value ≤ 0.05; **p-value ≤ 0.01 and ***p-value ≤ 0.001. Galanin showed significant accumulation of inositol phosphate above 0.1 μM (***p-value) whereas Ala^5-galanin (2–11) had (*p-value) accumulation of inositol phosphate at 0.1 μM and significant accumulation above 1uM (***p-value).

No significant change in IP production could be seen when Ala^5-galanin(2–11) was added to Bowes melanoma cells stably expressing GAL_1R nor Flp-In T-REx 293 with inducible expression of GAL_3R (data not shown).

3.4. Label free methods using EnSpire® and xCELLigence®

When using the optical based label-free real-time system, Enspire, the ligand Ala^5-galanin (2–11) was only able to generate a dynamic mass redistribution (DMR) response in the SH-SY5Y cells stably expressing GAL_2R with an EC_{50}-value of 3.23 ± 0.4 μM. Full-length human galanin was used as control and had in this study an EC_{50}-value of 1.08 ± 3.19 nM at SH-SY5Y cells expressing GAL_1R and 47.2 ± 5.3 nM for SH-SY5Y cells expressing GAL_2R.

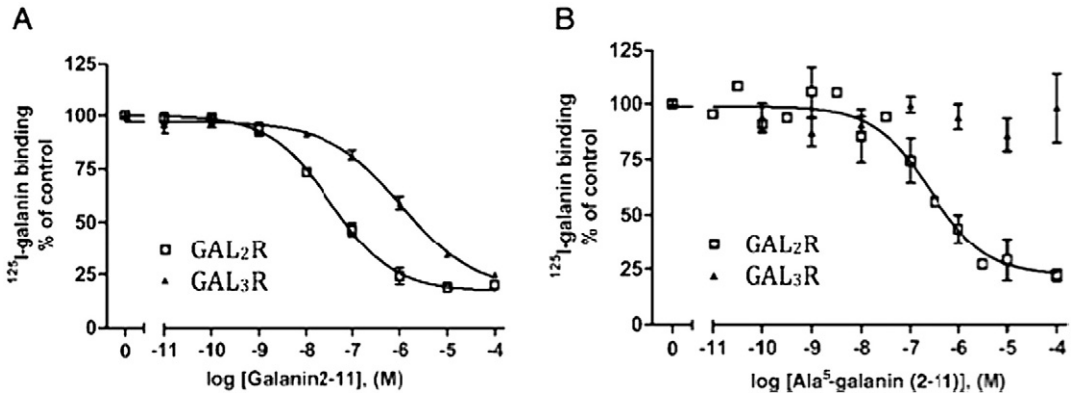


Fig. 1. Displacement studies of porcine-[^{125}I]-galanin on CHO K1 cells stably expressing human GAL_2R, presented in open squares, and Flp-In T-REx 293 cells with inducible expression of human GAL_3R, presented in filled triangles, using the non-GAL_1R ligand galanin (2–11) (A) and the specific GAL_2R ligand Ala^5-galanin (2–11) (B). Data is presented as mean ± SEM of three individual experiments performed in duplicates.

When using the impedance based label-free real-time assay, xCELLigence, Ala^5-galanin (2–11) was only able to generate a dose-response curve in CHO K1 cells stably expressing GAL_2R. The EC_{50}-value was 191 ± 49 nM (Table 4). No response could be observed when Ala^5-galanin (2–11) was added to Bowes Melanoma Cells stably expressing GAL_1R nor Flp-In T-REx 293 cells with tetracycline induced expression of GAL_3R (data not shown). Full-length rat galanin were used as a control and had in this study EC_{50}-values of 1.1 ± 0.11 nM for GAL_1R, 8.26 ± 1.9 nM for GAL_2R and 412 ± 38 nM for GAL_3R.

