Neuroanatomical characterization of the G protein-coupled receptor activity evoked by galanin-related ligands

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ABSTRACT

Galanin neuropeptide is distributed throughout the mammalian nervous system modulating a plethora of diverse physiological functions, including nociception, cognition and neuroendocrine regulation. The regulation of the galaninergic system is an interesting approach for the treatment of different diseases associated to those systems. Nevertheless, the pharmacological selectivity and activities of some galanin receptor (GalR) ligands are still in discussion and seem to depend on the dose, the receptor subtype and the second messengers to which they are coupled at different brain areas. The activity of different GalR ligands on $G_{i/o}$ proteins, was evaluated by the guanosine 5′-(γ-[^{35}S]thio)triphosphate ([^{35}S]GTPγS) autoradiography in vitro assay applied to rat brain tissue slices in the presence of galanin, M15, M35, M40, gal(2−11) or galnon. The enhancement of the [^{35}S]GTPγS binding induced by the chimerical peptides M15, M35 and M40 was similar to that produced by Gal in those brain areas showing the highest stimulations, such as dorsal part of the olfactory nucleus and ventral subiculum. In contrast to these peptides, using gal(2−11) no effect was measured on $G_{i/o}$ protein coupling in areas of the rat brain with high GalR$_1$ density such as posterior hypothalamic nucleus and amygdala, indicating low selectivity for GalR$_1$ receptors. The effects evoked by the non-peptide ligand, galnon, were different from those induced by galanin, behaving as agonist or antagonist depending on the brain area, but the stimulations were always blocked by M35. Thus, the activity of most used GalR ligands on $G_{i/o}$ protein mediated signalling is complex and depends on the brain area. More selective and potent GalR ligands are necessary to develop new treatments aimed to modulate the galaninergic system.

1. Introduction

Galanin is a neuropeptide distributed throughout the mammalian central nervous system (CNS). Galanin is particularly present in nucleus basalis of Meynert, some areas of the hypothalamus, hippocampus and amygdala, and is also expressed in the raphe and locus coeruleus nuclei (Melander et al., 1985; Gentleman et al., 1989; Skofitsch et al., 1986). This neuropeptide exerts its physiological effects by the interaction with G protein-coupled receptors (GPCR). To date, three different galanin receptor (GalR) subtypes (GalR$_1$, GalR$_2$ and GalR$_3$) have been identified and cloned (for review Branchek et al., 2000). All of them inhibit adenylyl cyclase activity by the coupling to $G_{i/o}$ proteins (Wang et al., 1998), even if GalR$_2$ can be also coupled to $G_{q/11}$ promoting the activation of phospholipase C (Duan et al., 2022; Smith et al., 1997; Wang et al., 1998). Galanin is not the unique endogenous ligand for GalR; at

present, three other peptides belonging to the galanin family have been described: galanin message-associated protein (GMAP), the galanin-like peptide (GALP) and alarin, although both GMAP and alarin show very low affinity to galanin receptors (Boughton et al., 2010; Lundkvist et al., 1995; Ohtaki et al., 1999; Santic et al., 2006; Wang et al., 1997). Recently, another endogenous ligand for GalR2 and GalR3, called spexin, has been described by synteny, examining the evolutionary connection of the physical co-localization of SPX, GAL and KISS genes in some vertebrate chromosomes (Kim et al., 2014).

Galanin in involved in the modulation of many physiological processes including nociception, cognition, neuroendocrine regulation, and, due to it expression with serotonin and noradrenaline, the possible role of the peptide in mood regulation (Bellido et al., 2002; Liu et al., 2001; McDonald et al., 1998; Melander et al., 1987). Galanin has been also involved in the physiopathology of different neurodegenerative and

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neuropsychiatric diseases, including Alzheimer's disease or anxiety, respectively. Regulations on galanin synthesis or the expression of this neuropeptide itself as well on GalR have been reported in those neurological diseases (Chan-Palay, 1988; Möller et al., 1999; Zhao et al., 2013). Thus, the synthesis of selective and potent drugs that act on GalR could help to develop new therapeutic approaches. However, most of ligands showing affinity for GalR subtypes have a low potency and selectivity. Mainly, GalR ligands are fragments or peptides derived from galanin, owing to the fact that the first 16 amino acids of galanin show high affinity for GalR and are critical for the binding of this neuropeptide to its receptors (Langel and Bartfai, 1998). Indeed, different galanin fragments have been used as GalR agonists, although their pharmacological profile differ from that produced by galanin; e.g., Gal (1−15) seems to have stronger effects than observed with galanin in behavioral tests related to anxiety-like behaviors (Millón et al., 2014). In this sense, Gal(1−15) has been assayed for possible therapeutic applications as antidepressant in an augmentation strategy in combination with SSRI-type compounds (Flores-Burgess et al., 2017, 2019, 2022). On the other hand, Gal (1−16) fragment shows a different pharmacological profile from that produced by galanin in dorsal hippocampus (Fisone et al., 1989; Girotti et al., 1993; Givens et al., 1992). These discrepancies indicate that the galanin N-terminal fragment is particularly relevant for the GalR subtypes selectivity (Hedlund et al., 1994). Another galanin fragment proposed as $GalR_2$ agonists is gal(2−11), also named as AR-M1896, but it has been detected that gal(2−11) also recognizes $GalR_3$ subtype with a similar affinity (Liu et al., 2001; Lu et al., 2005).

Chimerical peptides derived from galanin N-terminal fragment have also been synthesized. For example: M15 [gal(1−13)-substance P (5−11)] (Bartfai et al., 1991), M32 [gal(1−13)-neuropeptide Y(25−36)] (Arvidsson et al., 1993); M35 [gal(1−13)-bradykinin(2−9)] (Ogren et al., 1992), C7 [gal(1−13)-spantide I] (Crawley et al., 1993), galparan [gal(1−13)-mastoparan] (Langel et al., 1996), gal(1−13)-[Ala^{10,11}]ET-I (6−21)-NH$_2$ (Ruczynski et al., 2005), M38 [gal(1−13)-(Ala-Leu)$_3$-Ala-NH$_2$] (Xu et al., 1995) and M40 [gal(1−13)-Pro-Pro-(Ala-Leu)$_3$-Ala-NH$_2$] (Leibowitz and Kim, 1992). Several studies have showed GalR antagonist-like properties for these molecules, but experimental evidences seem to point out that some of these ligands could behave also as agonists depending on the dose, type of tissue or GalR subtype (Bartfai et al., 1993; Fathi et al., 1997; Korolkiewicz et al., 2002; Wang et al., 1999). Thus, M35 acts as an antagonist in many experimental models such as the flexor reflex and chronic constriction injury of the sciatic nerve in rat, but in galanin knockout (KO) mice, this peptide would have an agonist effect enhancing neurite outgrowth from cultured adult dorsal root ganglia neurons (Mahoney et al., 2003; Xu et al., 1997).

