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Preferential activation by galanin 1–15 fragment of the GalR1 protomer of a GalR1–GalR2 heteroreceptor complex

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ABSTRACT

The three cloned galanin receptors show a higher affinity for galanin than for galanin N-terminal fragments. Galanin fragment (1–15) binding sites were discovered in the rat Central Nervous System, especially in dorsal hippocampus, indicating a relevant role of galanin fragments in central galanin communication. The hypothesis was introduced that these N-terminal galanin fragment preferring sites are formed through the formation of GalR1–GalR2 heteromers which may play a significant role in mediating galanin fragment (1–15) signaling. In HEK293T cells evidence for the existence of GalR1–GalR2 heteroreceptor complexes were obtained with proximity ligation and BRET^2 assays. PLA positive blobs representing GalR1–GalR2 heteroreceptor complexes were also observed in the raphe-hippocampal system. In CRE luciferase reporter gene assays, galanin (1–15) was more potent than galanin (1–29) in inhibiting the forskolin-induced increase of luciferase activity in GalR1–GalR2 transfected cells. The inhibition of CREB by 50 nM of galanin (1–15) and of galanin (1–29) was fully counteracted by the non-selective galanin antagonist M35 and the selective GalR2 antagonist M871. These results suggested that the orthosteric agonist binding site of GalR1 protomer may have an increased affinity for the galanin (1–15) vs galanin (1–29) which can lead to its demonstrated increase in potency to inhibit CREB vs galanin (1–29). In contrast, in NFAT reporter gene assays galanin (1–29) shows a higher efficacy than galanin (1–15) in increasing Gq/11 mediated signaling over the GalR2 of these heteroreceptor complexes. This disbalance in the signaling of the GalR1–GalR2 heteroreceptor complexes induced by galanin (1–15) may contribute to depression-like actions since GalR1 agonists produce such effects.

1. Introduction

It was discovered that galanin in nanomolar concentrations can reduce the affinity of postjunctional [^{3}H]-5HT1A agonist binding sites in limbic membrane preparations which may reduce 5-HT1A recognition and probably signaling in the limbic system via antagonistic receptor–receptor interactions [1,2]. This suggested that galanin via such effects can contribute to development of depression, since postjunctional 5-HT1A receptors are one of the major targets for several antidepressant drugs [3]. The above results represented the first indications that brain GalR1–5-HT1A heteroreceptor complexes exist in which the GalR1 protomer inhibits postjunctional 5-HT1A recognition via allosteric receptor–receptor interactions. By means of Fluorescent Resonance Energy Transfer the GalR1–5-HT1A heteromers could subsequently be demonstrated in cellular models [4]. It was in these experiments also possible to demonstrate with CRE-luciferase and SRE-luciferase reporter gene assays an antagonistic allosteric receptor–receptor interactions in the regulation of the signaling of the 5-HT1A and GalR1 protomers which leads to transinhibition of the Adenylyl Cyclase (AC) and Mitogen Activated Protein Kinase pathways.

The three cloned galanin receptors (GalR1, Gi/o coupled; GalR2, Gq coupled; and GalR3, Gi/o coupled) show a higher affinity for galanin than for galanin N-terminal fragments like Gal (1–15) [5]. A substantial further development was therefore the demonstra-

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tion of specific N-terminal galanin fragment (1–15) binding sites in the rat Central Nervous System indicating a relevant role of galanin fragments in central galanin communication, especially in dorsal hippocampus, neocortex and striatum which show only few high affinity galanin (1–29) binding sites [6]. We have therefore introduced the hypothesis that these N-terminal galanin fragment (1–15) preferring sites may be formed through the formation of GalR1–GalR2 heteroreceptor complexes which leads to conformational changes in their galanin recognition sites converting GalR1 and/or GalR2 into galanin fragment preferring binding sites with reduced affinity for galanin (1–29) [3].

In the current work evidence is provided by Bioluminescence Resonance Energy Transfer ($BRET^2$) and *in situ* Proximity Ligation Assay (*in situ* PLA) analyses of the existence of GalR1–GalR2 heteroreceptor complexes in cotransfected HEK cells and in the hippocampal formation and parts of the raphe dorsalis and raphe medianus of the midbrain. Furthermore, in CRE-luciferase reporter gene assays Gal(1–15) is found to be substantially more potent than Gal(1–29) in increasing Gi/o mediated signaling in the GalR1–GalR2 heteroreceptor complexes in the HEK cells in line with the hypothesis.

2. Materials and methods

2.1. Plasmid constructs, cell culture, transfection and confocal microscopy

The constructs presented herein were made using standard molecular biology as described previously [4]. HEK293T cells were grown and transiently transfected as depicted in Borroto-Escuela et al. [4].