4. Discussion

The usage of knockout (KO)-animals, overexpression (OE)-animals and the use of a few available selective ligands for the different galanin receptor subtypes indicate specific involvement of galanin receptor subtypes in different diseases (for review see Webling et al., 2012). GAL_2R agonists are for example considered to be neuroprotective in both Alzheimer's disease (Pirondi et al., 2010) and in kainic acid induced cell death (Webling et al., 2015), antidepressant and anxiolytic (Kuteeva et al., 2008), and able to reduce the severity of seizures in epilepsy (Robertson et al., 2010; Mazarati et al., 2004) (for review see Webling et al., 2012). This has generated an increasing interest to generate and use specific ligands to delineate the effects of the galanin receptors, but the pharmacological tools available are still limited. The first specific galanin ligand presented was galanin (2–11) (Liu et al., 2001) and even though the finding that galanin (2–11) binds with similar affinity to both GAL_2R and GAL_3R (Lu et al., 2005a, 2005b), it is commonly used as an non-GAL_1R ligand.

Even though the N-terminal amino acid sequence of galanin is highly conserved among species and considered important for receptor binding, some N-terminal alterations have been shown to retain receptor binding affinity. Bulaj and colleagues reported a N-terminal methylated galanin ligand where the first glycine was replaced by sarcosine along with cationic amino acids C-terminally together with a fatty acid to improve stability (Bulaj et al., 2008). The presented ligand, named NAX 5055, had nM affinity for both GAL_1R and GAL_2R with a 14–fold preference for GAL_1R over GAL_2R. Unfortunately, no data for NAX 5055 and GAL_3R affinity have been reported to date. The first GAL_2R specific ligand M1145 presented by Runesson et al. (2009) was designed with a four amino acid sequence from the GAL_2R/GAL_3R selective galanin-like peptide (GALP) added to the N-terminus to improve the GAL_2R selectivity alongside replacement of the first glycine to asparagine to minimize binding to GAL_1R (Runesson et al., 2009) (Table 1). M1145 retained a nM binding affinity for GAL_2R and had a 90-fold preference for GAL_2R over GAL_1R and a 76-fold preference for GAL_2R over GAL_3R (Runesson et al., 2009). Based on the M1145 sequence, a new ligand with improved specificity for GAL_2R was designed and presented by Saar et al., 2011,

Table 3
Experimental K_i determined by displacement studies of porcine-[^{125}I]-galanin on Bowes Melanoma Cells expressing human GAL_1R, CHO K1 cells stably transfected with human GAL_2R or Flp-In T-REx 293 cells with inducible expression of human GAL_3R. The highest concentration used in this study served as a K_i-value when no experimentally K_i-value could be determined. Data are presented as mean values ± SEM of at least three individual experiments each performed in duplicates. When K_i-values could not be determined was the K_i presented as above the highest concentration experimentally used.

	K_i (nM)		
Name	GAL_1R	GAL_2R	GAL_3R
Rat galanin	1.70 ± 1.2	2.71 ± 0.9	4.33 ± 0.6
Galanin (2–11)	>100,000	14.6 ± 2.2	186 ± 74
Ala^5-galanin (2–11)	>100,000	258 ± 68	>100,000