Other two GalR ligands are the M617 [gal(1−13)-Gln^{14}-bradykinin (2−9)-NH$_2$], proposed as a $GalR_1$ agonist, and the M871 [gal(2−13)-Glu-His-(Pro)$_3$-(Ala-Leu)$_2$-Ala-NH$_2$], considered as a $GalR_2$ antagonist (Sollenberg et al., 2006 and, 2010). However, there is no information available about their selectivity for $GalR_3$ subtype.

More recently, other peptidergic galanin analogues have been synthesized, like the agonist Gal-B2 with slight selectivity for the $GalR_2$ than for $GalR_1$ (Robertson et al., 2010); J18, a non-specific agonist (Saar et al., 2013); and Ala^5-galanin (2−11), a full agonist for $GalR_2$ (Webling et al., 2016). In addition, some non-peptidical GalR ligands have been synthetized, such as spirocumaranon (Sch 202596) (Min et al., 1997), [2,3-dihydro-2-(4-methylphenyl)− 1,4-dithiepine-1,1,4,4-tetroxide] (Scott et al., 2000), galnon [(7-((9-fluorenyl-methoxycarbonyl) ciclo-hexylalanyllysyl) amino-4-methylcoumarin)] (Saar et al., 2002) and galmic (Bartfai et al., 2004). These molecules display low-affinity for GalR and they do not discriminate subtypes. However, two compound based on the structure of 3-arylimino-2-indolones, have been proposed as $GalR_3$ specific antagonists: SNAP 37889 [1-phenyl-3-[[3-(-trifluoromethyl)pheny-l]imino]− 1 H-indol-2-one], SNAP 398299 [1-[3-(2-pyrro-lidinylethoxy)phenyl]− 3-{[3-trifluoromethyl)phenyl] aza-methylene}benzo[*d*]azolin-2-one] (Swanson et al., 2005) and [3-(3, 4-diclorophenylimino)− 1-(6-methoxypyridin-3-yl)indolin-2-one (Barr

et al., 2006). Finally, a novel $GalR_1$-specific agonist has recently been described, a methyllanthionine-stabilized GalR agonist, called M89b (Kuipers et al., 2021).

The results of the present study further contribute to elucidate the activity triggered by some of the most used GalR ligands: M15, M35, M40, gal(2−11) and galnon, by mapping the functional coupling to $G_{i/o}$ proteins at different areas of the rat brain. For this purpose, these compounds have been assayed, alone or in combination, on rat brain sections using the guanosine 5′-(γ-[^{35}S]thio)triphosphate ([^{35}S]GTPγS) autoradiography method.

2. Material and methods

2.1. Chemicals

[^{35}S]GTPγS (1250 Ci/mmol) was purchased from PerkinElmer (Boston, MA, USA). Rat galanin, M15 (galantide) and gal(2−11) were acquired from American Peptide Company (Sunnyvale, CA, USA). Galnon, M35 and M40 were obtained from Bachem (Weil am Rhein, Germany). All other chemicals were obtained from standard sources and were of the highest purity commercially available.

2.2. Rat tissue

Eight-week-old male Sprague-Dawley rats (Harlan, Spain) weighting 250–275 g were used. After being anaesthetised, their brains were rapidly removed and frozen at –80 ºC. Then, sections of 20 μm were cut at −20 °C in a cryostat, mounted on gelatine-coated slides and stored at −20 °C until assay.

The present study covers a large number of areas of the entire brain. Thus, the most representative brain levels have been used to measure each of the areas under study. As an example, the 2.70 mm level from Bregma has been used to measure the intrabulbar part of the anterior commissure and the minor forceps of the corpus callosum. On the other hand, the sagittal slices were obtained at 1.90 mm lateral, following the Paxinos and Watson rat brain atlas stereotaxic coordinates (Paxinos and Watson, 1998).

All procedures were performed in accordance with European animal research laws (Directive 2010/63/EU) and the Spanish National Guidelines for Animal Experimentation (RD 53/2013, Law 32/2007). All experimental protocols were approved by the Local Ethics Committee for Animal Research of the University of the Basque Country (UPV/EHU) (CEEA 388/2014).

2.3. [^{35}S]GTPγS autoradiography

Tissue sections were air-dried for 15 min and then incubated in a buffer containing 50 mM Tris-HCl, 3 mM MgCl$_2$, 0.2 mM EGTA, 100 mM NaCl and 3 mU/ml adenosine deaminase (pH 7.4) for 20 min at room temperature. Then, a second incubation was carried out with the same buffer but supplemented with 2 mM GDP and 1 mM DTT for 20 min. Finally, a third incubation was performed in [^{35}S]GTPγS (0.04 nM) for 2 h at 30ºC in absence of ligands (to calculate the basal binding). The affinities of the peptide compounds used differs in terms of pKi in up to four orders of magnitude with that described for galnon (galanin pKi 9.5; M15 pKi 9.6; M35 pKi 10; M40 pKi 8.6 vs galnon pKi 4.9) (IUPHAR/BPS Guide to Pharmacology, 2022). Therefore, to achieve the optimal stimulations of the $GALR_1$ receptor the following concentrations were used: galanin (1 μM), gal(2−11) (1 μM), M15 (1 μM), M35 (1 μM), M40 (1 μM) and galnon (100 μM), as well as in the presence of the galnon/M35 and galnon/M40 mixtures, at the same molarities when used by separate. The non-specific binding was determined in the presence of GTPγS (10 μM). Slides were washed twice in a buffer 50 mM Tris-HCl, dried under a cold airflow and exposed to β radiation-sensitive films (Kodak Biomax MR) with a set of standards, [^{14}C]-microscales (Amersham, UK).

2.4. Quantitative image analysis of autoradiograms

The films were developed, scanned and quantified by transforming the optical densities into nCi/g tissue equivalent (nCi/g t.e.) (Image J-FIJI software, Bethesda, MA, USA). The slide background and non-specific densities were subtracted. The percentages of stimulation were calculated from the basal and ligand-stimulated [^{35}S]GTPγS binding densities according to the formula (stimulated x 100/basal) − 100. Data were expressed as mean values ± SEM of 7 animals. Statistical analyses were carried out using a one-way ANOVA for repeated measures. The criterion for statistical significance was $p < 0.05$ in all statistical evaluations.

