Effects of galanin subchronic treatment on memory and muscarinic receptors

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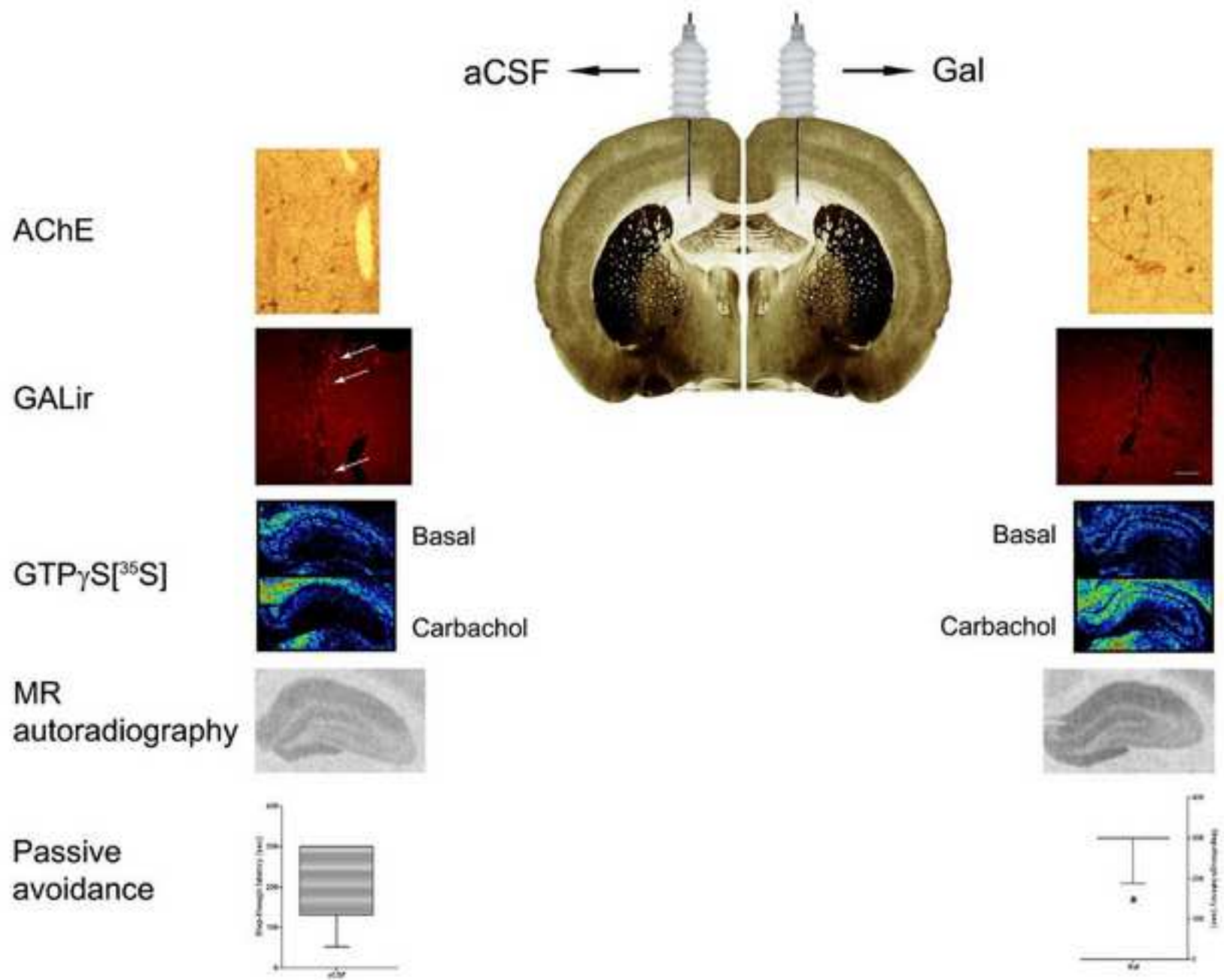
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ABBREVIATIONS

AD: Alzheimer's disease

CNS: Central Nervous System

aCSF: artificial cerebrospinal fluid

GAL: galanin

[^{3}H]-NMS: [^{3}H]-N-methyl-scopolamine

i.c.v.: intracerebroventricular

MR: muscarinic receptor

[^{35}S]GTPγS: guanosine 5'-(γ-[^{35}S]thio)triphosphate

- Galanin (GAL) 1.5 mmol x 6 days reverted cognitive deficits induced by i.c.v. aCSF.
- The number of AChE neurons decreased in nbM of both GAL and aCSF-treated animals.
- GAL prevented changes in muscarinic receptor (MR) densities and M_2 activity.
- The increase of GAL receptors in the aCSF group was avoided by GAL treatment.
- GAL improves memory modulating MR density and efficacy in hippocampal areas.

AChE

GALir

GTPγS[^{35}S]

Basal

Carbachol

MR autoradiography

Passive avoidance

aCSF

Gal

Basal

Carbachol

ABSTRACT

The cholinergic pathways, which originate in the basal forebrain and are responsible for the control of different cognitive processes including learning and memory, are also regulated by some neuropeptides. One of these neuropeptides, galanin (GAL), is involved in both neurotrophic and neuroprotective actions. The present study has evaluated in rats the effects on cognition induced by a subchronic treatment with GAL by analysing the passive avoidance response, and the modulation of muscarinic cholinergic receptor densities and activities. [^{3}H]-N-methyl-scopolamine, [^{3}H]-oxotremorine, [^{3}H]-pirenzepine were used to quantify the density of muscarinic receptors and the stimulation of the binding of guanosine 5'-(γ-[^{35}S]thio)triphosphate by the muscarinic agonist, carbachol, to determine their functionality.