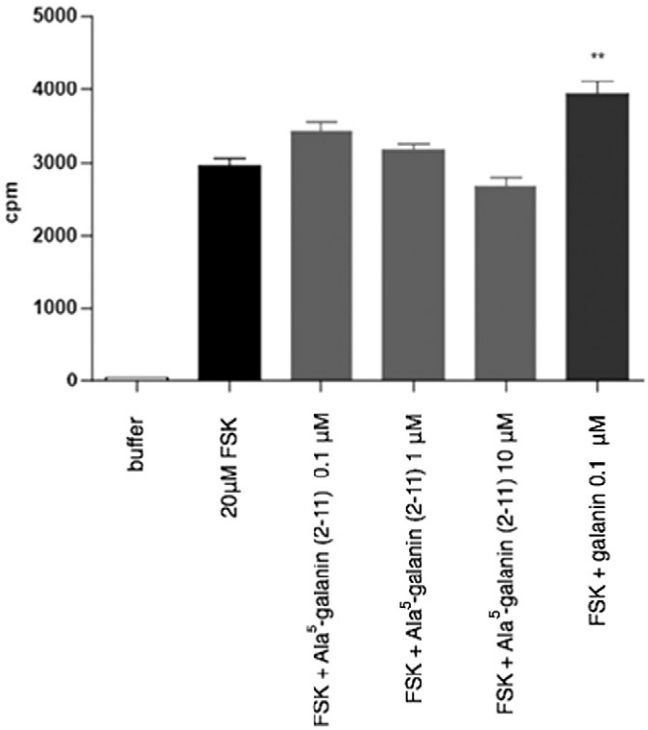


Fig. 2. Signal transduction using cyclic adenosine-monophosphate stimulation. Cyclic adenosine-monophosphate, cAMP, production was measured radiometrically. Ala^5-galanin (2–11) did not significantly stimulate cAMP production in SH-SY5Y cells stably expressing human GAL_2R. Full-length human galanin (0.1 μM) together with forskolin (FSK) were used as control. Data shown are mean values of triplicates ± SEM of one experiment. **p-Value ≤ 0.01, ANOVA Bonferroni's multiple comparison test.

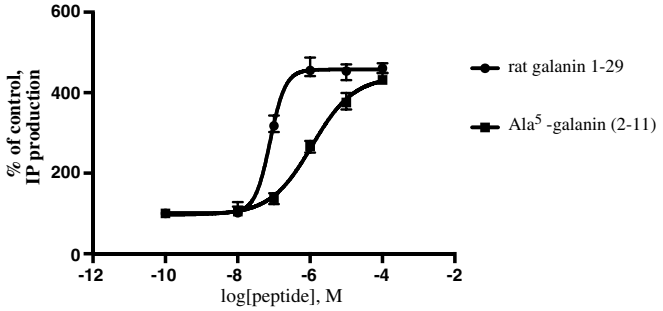


Fig. 3. Signal transduction via inositol phosphate accumulation. CHO K1 cells stably expression human GAL_2R were pre-incubated with [^{3}H]-myo-inositol for 24 h previous to galanin ligand addition at different concentrations (10^{-10}–10^{-4} M). The dose response curve of Ala^5-galanin (2–11) is plotted using filled squares and full-length rat galanin plotted as filled circles. Data are presented as % of control when no ligand was added as mean ± SEM of six individual experiments each performed in duplicates.

named M1153 (Table 1) (Saar et al., 2011). The C-terminal branching produced a ligand that retained nM affinity for GAL_2R with a 380-fold preference for GAL_2R over GAL_1R and a 46-fold preference for GAL_2R over GAL_3R. The seventh amino acid in the M1145 sequence, threonine, was deleted to reduce the affinity for GAL_3R according to mutagenesis studies resulting in an even more selective GAL_2R specific ligand, named M1160 (for sequences see Table 1) (Saar et al., 2013b). M1160 had a 465-fold preference for GAL_2R over GAL_1R and more than a 1000-fold preference for GAL_2R over GAL_3R. In Ala^5-galanin (2–11), the substitution of serine to an alanine at position five in galanin (2–11) reduced one hydroxyl group in the peptide. The re-evaluation of an alanine scan to identify important pharmacophores for GAL_2R showed a 6-fold reduction in EC_{50}-value while replacing the serine at position five in galanin (2–11) (Lundström et al., 2005). Land et al. (1991) showed a similar 6-fold reduction in K_D while comparing galanin (1–16) to Ala^6-galanin (1–16) (Land et al., 1991). In an in silico study using the short galanin fragment, galanin 2–6, for docking to GAL_3R highlighted serine as important for forming a hydrogen bond to Ala92$^{3.22}$ (Runesson et al., 2010). In concordance with the experimental result presented herein, where the importance of serine at position five were highlighted by replacing serine with alanine resulted in a ligand unable to bind to GAL_3R even at high concentrations of 0.1 mM.