The comparative study between different ligands of the GalR

functional coupling to $G_{i/o}$ proteins was analysed using Pearson linear correlations, when the data followed a normal correlation or Spearman's when they did not. The correlation coefficients obtained in these analyses indicate the direction and magnitude of the relationship between the variables (r; interval between −1 and +1), determining the values of statistical probability.

3. Results

3.1. Galanin receptor ligands activity

The highest values of [^{35}S]GTPγS binding stimulation induced by galanin, M15, M35 and M40 (1 μM) were obtained in the dorsal part of
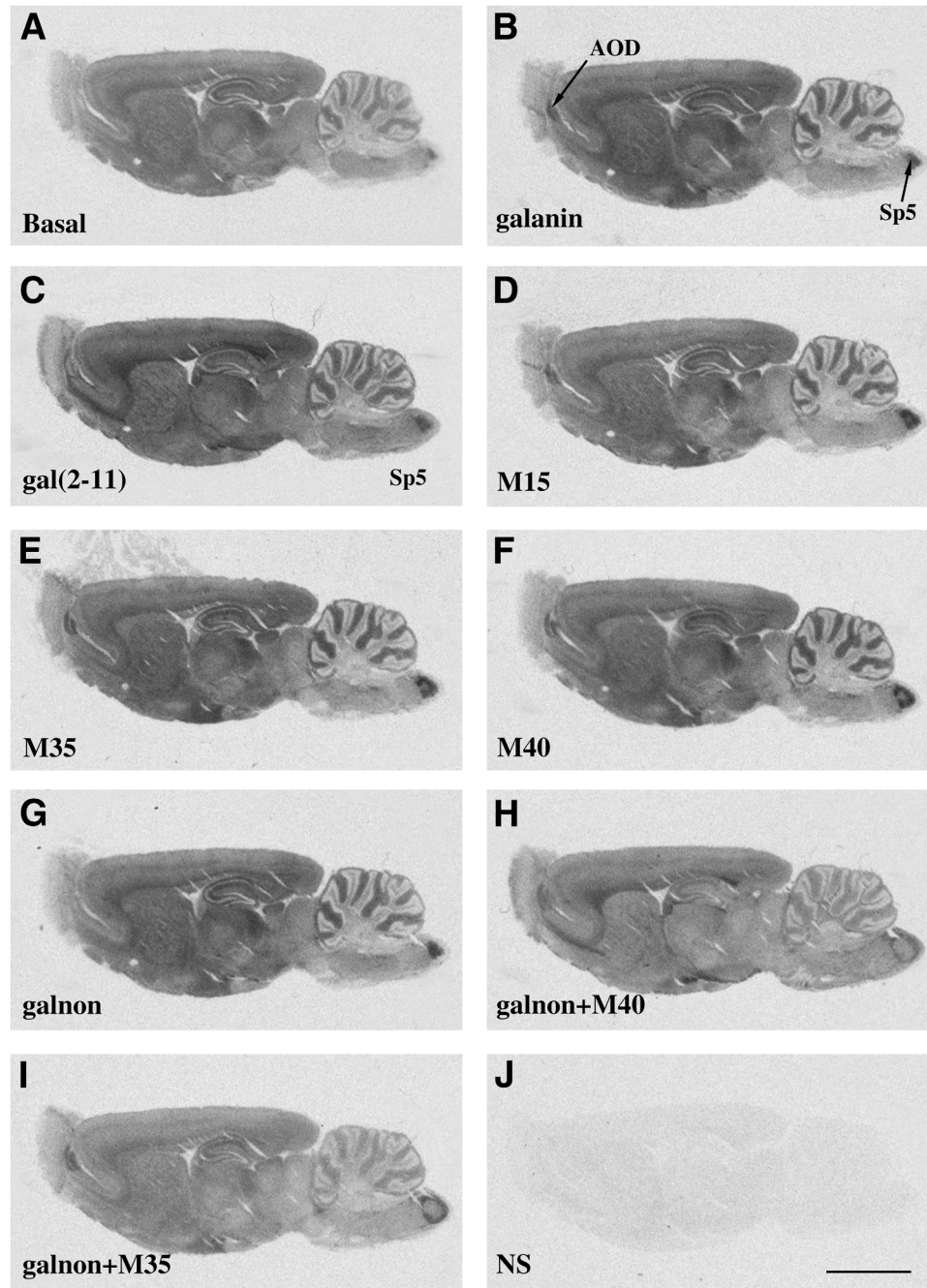
Fig. 1. [^{35}S]GTPγS autoradiographic images of sagittal tissue sections from the rat brain in absence of ligand (A) and in the presence of 1 μM galanin (B), 1 μM gal (2−11) (C), 1 μM M15 (D), 1 μM M35 (E), 1 μM M40 (F), 100 μM galnon (G), 100 μM galnon and 1 μM M40 (H), 100 μM galnon and 1 μM M35 (I). The non-specific binding was determined in the presence of 10 μM GTPγS (J). AOD: anterior olfactory nucleus; Sp5: spinal trigeminal tract. Scale bar = 5 mm.

the anterior olfactory nucleus (Fig. 1). However, neither gal(2−11) (1 μM) nor galnon (100 μM) were able to mimic this effect over the basal binding (Table 1, Fig. 1).

In the ventral subiculum and in the lateral olfactory tract, where galanin increased the [^{35}S]GTPγS binding in a 40% (p = 0.04 and p = 0.003, respectively) over the basal, the chimerical peptides M15 and M35 stimulated about a 30% (p = 0.05 an p = 0.001, respectively) (Table 1). Nevertheless, the M40 peptide only enhanced the basal binding in a relevant way in the lateral olfactory tract (12%, p < 0.05). A similar effect was also observed in the posterior hypothalamic nucleus, where galanin induced a stimulation of 44% (p = 0.026), where these chimerical peptides did not exceed the 15% (Table 2).

A moderate stimulation was produced by galanin in the internal capsule (26%, p = 0.02), dorsal horn layers I and II of the spinal cord (22%, p = 0.018), medial (13%, p = 0.003) and central amygdaloid nucleus (20%, p = 0.034) and the spinal trigeminal tract (12%, p = 0.033). The M35 peptide induced a similar stimulation of the [^{35}S]GTPγS binding in most of those areas with the exception of amygdala (Tables 1 and 2). However, M15 only slightly enhanced the coupling of GalR to $G_{i/o}$ proteins in the spinal trigeminal tract (10%, p = 0.005), while M40 and gal(2−11) induced a non-significant stimulation of 12% in the layers I and II of the spinal cord (Fig. 1; Table 2). In addition, gal(2−11) weakly increased the [^{35}S]GTPγS binding in white matter areas such as internal capsule (10%, p < 0.05).