2.2. In situ Proximity Ligation Assay

In situ Proximity Ligation Assay (*in situ* PLA) was performed as described previously [7–9]. Free-floating brain sections from formalin perfused rats and HEK293 cell cultures were employed using the following primary antibodies: goat monoclonal anti-GalR1 (Abcam, Sweden) and rabbit monoclonal anti-GalR2 (VTG, Sweden). Control experiments employed only one primary antibody or cells transfected with cDNAs encoding only one type of receptor. PLA signal was visualized and quantified by using a confocal microscope Leica TCS-SL confocal microscope (Leica, USA) and the Duolink Image Tool software.

2.3. $BRET^2$ saturation assay

Forty-eight hours after transfection, HEK293T cells transiently transfected with constant (1 μg) or increasing amounts (0.12–5 μg) of plasmids encoding for $GalR1^{Rluc}$ and $GalR2^{GFP2}$ respectively, were rapidly washed twice in PBS, detached, and resuspended in the same buffer. Cell suspensions (20 μg protein) were distributed in duplicate into the 96-well microplate black plates with a transparent bottom (Corning 3651) (Corning, Stockholm, Sweden) for fluorescence measurement or white plates with a white bottom (Corning 3600) for BRET determination. For $BRET^2$ measurement, coelenterazine-400a also known as *DeepBlue*™C substrate (VWR, Sweden) was added at a final concentration of 5 μM. Readings were performed 1 min after using the POLARstar Optima plate-reader (BMG Labtechnologies, Offenburg, Germany) that allows the sequential integration of the signals detected with two filter settings [410 nm (with 80 nm bandwidth) and 515 nm (with 30 nm bandwidth)]. The $BRET^2$ ratio is defined as previously described [10,11].

2.4. $BRET^2$ competition assay

Forty-eight hours after transfection, HEK293T cells transiently transfected with constant amounts (0.75 μg) of plasmids encoding for $GalR1^{Rluc}$ and $GalR2^{GFP2}$ and increasing amounts (0.1–8 μg) of plasmids encoding for wild-type GalR1 or GalR2, 5-HT1A and the mock pcDNA3.1+; respectively. The energy transfer was determined as described for the $BRET^2$ saturation assay.

2.5. Luciferase reporter gene assay

We used a dual luciferase reporter assay to indirectly detect variations of cAMP levels, PLC-β activity, or activations of MAPK pathway in transiently transfected cell lines treated with different compounds in a range of concentrations (typically 10 nM to 1 μM). For luciferase assays, 24 h before transfection, cells were seeded at a density of 1×10^6 cells/well in 6-well dishes and transfected with TransFectin. Cells were co-transfected with plasmids corresponding to three constructs as follows (per 6-well): 2 μg firefly luciferase-encoding experimental plasmid (pGL4-CRE-luc2p or pGL4-NFAT-luc2p; Promega, Stockholm, Sweden), 1 μg of GalR1 plus GalR2 expression vectors and 0.5 μg *R*luc-encoding internal control plasmid (phRG-B; Promega). Approximately 46 h post transfection, after the cells were incubated with appropriate ligands and harvested with passive lysis buffer (Promega), the luciferase activity of cell extracts was determined using a luciferase assay system according to the manufacturer's protocol in a POLARstar Optima plate reader (BMG Labtechnologies, Offenburg, Germany) using a 535 nm filter with a 30-nm bandwidth. Firefly luciferase was measured as firefly luciferase luminescence over a 15 s reaction period. The luciferase values were normalized against *R*luc luminescence values.

2.6. Statistical analysis

The number of samples (n) in each experimental condition is indicated in Figure legends. All data were analyzed using GraphPad PRISM 4.0 (GraphPad Software, USA). When two experimental conditions were compared, statistical analysis was performed using an unpaired t test. Otherwise, statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Tukey's Multiple Comparison post-test. BRET isotherms were fitted using a nonlinear regression equation assuming a single binding site, which provided BRETmax and BRET50 values.

3. Results

3.1. Cellular models

3.1.1. Colocation of GalR1/GalR2

By confocal microscopy analysis $GalR1^{YFP}$ (pseudocolor in red) was found to be colocated with $GalR2^{GFP2}$ (pseudocolor in green) in the plasma membrane of HEK293T cells as shown from the development of a yellowish fluorescence (Supplementary Fig. 1A).