The C-terminus of galanin has been reported as an important sequence for binding to the GAL_3R even though the GAL_3R binding pocket has been described as tighter and more narrow as compared to GAL_1R and GAL_2R (Runesson et al., 2010). Our new GAL_2R specific ligand, Ala^5-galanin (2–11), behaves as a full agonist for GAL_2R when examined by ^{125}I-galanin competitive binding studies as well as IP turnover studies (Table 3; Figs. 1 and 2). Ala^5-galanin (2–11) has a lower affinity for the receptor compared to other published GAL_2R selective ligands, but is the shortest peptide fragment that shows excellent receptor specificity with no detectable binding to neither GAL_1R nor GAL_3R even at doses as high as 0.1 mM. In concordance with previously published specific GAL_2R agonists (M1145 (Runesson et al., 2009), M1153 (Saar et al., 2011) and M1160 (Saar et al., 2013b)) is the binding affinity for GAL_2R lower than for full-length galanin and galanin (1–16). Our new GAL_2R specific ligand, Ala^5-galanin (2–11), could be used as a research tool to delineate the galaninergic system and the role of GAL_2R in several studies where galanin (2–11) has been used to exclude effects caused by interactions with GAL_3R.

Limitations for this ligand, among most peptides, are the fast degradation when used in animal studies along with problematic administration routes were i.c.v injections are often used since unmodified peptide ligands are unable to pass the blood-brain barrier (BBB). Bulaj and co-workers presented a new modification that increased the half-life of galanin analogues from minutes to several hours by adding several positive amino acids along with a fatty acid at the C-terminus (Bulaj et al., 2008; Robertson et al., 2010). A study by Saar et al. (2013a) have adopted the modifications from Bulaj and co-workers to produce stable GAL_2R selective ligands that could pass the BBB when administrated intravenously and exert anti-depressant affects in mice (Saar et al., 2013a). Recent studies by White and colleagues presents a C-terminally monodisperse oligoethyleneglycol (dPEG) modified GAL_2R selective galanin ligand, named NAX 409-9, that is stable and peripherally active with restricted BBB penetration (Zhang et al., 2013; Metcalf et al., 2015).

Recently, a 14 amino acid long peptide named spexin was reported as a $GAL_{2/3}R$ ligand with a higher potency for GAL_3R than galanin (for sequence see Table 1) (Kim et al., 2014). Human spexin is C-terminally amidated like galanin from most species (human and macaque being exceptions) and share Trp^2, Thr^3, Tyr^9, Leu^{10} and Gly^{12} (Mirabeau et al., 2007; Schmidt et al., 1991; Cunningham et al., 2002). Based on the spexin sequence, Reyes-Alcaraz et al. (2016) reported several new GAL_2R specific ligands when replacing amino acids in the spexin sequence by the corresponding amino acid from galanin and highlighted the importance of Gln^5, Met^7, Lys^{11} and Ala^{13} in spexin for GAL_3R binding (Reyes-Alcaraz et al., 2016). The presented GAL_2R specific ligands, Asn^5-spexin, Ala^7-spexin, Phe^{11}-spexin, Pro^{13}-spexin and Asn^5, Ala^7, Phe^{11}, Pro^{13}-spexin, all retained similar binding affinity to GAL_2R as spexin but no binding to neither GAL_1R nor GAL_3R could be detected even at concentrations of 10 μM (Reyes-Alcaraz et al., 2016). Additionally, the spexin based GAL_2R specific ligands were chemically stabilized by either addition of Fmoc, N-terminal PEGylation, C-terminal or N-terminal substitution of $_D$-amino acids which resulted in an up to four times improved serum stability compared to spexin (Reyes-Alcaraz et al., 2016).

The signaling cascade of GPCRs has been shown to involve both G protein-dependent and -independent pathways. For GAL_2R, it has been shown that the Gα subtypes used upon activation are mainly $G_{q/11}$, but $G_{i/o}$, and $G_{12/13}$, are also involved (for review see Lang et al., 2015; Wittau et al., 2000). Thus to get a summarized picture using classical signaling assays is very time consuming. Signaling through G_q causes an increase in IP production whereas signaling through $G_{i/o}$ is classically accompanied by decreasing cAMP levels. The contribution of $G_{12/13}$ is more difficult to access using classical assays (Wettschureck, 2005).