On the contrary, galnon evoked a 71% (p = 0.004) of stimulation not only in the internal capsule but also in other white matter areas mainly constituted of fiber tracts, such as pyramidal tract, corpus callosum, longitudinal fasciculus of the pons, anterior part of the anterior commissure and subfornical organ. However, galanin failed to stimulate the [^{35}S]GTPγS binding in those white matter areas. A similar effect was observed with M35 in the solitary tract nucleus, where this ligand only slightly enhanced the [^{35}S]GTPγS binding (14%, p = 0.05), although galanin did not increased it significantly (Table 2).

On the other hand, gal(2−11), M15, M35, M40 and galnon induced an statistically significant reduction of the [^{35}S]GTPγS basal binding (inverse agonist effect) in the dorsal part of the dentate gyrus, in the centromedial thalamic nucleus and in the medial hypothalamic nucleus, where galanin did not. In addition, the chimerical peptide M15 also behaved as an inverse agonist in the nucleus of the solitary tract and in the olfactory tubercle. M40 and galnon showed the same effect in this area, as well as in the striatum. Furthermore, M40 evoked a moderate reduction of the basal binding in the corpus callosum, while gal(2−11) decreased it in the ventral subiculum, lateral parabrachial nucleus and amygdaloid nuclei (Tables 1 and 2).

Galnon produced a similar action in the parabrachial nucleus and in the amygdaloid nuclei. In fact, this molecule acted as an inverse agonist in most of the analysed brain areas, being this effect more relevant in the *accumbens* nucleus and central gray. Nevertheless, the galnon and M35 mixture enhanced the inverse agonism effect of both ligands in all analysed areas, with the exception of basal part of the anterior olfactory nucleus, I and II layers of the spinal cord and posterior hypothalamic nucleus. Moreover, M35 reduced the galnon stimulation in these areas, even decreasing the [^{35}S]GTPγS binding under the values of the basal binding (inverse agonism) (Fig. 2). By contrast, galnon and M40 mixture did not reach a high decrease in the [^{35}S]GTPγS basal binding. Although M40 partially blocked the stimulation induced by galnon in grey matters areas, in white matter areas such as internal capsule (50%, p = 0.008), corpus callosum (28%, p = 0.004) and anterior commissure (39%, p = 0.037), a significant stimulation was observed (Tables 1 and 2).

3.2. Comparative correlation study of the activity elicited by GalR ligands

The stimulation of the [^{35}S]GTPγS binding induced by the different GalR ligands was compared between them, using correlation analysis. The following significant correlations were observed between the stimulation evoked by galanin and that produced by gal(2−11) (r = 0.3903; p = 0.0186), M15 (r = 0.5039; p = 0.0017), M35 (r = 0.8090; p < 0.0001), M40 (r = 0.5278; p = 0.0009) and the mixture of galnon/M35 (r = 0.4998; p = 0.0019) and galnon/M40 (r = 0.4050; p = 0.0143), but not with the galnon stimulation (Figs. 3 and 4). In addition, the M40 stimulation was correlated with the M15 (r = 0.6787; p < 0.0001), M35 (r = 0.6906; p < 0.0001), galnon/M35 (r = 0.6614; p < 0.0001) and galnon/M40 (r = 0.5116; p = 0.0014) stimulations.

The activity induced by M35 showed a correlation with that produced by M15 (r = 0.5934; p < 0.0001) and the mixtures of galnon/M35 (r = 0.5686; p = 0.0003) and galnon/M40 (r = 0.5306; p = 0.0009). However, the M15 stimulation was more similar to the mixtures of galnon/M35 (r = 0.5245; p = 0.0010) and galnon/M40 (r = 0.6264; p < 0.0001).

Finally, the activity induced by galnon (100 μM) only correlated with that observed in the presence of this molecule with other peptides: galnon/M35 (r = 0.4242; p = 0.0111; excluding the anterior olfactory nucleus) and galnon/M40 (r = 0.8022; p < 0.0001; excluding the anterior olfactory nucleus) (Fig. 4). In fact, galnon/M35 mixture showed a positive correlation with galnon/M40 mixture (r = 0.7402; p < 0.0001).

Table 1

Autoradiographic densities for the specific binding of [^{35}S]GTPγS induced by galanin, M15, M35, M40, gal(2–11) (1 μM), galnon (100 μM), and galnon/M35 and galnon/M40 combinations in the rinencephalon and telencephalon of the rat.

Brain region	Percentages of stimulation over [^{35}S]GTPγS basal binding (%)							
	galanin	M15	M35	M40	gal (2–11)	galnon	galnon+M35	galnon+M40
Rinencephalon								
Anterior olfactory nucleus, dorsal part	156 ± 34 **	110 ± 27	136 ± 46	108 ± 39	9 ± 4	0 ± 56	69 ± 60	151 ± 20 *
Lateral olfactory tract nucleus	41 ± 10 **	35 ± 8 **	30 ± 6 *	12 ± 6 *	1 ± 2	-21 ± 8	-25 ± 7 *	-7 ± 9
Olfactory tubercle	-1 ± 12	-14 ± 4	1 ± 8	-22 ± 4	-8 ± 4	-36 ± 11 *	-53 ± 6	-38 ± 9
Telencephalon								
Striatum	-4 ± 12	-11 ± 5	-15 ± 9	-19 ± 4 *	-11 ± 6	-30 ± 11 *	-55 ± 2 **	-48 ± 4 **
Accumbens nucleus	5 ± 12	-13 ± 6	-0 ± 12	-1 ± 8	-7 ± 8	-44 ± 13 *	-49 ± 2 **	-45 ± 6 **
Anteriro commissure, anterior part	6 ± 12	1 ± 1	-2 ± 3	-4 ± 1	-4 ± 2	51 ± 18	-45 ± 3 **	39 ± 12 *
Corpus callosum	1 ± 9	-6 ± 7	-9 ± 6	-13 ± 2 **	-2 ± 2	67 ± 10 **	-43 ± 5 **	28 ± 12 *
Central amygdaloid nucleus	20 ± 7 *	2 ± 8	6 ± 9	3 ± 4	-25 ± 9 *	-34 ± 2 *	-40 ± 5 **	-32 ± 4 **
Medial amygdaloid nucleus	13 ± 3 **	-5 ± 6	6 ± 7	0 ± 3	-28 ± 10 *	-34 ± 2 *	-33 ± 8 **	-33 ± 7 *
Subfornical organ	0 ± 4	-4 ± 8	-10 ± 6	-6 ± 3	-3 ± 6	31 ± 8 *	-39 ± 3 **	-15 ± 8
Ventral subiculum	41 ± 12 *	36 ± 13 *	25 ± 4 ***	6 ± 14	-30 ± 4	-12 ± 9	-21 ± 6	-3 ± 14
Granular layer of the dorsal dentate gyrus	-14 ± 8	-29 ± 4 **	-29 ± 12 *	-27 ± 7 *	-14 ± 14	-34 ± 5 **	-43 ± 4 **	-28 ± 4 **
Molecular layer of the dorsal dentate gyrus	-12 ± 6	-14 ± 8 **	-26 ± 7 *	-20 ± 6 *	-18 ± 8	-37 ± 6 **	-48 ± 1 ***	-40 ± 3 **