3.1.2. GalR1–GalR2 heteromerization

Red PLA positive clusters (blobs) were observed in HEK293T cells transiently co-expressing GalR1 and GalR2 but not after single GalR1 expression representing GalR1–GalR2 heteroreceptor complexes (Supplementary Fig. 1B). The PLA positive blobs were observed mainly in the cell surface membrane. $BRET^2$ assay was performed on HEK293T cells co-transfected with a constant amount of the $GalR1^{Rluc}$ construct while increasing the concentrations of the $GalR2^{GFP2}$ plasmid. As shown in Fig. 1B, significant, strong and saturable $BRET^2$ signals were found for the

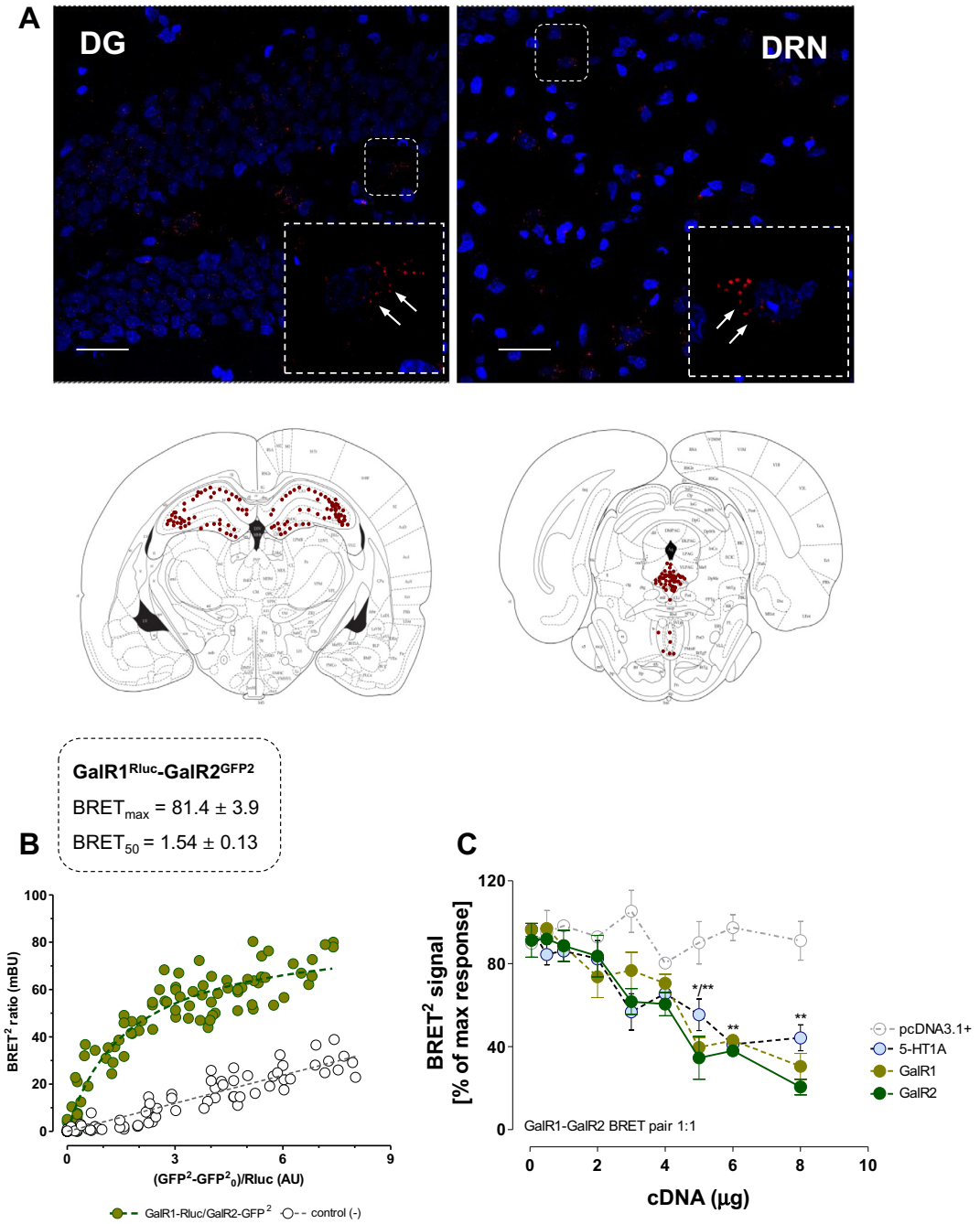

Fig. 1. (A) GalR1–GalR2 heteroreceptor complexes in hipocampal and median raphe sections of rat detected by PLA. *In situ* PLA was performed using primary antibodies of different species directed to GalR1 and GalR2 (see Section 2). The detected heteroreceptor complexes are seen as red clusters indicated by arrows. Specific GalR1–GalR2 clusters are visualized within discrete regions of the hippocampus and median raphe. They were almost absent in the corpus callosum (cc). The relative densities of PLA cluster distributions are schematically illustrated by the density of red puncta. Nuclei are shown in blue (DAPI). Scale bars are shown in the right low part of each panel. Three independent experiments (three rats) were performed. Bregma level −1.7 and −7.64. (B) $BRET^2$ saturation curves for the GalR1–GalR2 heteromers with increasing expression levels of the GFP^2 tagged GalR2 receptor. Cells individually expressing $GalR1^{Rluc}$ were mixed prior to exposition to coelenterazine-400 with cells individually expressing $GalR2^{GFP2}$ as a negative control. Plotted on the *X*-axis is the fluorescence value obtained from the GFP^2, normalized with the luminescence value of $D_{2L}R^{Rluc}$ expression 10 min after coelenterazine incubation. Mean ± S.E.M.; $n = 10$, in triplicate. The GalR1–GalR2 curve fitted better to a saturation curve than to a linear regression as found with mixed pool of cell from cell individually expressing $GalR1^{Rluc}$ + $GalR2^{GFP2}$ (*F* test ($P < 0.01$). Data are means ± S.E.M. ($n = 6$). (C) $BRET^2$ competition experiment for the GalR1–GalR2 heteromers. A fixed ratio (1:1) of expression levels of the $GalR1^{Rluc}$/$GalR2^{GFP2}$ tagged receptors was used in presence of increasing concentrations of wild-type receptors. Plotted on the *X*-axis is the concentration of cDNA transfected per competitor. Mean ± S.E.M.; $n = 10$ in triplicate. *: 5HT1A group (5 µg cDNA) is significantly different compared to pcDNA3 + group ($P < 0.05$); **: GalR1 and GalR2 groups (5 µg cDNA each) are significantly different compared to pcDNA3 + group ($P < 0.01$); **: 5HT1A, GalR1 and GalR2 groups are significantly different compared to pcDNA3 + group in the range from 6 to 8 µg cDNA ($P < 0.01$) by two-way analysis of variance (ANOVA). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