In the IP turnover study classically measuring G_q signaling, Ala^5-galanin (2–11) behaved as a full agonist compared to full-length rat galanin on CHO K1 cells overexpressing GAL_2R in agreement that G_q is the main signaling pathway for GAL_2R. By comparing the classical signaling assays with the new real time label free techniques, full-length galanin showed similar values to GAL_1R and GAL_2R, with 1.1 ± 0.11 nM and 8.26 ± 1.9 nM, respectively, while the EC_{50} towards GAL_3R was 412 ± 38 nM. The xCELLigence system has similar or improved sensitivity when compared to traditional endpoint assays performed in the same laboratory. The relative low EC_{50} for full-length galanin at GAL_3R could reflect intrinsic receptor properties or might be related to the utilized cell clone. Runesson et al. (2009) reported an EC_{50}-value of 530 nM for galanin on GAL_3R using a GTPγ-assay and the receptor expression of GAL_3R is similar to those of GAL_1R and GAL_2R used in this study (Runesson et al., 2009).

Table 4
Galanin receptor signaling studies using a real-time label-free technique based on impedance - the xCELLigence system.
EC_{50} values obtained from the impedance based (RT-CES) xCELLigence system using Bowes Melanoma Cells stably expressing GAL_1R, CHO K1 cells stably expressing GAL_2R, and Flp-In T-REX 293 cells with tetracycline induced expression of GAL_3R. Dose-response curves were generated and calculated by area-under-curve between 0 and 3600 s after peptide addition, and plotted against the ligand concentration. Data from at least three representative experiments performed in duplicates, are presented as mean ± SEM.

	EC_{50} (nM)		
Name	GAL_1R	GAL_2R	GAL_3R
Rat galanin 1–29	1.1 ± 0.11	8.26 ± 1.9	412 ± 38
Ala^5-galanin (2–11)	>100,000	191 ± 49	>100,000

In this study, the lack of statistically relevant results by Ala[5]-galanin (2–11) in the cAMP stimulation assay using cells expressing GAL_2R at a 10-fold lower concentration compared to the CHO K1 cell line could be due to Ala[5]-galanin (2–11) being a biased ligand i.e. a ligand that prefers one signaling pathway over several others and the lower receptor expression might be difficult to evaluate using this assay. Signaling through $G_{i/o}$ will decrease cAMP levels and therefore forskolin was added to stimulate the cells. However, full-length galanin caused a significant increase in cAMP. Increased cAMP levels are classically related to activation through G_s, but GAL_2R has not been reported to signal through this G-protein (Lang et al., 2015; Lang et al., 2007; Wittau et al., 2000). However, Gu et al., (1994) reported that full-length galanin added to dispersed smooth muscle cells from guinea pig stomach caused increased levels of cAMP (Gu et al., 1994). Signaling through G_q activates protein kinase C (PKC) and causes an increase in intracellular Ca^{2+} concentration via IP (Lang et al., 2015), both PKC and Ca^{2+} have been reported to stimulate adenylyl cyclase (AC) which in turn leads to increased cAMP levels (Mons et al., 1998). This might explain the small change in cAMP concentrations for full-length galanin since both cAMP stimulating as well as cAMP decreasing routes could be activated. If comparing classical signaling alongside the new label-free real-time assays according to cell line used it is obvious that similar or somewhat better sensitivity can be observed using label-free assays. The signaling cascade can be further evaluated using the real-time label-free techniques together with inhibitors, as shown by Bouvier and colleagues that presented an additional signaling route for the β_2-adrenergic receptor (Stallaert et al., 2012). These techniques can also be used to evaluate primary cell cultures, an interesting aspect since GPCRs recently have been reported to exist as heteromers in vivo (Borroto-Escuela et al., 2014; Fuxe et al., 2012; Narvaez et al., 2015). But in this study, the real-time label-free techniques were used to evaluate their potential use as a screening method for novel galanin receptor ligands.

5. Conclusions

Ala[5]-galanin (2–11) was found to be the shortest GAL_2R specific agonist and the importance of serine at position five from a previous in silico study of GAL_3R was here experimentally verified. Two classical second messenger assays for GPCR signaling was compared with two label-free real-time techniques on both high receptor expressing cells as well as a lower receptor expressing cell lines resulting in similar or somewhat better sensitivity observed for the label-free techniques.

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