Data are mean ± SEM values of four to seven animals. ANOVA followed by Bonferroni's test. * p < 0.05; ** p < 0.01; *** p < 0.001

Table 2

Autoradiographic densities for the specific binding of [^{35}S]GTPγS induced by galanin, M15, M35, M40, gal(2–11) (1 μM), galnon (100 μM), and galnon/M35 and galnon/M40 combinations in the diencephalons, mesencephalon, rhombencephalon and spinal cord of the rat.

Brain region	Percentages of stimulation over [^{35}S]GTPγS basal binding (%)							
	galanin	M15	M35	M40	gal (2–11)	galnon	galnon+M35	galnon+M40
Diencephalon								
Paraventricular thalamic nucleus, anterior part	4 ± 7	-4 ± 9	4 ± 4	1 ± 11	-9 ± 7	-27 ± 8	-48 ± 7 *	-33 ± 6 *
Centromedial thalamic nucleus	-7 ± 7	-15 ± 6	-22 ± 4 * *	-10 ± 7	-5 ± 1	-30 ± 8 *	-48 ± 3 **	-46 ± 4 **
Medial hypothalamic nucleus	-1 ± 6	-14 ± 4 *	-16 ± 3 *	-12 ± 7	-27 ± 6 * *	-32 ± 9 *	-43 ± 9 *	-41 ± 9 *
Posterior hypothalamic nucleus	44 ± 14 *	14 ± 9	10 ± 8	7 ± 7	5 ± 5	-22 ± 7	-6 ± 4	-6 ± 10
Medial mammillary nucleus, mediolateral part	4 ± 2	-30 ± 14	-4 ± 8	-18 ± 14	-29 ± 13	-29 ± 4	-44 ± 4	-39 ± 3
Mesencephalon								
Central gray	2 ± 5	-6 ± 2	2 ± 4	3 ± 6	-11 ± 6 * *	-37 ± 2	-45 ± 4 **	-39 ± 3 **
Internal capsule	26 ± 8 *	5 ± 10	20 ± 5	-3 ± 4	10 ± 6 *	71 ± 12 **	-24 ± 7 *	50 ± 10 **
Rhombencephalon and spinal cord								
Longitudinal fasciculus of the pons	-6 ± 7	5 ± 8	0 ± 8	21 ± 8	-15 ± 6	55 ± 13 *	-30 ± 5 **	40 ± 9 *
Lateral parabrachial nucleus	-6 ± 8	-11 ± 7	0 ± 11	5 ± 11	-23 ± 6	-34 ± 4 **	-35 ± 7 **	-32 ± 4 **
Pyramidal tract	-1 ± 7	0 ± 9	-3 ± 2	2 ± 8	-13 ± 1	104 ± 23 *	-21 ± 3	113 ± 28 **
Solitary tract nucleus	8 ± 4	-20 ± 4	14 ± 5 *	8 ± 5	-10 ± 8	-16 ± 9 **	-30 ± 5 * *	-13 ± 8
Spinal trigeminal tract	12 ± 4	10 ± 1 * *	12 ± 1	7 ± 5	3 ± 7	-18 ± 7 **	-22 ± 4 *	-101 ± 3
Layers I and II of the spinal cord	22 ± 4 *	1 ± 10	17 ± 8	11 ± 8	14 ± 7	-24 ± 1 **	-13 ± 11	-14 ± 6

Data are mean ± SEM values of four to seven animals. ANOVA followed by Bonferroni's test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

4. Discussion

In this study, we have analyzed the differences in the [^{35}S]GTPγS binding evoked by some of the most used galanin receptors ligands. For this purpose, an exhaustive anatomical analysis of the activation of galanin GPCRs was performed in rat brain slices, since this is a valid animal model for the study of the activation of galanin receptors by functional autoradiography (Barreda-Gómez et al., 2014). Initially, the stimulation of GalRs evoked by galanin itself was analyzed and, as we previously described, the highest stimulations induced by this neuropeptide were observed in the dorsal part of the anterior olfactory nucleus and in the lateral olfactory tract (Barreda-Gómez et al., 2005). This effect was also mimicked by M15, M35 and M40, but not by gal(2−11). However, the non-peptide ligand, galnon reduced the [^{35}S]GTPγS basal binding in the lateral olfactory tract but it did not modify the stimulation induced by M35 and M40 in these olfactory nuclei. Therefore, galnon does not seem to recognize GalR$_1$, since both areas showed a high expression of this GalR subtype (ODonnell et al., 1999). In this context, the low expression of GalR$_2$ could explain the absence of response to gal(2−11) in these nuclei, because this ligand is considered as an agonist of GalR$_2$ and GalR$_3$ (ODonnell et al., 1999; Lu et al., 2005).

Early studies analysing the distribution of galanin in the CNS already suggested that this neuropeptide could play an important role in the control of memory, attention and learning, when it was shown that certain cholinergic cells in the basal forebrain of the rat expressed galanin (Rökaeus et al., 1984). This group of galanin/choline acetyltransferase (ChAT)-positive neurons together with another cholinergic neurons project to the hippocampus (Melander et al., 1985). Further experimental evidence of the interaction between the cholinergic system and galanin are that this neuropeptide inhibits scopolamine-induced acetylcholine release in both the cerebral cortex (Wang et al., 1999) and the ventral hippocampus (Fisone et al., 1987). Accordingly, the high GalR$_1$ expression in the subiculum of the ventral hippocampus (ODonnell et al., 1999), could fit with the observed high activity elicited by galanin, M15, M35 and M40, but not by galnon, and gal(2−11) that behaved as inverse agonists in this area. This inverse agonist behaviour of gal(2−11) was also observed in diverse brain nuclei, such as amygdala, medial hypothalamic, medial mammillary and lateral parabrachial, and in different layers of the dorsal dentate gyrus. Indeed, all of tested ligands, included galanin, reduced the basal binding in this hippocampal region. A possible explanation for this effect could be the result of promoting the coupling of GalR$_2$ to G$_{q/11}$, considering that this subtype is highly expressed in the dentate gyrus of the rat (ODonnell et al., 1999). In this context, A. Mazarati and co-workers observed