GalR1–GalR2 pair, giving high $BRET^2$ maximal values ($BRET^2_{max}$ of 81.4 ± 3.9 mBU). No specific $BRET^2$ signal was obtained from a mixture of singly expressing $GalR1^{Rluc}$ cells and $GalR2^{GFP2}$ cells (Fig. 1B, control (−)). In all cases $BRET^2$ signaling in cotransfected cells increased as a hyperbolic function to the increasing concentration of the GFP^2 fusion construct, reaching an asymptote at the highest

concentrations used. Thus, since the negative control led to a quasi-linear curve, the specificity of the saturation (hyperbolic) assay for the GalR1^{Rluc} and GalR2^{GFP2} pair could be established. The specificity of this interaction was also indicated in Fig. 1C where increasing concentrations of expressed WT receptor (GalR1 and GalR2) in combination with the protomers of the BRET pair (constant ratio 1:1) led to a concentration dependent disappearance of the BRET^2 signal. The same was also true for increasing concentrations of expressed 5-HT1A receptor known to form heteromers with GalR1 [4]. Instead co-transfection of pcDNA3.1+ in the same concentration range failed to diminish the signal.

3.1.3. Effects of N-terminal galanin (1–15) vs galanin (1–29) on the signaling of the GalR1–GalR2 heteroreceptor complexes

A differential role of galanin 1–29 and the galanin fragment 1–15 in the signaling cascades Gi/o-AC-PKA and Gq-PLC-PKC/Ca was determined in CRE and NFAT-luciferase reporter gene assays in heterologous HEK293T cells coexpressing the GalR1 and/or GalR2 receptors.

As seen from the concentration–response curves in Fig. 2A, galanin fragment (1–15) was more potent than galanin (1–29) in inhibiting the forskolin-induced increase of luciferase activity in GalR1–GalR2 transfected cells. (EC_{50} (galanin 1–15) ~0.92 nM and EC_{50} (galanin 1–29) ~35.8 nM).

The inhibition of CREB by 50 nM of galanin 1–15 and of galanin1–29 was fully counteracted by the non-selective galanin antagonist M35 and the selective GalR2 antagonist M871 (Fig. 2B). After single transfection with GalR2, galanin fragment1–15 and galanin 1–29 lacked at 50 nM failed to inhibit CREB (Fig. 2B). Taken together, these results suggest that the orthosteric agonist binding site of GalR1 may have an increased affinity for the galanin 1–15 fragment vs galanin 1–29 which can lead to its demonstrated increase in potency to inhibit CREB vs galanin 1–29.

In GalR1–GalR2 transfected cells, Gal1–29 at 50 nM produced a large and highly significant increase in NFAT levels, while Gal1–15 failed to significantly increase NFAT levels (Fig. 3A). The effects of galanin1–29 were significantly counteracted by both M35 and M871 (1 μM) and more markedly by the selective GalR2 antagonist. In GalR2 singly transfected cells similar results were obtained but here the effect of the Gal1–15 fragment became significant (Fig. 3B). The increases in NFAT levels observed with Gal1–29 and Gal1–15 were both fully counteracted by M871 (1 μM).