different effect of GalR$_2$ depending on the G protein subtype activation and they proposed that the coupling of GalR$_2$ to G$_{i/o}$ proteins in the hippocampus induced antiepileptogenic actions, whilst the coupling to G$_{q/11}$ proteins activated the proconvulsant pathway (Mazarati et al., 2006). Regarding the ventral hippocampus, a brain region mainly involved in stress, emotions and affective characteristics of cognition; the regulation of the galanin response on cholinergic neurotransmission has been studied (Yoshitake et al., 2011), but also in other related areas such as the bed nucleus of *stria terminalis* (Miller et al., 1997). This nucleus whose neurons co-express galanin and GalR$_1$, projects to the septal complex and ventral hippocampus. Bed nucleus of *stria terminalis*, as well as diverse telencephalic areas, such as septal nuclei, diagonal band, central amygdaloid nucleus and stria terminalis, showed a low, but statistically significant, galanin stimulations. This weak stimulation agrees with the moderate [^{125}I]-galanin binding reported in previous studies and probably is due to the activation of GalR$_1$, which is mainly expressed in these areas (Melander et al., 1988; Mennicken et al., 2002; ODonnell et al., 1999; Skofitsch et al., 1986).

Another area with high presence of galaninergic neurons is the amygdala (Skofitsh et al., 1986). This nucleus is intimately related to stress-related disorders (Barnabas et al., 2016). Nevertheless, no relevant increase in the [^{35}S]GTPγS basal binding was observed by any of the analysed GalR ligands in the amygdaloid nucleus, despite the high density of GalR in this area. Moreover, galnon, as well as gal(2−11), reduced significantly the basal activity, without being altered by the presence of M35 and M40. Thus, at least in these nuclei, galnon and gal(2−11) seem to act over the same type of receptors, which are not recognised by the chimerical peptides M35 and M40. The amygdala and the ventral hippocampus are two regions involved in the olfactory signalling pathway and participate in the processes of establishing and recalling olfactory memory, as well as in the association of olfactory memory with other events. In rat, these regions have a high density of [^{125}I]-galanin binding sites and immunoreactive fibres for this neuropeptide (Skofitsch et al., 1986, Melander et al., 1986, 1988, Jacobowitz et al. 2004). In addition, both the amygdala and the subiculum and CA1 area of the ventral hippocampus, have high GalR expression, especially of the GalR$_1$ subtype that couples to G proteins of the G$_{i/o}$ family (O'Donnell et al., 1999; Waters and Krause, 2000). Considering that other areas of the olfactory system, such as the basal nucleus of the *stria terminalis*, olfactory bulb and tract, diagonal band nucleus, piriform cortex and entorhinal cortex, are also rich in galanin and its receptors, we can assume that this neuropeptide is actively involved in the regulation of olfactory signalling pathways in the CNS (Jacobowitz et al., 2004; Melander et al., 1988;).
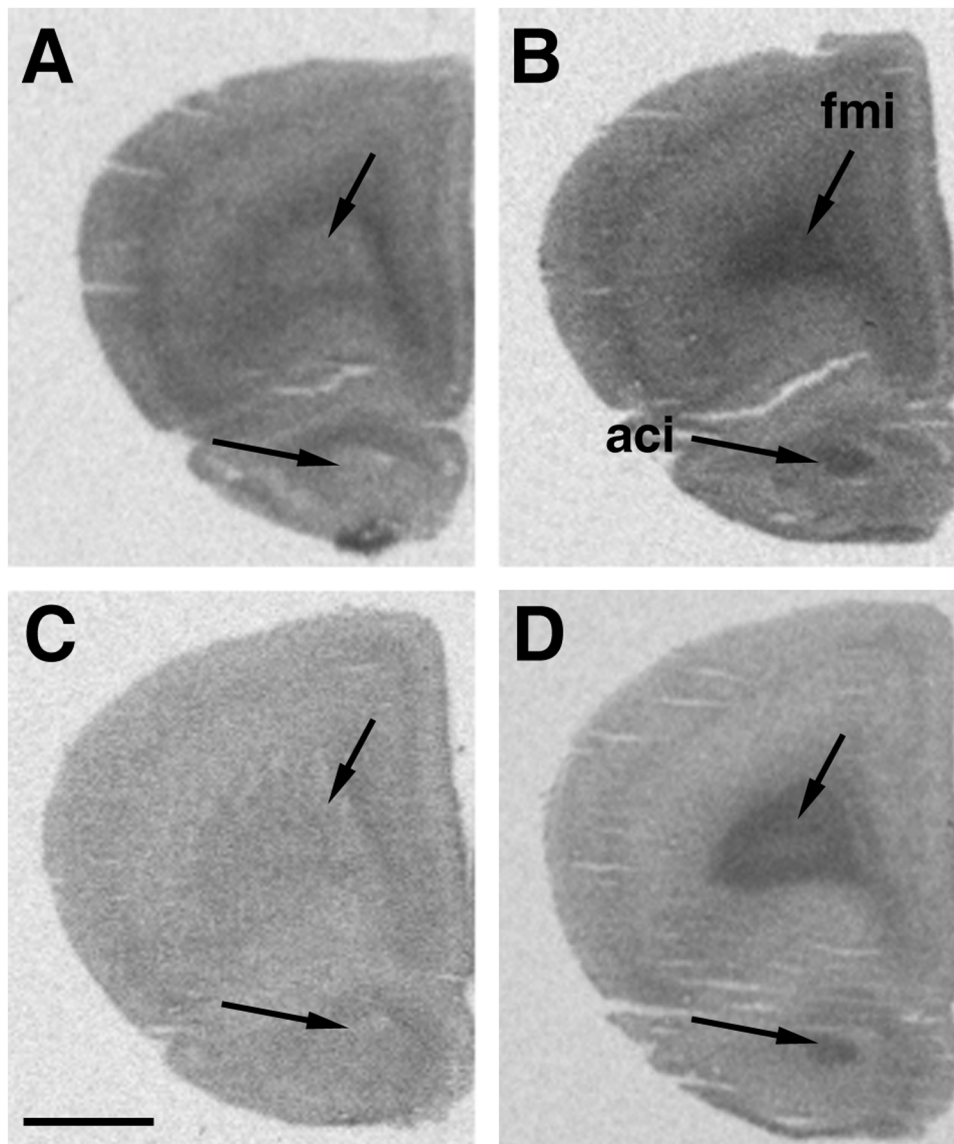
Fig. 2. [^{35}S]GTPγS autoradiographic images of coronal tissue sections from the rat brain in absence of ligand (A) and in the presence of 100 μM galnon (B), 100 μM galnon and 1 μM M35 (C), 100 μM galnon and 1 μM M40 (D). aci: anterior commissure, intrabulbar part; fmi: forceps minor of the corpus callosum. Scale bar = 2 mm.