3.1.4. GalR1–GalR2 heteromerization in the rat brain

PLA positive blobs representing GalR1–GalR2 heteroreceptor complexes were observed in several discrete regions of rat hippocampal formation at Bregma level −1.7 using free floating transverse cryostat sections (30 μm). The highest density was

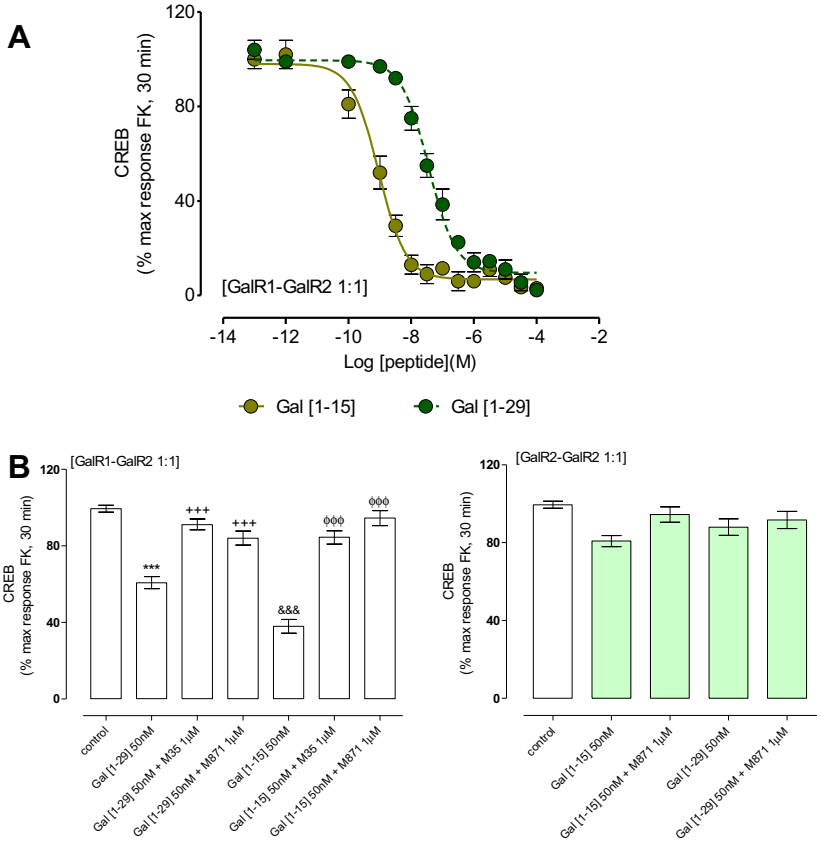

Fig. 2. Agonist-induced GalR1 and GaLR2 receptor activation in a forskolin-induced CRE-luciferase reporter gene assay. (A) Dose–response curves with N-fragment Gal 1–15 and Gal1–29 in GalR1–GalR2 co-transfected HEK293T cells. HEK293T cells were transiently co-transfected with 2 μg firefly luciferase-encoding experimental plasmid (pGL4-CRE-luc2p), 1 μg of both (GalR1 and GalR2) expression vectors and 0.5 μg *Renilla* luciferase-encoding internal control plasmid (phRG-B). Forty-six hours after transfection, cells were treated for 30 min with 2 μM forskolin (sub-maximal concentration value), agonist or antagonist (in presence of agonist) and the luciferase activity was measured after 4 h. Light emission is expressed as a percentage of the control forskolin-induced value. The data represent the mean ± S.E.M. of five independent experiments performed in quadruplicate (EC_{50} ~0.92 nM in Gal1–15 group and EC_{50} ~35.8 nM in the Gal 1–29 group). (B) HEK293T cells were transiently co-transfected with 2 μg firefly luciferase-encoding experimental plasmid (pGL4-CRE-luc2p), 1 μg of both (GalR1 and GalR2) expression vectors and 0.5 μg *Renilla* luciferase-encoding internal control plasmid (phRG-B). Forty-six hours after transfection, cells were treated for 30 min with 2 μM forskolin (sub-maximal concentration value), agonist or antagonist (in presence of agonist) and the luciferase activity was measured after 4 h. Light emission is expressed as a percentage of the control forskolin-induced value. The data represent the mean ± S.E.M. of six independent experiments performed in quadruplicate. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Tukey's Multiple Comparison post-test. The *P* value 0.05 and lower was considered significant. ***: Significantly different compared to control ($P < 0.001$); +++: Significantly different compared to Gal 1–29, 50 nM ($P < 0.001$); &&&: Significantly different compared to Gal 1–29, 50 nM ($P < 0.001$). ϕϕϕ: Significantly different compared to Gal 1–15, 50 nM ($P < 0.001$).