In the centromedial and paraventricular thalamic nuclei, galanin did not stimulate the GalR activity. This absence of effect contrasts with the elevated expression of GalR$_1$ and with the high density of [^{125}I]-galanin-binding sites observed in both nuclei (ODonnell et al., 1999; Melander et al., 1988). In a similar way, in other thalamic and epithalamic regions, such as *reuniens* nucleus and lateral habenula, no stimulation was produced by galanin or by any of the assayed compounds (ODonnell et al., 1999; Melander et al., 1988). The low stimulation of G$_{i/o}$ proteins activity observed in the piriform cortex and dorsal hippocampus could indicate that a compensatory mechanism occurred in these brain regions, so that the high density of GalR is accounting for the low efficiency in the coupling to G$_{i/o}$ proteins. Nevertheless, the possibility exist that the GalR would be coupled to G$_{q/11}$ proteins, which cannot be quantified using this technique.

Hypothalamus is another region with a high density of GalR coupled to G$_{i/o}$ proteins, mainly the magnocellular preoptic, posterior hypothalamic and arcuate nuclei (Barreda-Gómez et al., 2005). According to this observation, an elevated stimulation of the [^{35}S]GTPγS binding was quantified in the presence of galanin in the posterior hypothalamic nucleus, while the rest of analysed peptides generated lower

stimulations. This high stimulation by galanin in the hypothalamic nucleus is remarkable since this area is closely related to anxious-like behaviour and food intake, being highly expressed in several hypothalamic nuclei involved in appetite control such as the paraventricular nucleus (PVN) or the arcuate nucleus (Gundlach et al., 2001). Galanin has orexigenic effects, as both i.c.v. and direct administration into the PVN causes an increase in food intake, although this response is of lower intensity and duration than that produced by neuropeptide Y (Leibowitz, 2005). In addition, high-fat diets increase galanin levels in the PVN, in addition to increasing leptin, triglycerides, non-esterified fatty acids and glucose, while decreasing insulin and corticosterone levels (Leibowitz et al., 2004). Furthermore, both insulin and leptin have been shown to inhibit galanin and neuropeptide Y expression in the hypothalamus (Sahu, 1998; Wang and Leibowitz, 1997), as well as to inhibit also insulin release in the pancreas, thus establishing a feedback mechanism (Lindskog, Ahrén 1991; Wang and Leibowitz, 1997). The fact that the blockade of fatty acid oxidation leads to a decrease in galanin mRNA and an increase in GalR$_1$ expression in PVN supports the importance of galanin in modulating lipid metabolism (Gorbatyuk, Hökfelt 1998; Tang et al., 1997). Contrary to other galanin ligands

Fig. 3. Correlation between the stimulation of the [^{35}S]GTPγS binding induced by 1 μM galanin and the stimulation evoked by 1 μM M15 (A), 1 μM M35 (B) and 1 μM M40 (C) in different areas of the rat CNS. The discontinuous line is a representative correlation of 1:1. Note that these chimerical peptides present an agonist effect similar to that showed by galanin.

studied, galnon was able to decrease the [^{35}S]GTPγS basal binding. However, this inverse agonism effect was blocked by M35 and M40. Thus, galnon and the chimeric peptides M35 and M40 seem to act over the same receptors in the posterior hypothalamic nucleus but with different pharmacological properties.

In the rhombencephalon the highest stimulations were obtained in the spinal trigeminal nucleus and tract, as well as in the mesencephalic, reticular, lateral parabrachial and solitary tract nuclei. M35 and M40 enhanced the [^{35}S]GTPγS binding in the same proportion as galanin in the spinal trigeminal tract and solitary tract nucleus. On the contrary, galnon acted as inverse agonist in both nuclei. This effect induced by galnon was also observed in layers I and II of the spinal cord, where galanin increased the GalR coupling to G$_{i/o}$ proteins. In fact, M35, M40 and gal(2−11) slightly stimulated the [^{35}S]GTPγS binding in this area. These results agree with the high density of [^{125}I]-galanin binding sites and with the elevated expression of GalR$_1$ described in these layers of the spinal cord, although the presence of other GalR subtypes has also been reported (Mennicken et al., 2002; ODonnell et al., 1999; Skofitsch et al., 1986). Experimental evidence suggests that GalR$_1$ is found on glutamatergic interneurons in the posterior horn of the spinal cord, which would support the hypothesis that the antinociceptive effects exerted by galanin, via GalR$_1$, may be mediated by attenuation of excitatory tone in the spinal cord (Landry et al., 2006). However, this neuropeptide at low doses also exhibits pronociceptive actions, which are probably mediated by GalR$_2$ (Liu et al., 2001; Jimenez-Andrade et al., 2004). In line with this hypothesis, Jiménez-Andrade described that the GalR$_{2/3}$ agonist AR-M1896, increases capsaicin-induced neurogenic inflammation (Jimenez-Andrade et al., 2006). Moreover, the spinal cord and spinal ganglia have high levels of galanin during development (Xu et al., 1996) and after peripheral nerve damage in adults (Hökfelt et al., 1987). This pattern of expression in both the neuropeptide and its receptors suggests that galanin plays an important role in the control of pain, particularly neuropathic pain or pain following nerve injury, although its function is not yet clear.

The study of the correlations between the stimulations of the different drugs provided information on the pharmacological profile of the tested molecules. When the stimulations induced by several ligands correlate, it is inferred that this phenomenon is a consequence on acting on the same binding sites. In addition, the fact of the absence of connection indicates that they can act on different receptors and trigger different responses. Thus, M15, M35 and M40 induced a similar stimulation of basal binding of [^{35}S]GTPγS than galanin, indicating that these molecules are agonists of GalRs, but the fact that they show different degrees of correlation between them could indicate that they show selectivity for some of their subtypes, supporting previous studies (for review see Freimann et al., 2015). In addition, galanin induced activation of G$_{i/o}$ proteins does not correlate to that produced by gal (2−11), probably due to the fact that GalR$_2$ is mainly coupled to G$_{q/11}$ proteins in brain tissue. Finally, galnon reduced the basal activity in most of the analysed areas and increased the [^{35}S]GTPγS binding in diverse white matter regions, where galanin does not induce any effect, suggesting that this non-peptide molecule acts as agonist or inverse agonist of GPCRs different from GalR. Moreover, GPCR expressed in white matter areas show affinity for M35 and M40, compounds that antagonize the stimulation generated by galnon. Therefore, M35 and M40 would be agonists for GalR, but also antagonist for those non-GalR recognised by galnon.

In conclusion, this anatomical study analysed the in vitro activity of drugs that have affinity for galaninergic receptors. Some galanin-like drugs usually used in experimental procedures show disparate efficacy and selectivity for the different galanin receptor subtypes. The present study using the [^{35}S]GTPγS technique, indicates different activities evoked by these ligands on G$_{i/o}$ protein coupled receptors, from agonist to antagonist effects depending on the drug used. Moreover, these differences seem to be specific of discrete brain areas or nucleus. Thus, the reported results contribute to the pharmacological characterization of
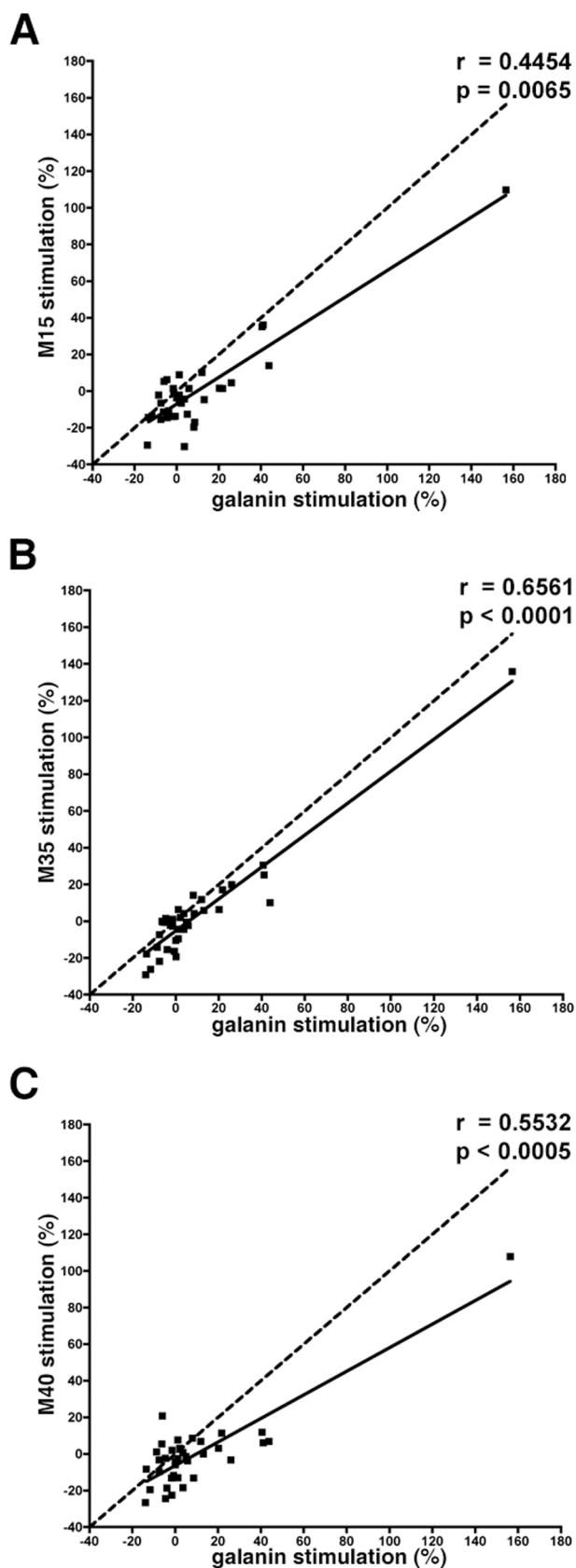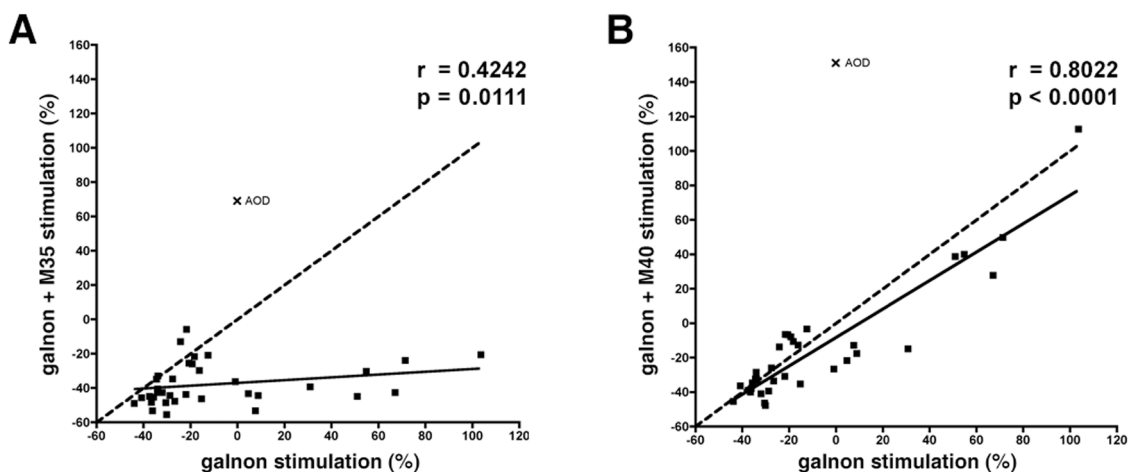
Fig. 4. Correlation between the stimulation of the [^{35}S]GTPγS binding induced by 100 μM galnon and the stimulation evoked by this molecule in the presence of 1 μM M35 (B) and 1 μM M40 (C) in diverse areas of the rat brain. The discontinuous line is a representative correlation of 1:1. Note that the chimerical peptide M35 antagonized the stimulation induced by galnon in most of analysed brain regions, excluding certain areas such as the anterior olfactory nucleus. AOD: anterior olfactory nucleus.

GalR ligands to further improve the design of more specific drugs to modulate the galanin mediated effects in selected brain areas, trying to avoid side effects.

CRediT authorship contribution statement

Gabriel Barreda-Gómez: Conceptualization, Methodology, Formal analysis, Writing – original draft. **Iván Manuel:** Data curation, Writing – original draft, Writing – review & editing. **Rafael Rodríguez-Puertas:** Conceptualization, Resources, Funding acquisition, Project administration. All authors have read and agreed to the published version of the manuscript.

Acknowledgements

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