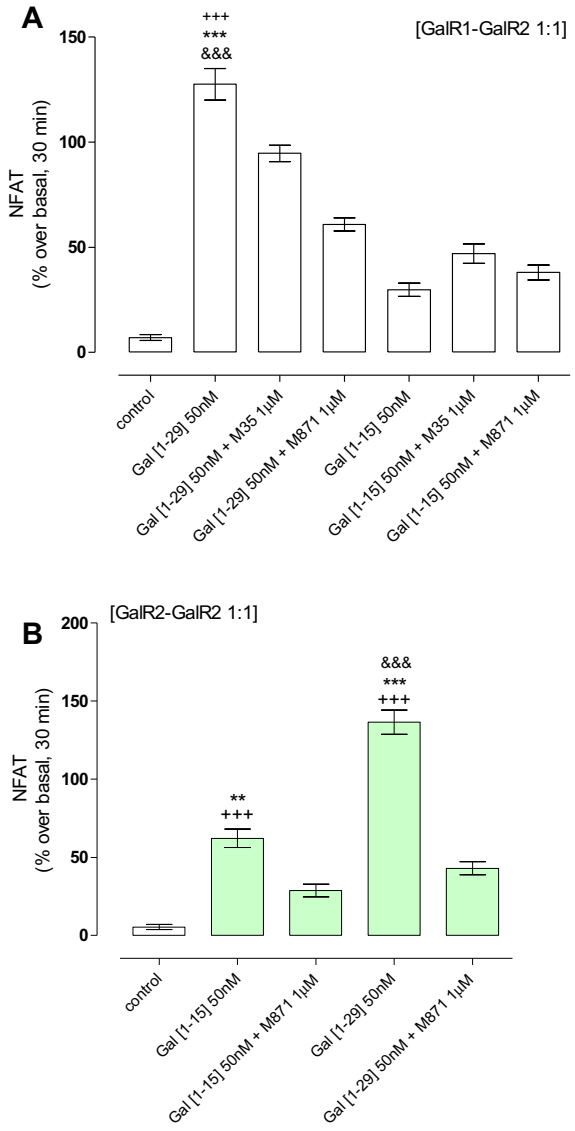

Fig. 3. Agonist-induced GalR1 and GaLR2 receptor protomer activation in an NFAT-luciferase reporter gene assay. (A) HEK293T cells were transiently co-transfected with 2 μg firefly luciferase-encoding experimental plasmid (pGL4-NFAT-luc2p), 1 μg of both (GalR1 and GalR2) expression vectors and 0.5 μg *Renilla* luciferase-encoding internal control plasmid (phRG-B). Forty-six hours after transfection, cells were treated for 30 min with agonist or antagonist (in presence of agonist) and the luciferase activity was measured after 4 h. Light emission is expressed as a percentage over basal values. The data represent the mean ± S.E.M. of four independent experiments performed in quadruplicate. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Tukey's Multiple Comparison post-test. The *P* value 0.05 and lower was considered significant. &&&: Significantly different compared to control group ($P < 0.001$); ***: Significantly different compared to Gal 1–29 + M35 group and Gal 1–29 + M871 group ($P < 0.001$); +++: Significantly different compared to Gal 1–15, 50 nM ($P < 0.001$). (B) HEK293T cells were transiently co-transfected with 2 μg firefly luciferase-encoding experimental plasmid (pGL4-NFAT-luc2p), 1 μg of GalR2 expression vector and 0.5 μg *Renilla* luciferase-encoding internal control plasmid (phRG-B). Forty-six hours after transfection, cells were treated for 30 min with agonist or antagonist (in presence of agonist) and the luciferase activity was measured after 4 h. Light emission is expressed as a percentage over basal value. The data represent the mean ± S.E.M. of four independent experiments performed in quadruplicate. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Tukey's Multiple Comparison post-test. The *P* value 0.05 and lower was considered significant. +++: Significantly different compared to control ($P < 0.001$); **: Significantly different compared to Gal 1–15 + M871 ($P < 0.01$). ***: Significantly different compared to Gal 1–29 + M871 ($P < 0.001$); &&&: Significantly different compared to Gal 1–15 ($P < 0.001$).

observed in the CA3 area with medium densities in CA1 and CA2 areas and parts of the dentate gyrus (ventral leaflet and the polymorphic layer) (Fig. 1A). Also no PLA positive clusters over background in negative controls were observed in the cortical regions analyzed and corpus callosum at this Bregma level. The densities of red spheres in the lowest left panel of Fig. 1A illustrate the relative densities of PLA GalR1–GalR2 positive blobs found in the different regions of the hippocampal formation.

High densities of PLA GalR1–GalR2 positive blobs were also observed in the ventral and ventrolateral subnuclei of the dorsal raphe but not in the dorsal and dorsolateral subnuclei as shown in Fig. 1A, especially in the lowest panel (right). Medium densities were found in parts of the median raphe.

4. Discussion

The experimental work in the current study gives evidence for the existence of GalR1–GalR2 heteroreceptor complexes in GalR1–GalR2 cotransfected HEK cells and in the rat hippocampal formation and in parts of the dorsal and median raphe of the midbrain based on BRET^2 analysis and *in situ* Proximity Ligation Assays. Their potential existence in the hippocampal formation has been postulated [3] to explain the much higher potency of galanin fragment (1–15) vs galanin (1–29) in reducing the affinity of the 5-HT1A agonist binding sites in the hippocampal formation [12]. This may be possible through allosteric receptor–receptor interactions in postulated GalR1–GalR2–5-HT1A heteroreceptor complexes increasing the affinity of the galanin fragment for this heteroreceptor complex [3,13]. The results obtained in cellular models in the current paper give support to this hypothesis through studies on the effects of galanin (1–15) and galanin (1–29) on Gi/o mediated signaling in GalR1–GalR2 heteroreceptor complexes in HEK cells using a CRE luciferase reporter gene assay.

Galanin (1–15) was found to be substantially more potent than galanin (1–29) to reduce CREB levels in GalR1–GalR2 cotransfected cells. Both galanin peptides lacked effects on CREB in the GalR2 alone transfected cells. Therefore, the GalR1 protomer may preferentially bind galanin (1–15) vs galanin (1–29) and activate its Gi/o mediated signaling leading to enhanced reductions of AC activity and CREB. Thus, the GalR1 protomer in the GalR1–GalR2 heteromer appears to be an N-terminal galanin fragment preferring receptor in line with the hypothesis [3,13].

The inhibitory effects on CREB by both galanin peptides were blocked by an unselective galanin receptor antagonist and unexpectedly also by a selective GalR2 antagonist M871. It therefore seems possible that the GalR1 protomer in the GalR1–GalR2 heteromer can also change its galanin receptor antagonist pharmacology and be blocked by the GalR2 antagonist M871 at the concentration used (1 μM).

In contrast to the GalR1 protomer, the GalR2 protomer was more effectively activated by galanin (1–29) than by galanin (1–15) at 50 nM as studied in the NFAT luciferase reporter gene assay which monitors the activity of the Gq/11 mediated PLC-PKC/Calcium signaling pathways. At 50 nM galanin (1–15) fragment did not even significantly increase NFAT levels. Similar marked and highly significant increases were obtained with galanin (1–29) in HEK cells singly transfected with GalR2 but here the galanin fragment (1–15) produced a highly significant but modest increase in NFAT. The actions of galanin (1–29) and galanin (1–15) were fully blocked by the selective GalR2 antagonist M871 in the singly transfected cells where GalR2 homomers were likely in dominance. In GalR1–GalR2 cotransfected cells containing the GalR1–GalR2 heteromers M871 substantially blocked the NFAT increase induced

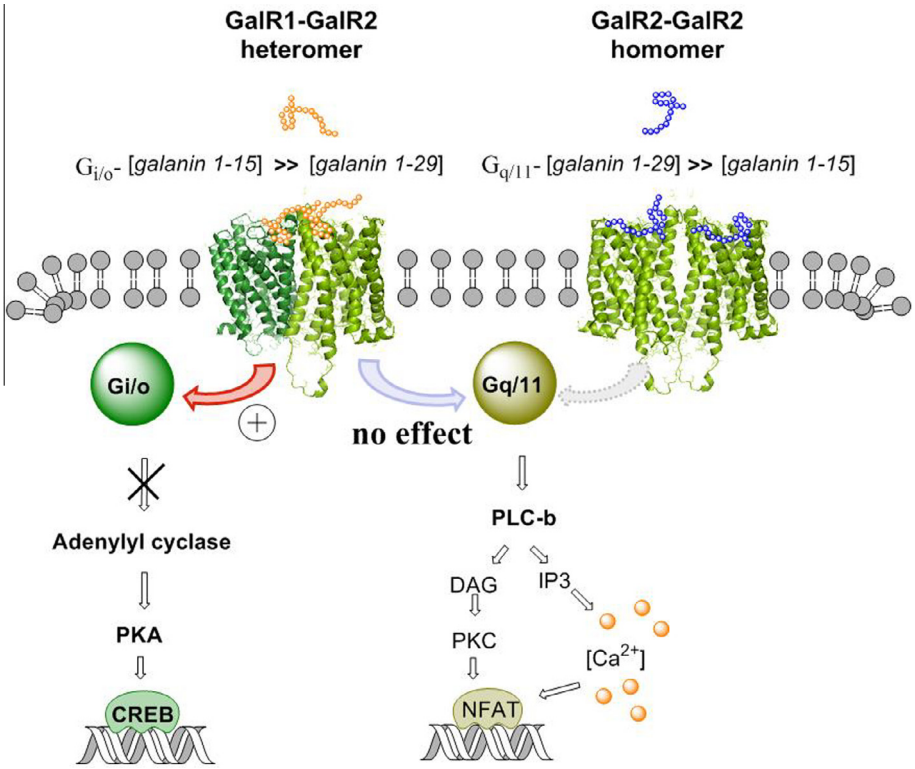

Fig. 4. Illustration of the actions of galanin (1–15) vs galanin (1–29) at the GalR1–GalR2 heterodimer including a comparison with their actions at the GalR2 homodimer. Galanin (1–15) is shown to have an increased potency vs galanin (1–29) to activate the Gi/o mediated signaling of the GalR1 protomer in the GalR1–GalR2 heterodimer using the CRE luciferase reporter gene assay. Instead galanin fragment (1–15) failed to produce a significant activation of the Gq/11 mediated signaling of the GalR2 protomer, while galanin (1–29) produced a marked increase of this signaling as studied in the NFAT luciferase reporter gene assay. At the GalR2 homodimer the galanin fragment (1–15) did produce a significant increase of the Gq/11 mediated signaling but showed a substantially lower efficacy than galanin (1–29). Thus, a unique feature of galanin (1–15) is its ability to produce a disbalance of the signaling of the GalR1–GalR2 heterodimer with enhanced activation of the Gi/o mediated signaling via the GalR1 protomer and no significant effects on the Gq/11 mediated signaling via the GalR2 protomer. It is expected to lead to enhanced depression like actions.

by galanin (1–29) while the unselective galanin antagonist produced only a small reduction of the NFAT increase induced by galanin (1–29) in the concentration tested (1 μM). The results suggest that galanin fragment (1–15) fails to activate the Gq/11 coupled GalR2 protomer in the GalR1–GalR2 heteromer while it has a significant ability to increase the Gq/11 mediated signaling in the GalR2 homomer. Thus, the galanin fragment (1–15) appears to preferentially target the GalR1 Gi/o coupled protomer in the GalR1–GalR2 heteromer without significantly increasing the Gq/11 mediated signaling in the GalR2 protomer. (Fig. 4).

With the Proximity Ligation Assay it was possible to demonstrate low to high densities of GalR1–GalR2 heteroreceptor complexes in the hippocampal formation in the dorsal and median raphe nuclei, areas known to be rich in galanin N-terminal binding sites [6,12]. Thus, galanin (1–15) fragment may produce its brain actions mainly by targeting the GalR1–GalR2 heteroreceptor complexes located especially in the hippocampal formation and the midbrain raphe nuclei. It should be noted that galanin fragment (1–15) also shows a higher potency and efficacy than galanin (1–29) in reducing the affinity of the 5-HT1A binding sites in ventral limbic cortex [14]. Based on the current findings in cellular models it seems likely that in the brain one major molecular mechanism for galanin fragment (1–15) may be its preferential activation of the GalR1 protomer Gi/o mediated signaling in the GalR1–GalR2 heteroreceptor complex. This signaling becomes dominant since according to the current findings in cellular models the galanin fragment (1–15) is not able to activate the GalR2 protomer Gq/11 mediated signaling. Furthermore, the GalR1 agonist M617 has been found upon intraventricular infusion to produce depression-like behavior [15]. Therefore, we propose that the depression-like actions of GalR1 agonists may to a substantial degree be due to an activation of the GalR1 protomer in the GalR1–GalR2 heteroreceptor complex located especially in the hippocampal formation and the midbrain raphe nuclei. It seems likely that galanin fragment (1–15) also may produce depression-like actions.

Taken together, GalR1–GalR2 heteroreceptor complexes have been discovered in cellular models and in the raphe-hippocampal system of the rat using BRET2 and PLA assays. In cellular models using CREB and NFAT luciferase reporter gene assays, the galanin fragment (1–15) vs galanin (1–29) was found to preferentially activate the GalR1 protomer Gi/o mediated signaling reducing the activity of the AC-PKA-CREB signaling pathway while failing to activate the GalR2 protomer Gq/11 mediated PLC-PKC/calcium signaling pathway. This disbalance in the signaling of the GalR1–GalR2 heteroreceptor complexes (Fig. 4) induced by galanin fragment (1–15) may lead to state of depression which is currently being investigated.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.08.061.

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