Some cognitive deficits that were induced by the administration of artificial cerebrospinal fluid (i.c.v. aCSF 2 µl/min, once a day for 6 days) were not observed in the animals also treated with GAL (i.c.v. 1.5 mmol in aCSF, 2 µl/min, once a day for 6 days). GAL modulates the changes in M_1 and M_2 muscarinic receptor densities observed in the rats treated with aCSF, and also increased their activity mediated by $G_{i/o}$ proteins in specific areas of dorsal and ventral hippocampus. The subchronic administration of the vehicle was also accompanied by an increased number of positive fibres and cells for GAL around the cortical tract of the cannula used, but that was not the case in GAL-treated rats. In addition, the increase of GAL receptor density in ventral hippocampus and entorhinal cortex in the aCSF group was avoided when GAL was administered. The number of acetylcholinesterase (AChE) positive neurons was decreased in the nucleus basalis of Meynert of both GAL and aCSF-treated animals. In summary, GAL improves memory-related abilities probably through the modulation of muscarinic receptor density and/or efficacy in hippocampal areas.

INTRODUCTION

Galanin (GAL) is a neuropeptide composed of 29 aminoacids in the rat, which is widely distributed in peripheral organs and in the Central Nervous System (CNS). GAL involvement in neuroprotective and neurotrophic actions has been reported in different models of brain damage in addition to its classical roles in other neuronal functions such as feeding, nociception, learning and memory (Lang et al., 2007). GAL immunoreactivity has been reported in cortical cholinergic innervation from the septum-basal forebrain complex (Melander et al., 1985), and also in monoaminergic and gabaergic neurons (Melander et al., 1986) Regarding the cholinergic system, GAL and GAL receptors have been found located in areas involved in cognitive processes (Hökfelt et al., 1987; Melander et al., 1988). The effects of exogenous GAL on the central cholinergic system differ according to the area studied. In vitro studies demonstrates that GAL inhibits both basal and K^+-stimulated acetylcholine (ACh) release in ventral hippocampus and cortical slices (Fisone et al., 1987; Wang et al., 1999). GAL increases ACh release in the hippocampus when it is infused in medial septum or in the nucleus of the diagonal band of Broca in freely moving rats (Elvander et al., 2004), probably by activation of cholinergic neurons (Jhamandas et al., 2002). Moreover, the perfusion of GAL by microdialysis increases striatal ACh levels in anaesthetized animals (Antoniou et al., 1997). The effects of GAL on ACh release in awake rats seem to depend on the site of administration (Ögren and Pramanik 1991; Amoroso et al., 1992; Antoniou et al., 1997) and also on the receptor type present in each area (Laplante et al., 2004).

The effects of GAL on memory also depend on the dose and the brain area studied (Sündstrom et al., 1988; Malin et al., 1992; Robinson and Crawley, 1993; Ukai et al., 1995; Ögren et al., 1996, 1999; Schott et al., 2000; Kinney et al., 2002, 2003; Elvander et al., 2004). GAL at low doses enhances acetylcholine release and improves cognition (Ögren

et al., 1996; Elvander et al., 2004), whereas high-medium doses had no effect or induce cognitive deficits (Ukai et al., 1995; Ögren et al., 1996). Intriguingly, both GAL over-expression and GAL gene silencing in genetically modified mice produce cognitive deficits and a reduction of cholinergic markers in the basal forebrain (O'Meara et al., 2000; Steiner et al., 2001). On the other hand, GAL is up-regulated in cholinergic forebrain neurons after hippocampal and/or cortical lesion (Cortés et al., 1990). GAL also reduces the activation of intracellular second messengers mediated by muscarinic receptors (MR) (Palazzi et al., 1991).

As has been stated above, the role of GAL in the ventral hippocampus has been widely studied. Indeed, the majority of cholinergic projections containing the neuropeptide have been found in the septo-hippocampal projection which innervates the ventral hippocampus and, to a lesser degree, the dorsal hippocampus. This is consistent with the greater number of high affinity sites for [^{125}I]-galanin in ventral hippocampus in comparison with dorsal hippocampus (Fisone et al., 1987; Melander et al., 1988).

An enhancement of galaninergic innervations within the basal forebrain is also present in certain neurodegenerative diseases such as Parkinson's and Alzheimer's (AD) (Chan-Palay, 1988; Beal et al., 1990; Mufson et al., 1993). GAL receptor density is increased in the hippocampus, amygdala, entorhinal cortex and nucleus basalis of Meynert (nbM) of AD patients, although its regulation seems to vary during the different stages of the disease (Rodríguez-Puertas et al., 1997a; Mufson et al., 2000; Pérez et al., 2002). Additionally, GAL immunoreactivity has also been reported in amyloid plaques and in their associated neurites (Diez et al., 2000; Mufson et al., 2005). Nevertheless, it is still unclear whether GAL hyperinnervation in AD patients contributes to cognitive deficits or, on the contrary, has a neurotrophic and neuroprotective effect on cholinergic neurons.

In the present work we study the interaction between the galaninergic and cholinergic systems by evaluating the effects of a subchronic treatment with a low dose of GAL on

learning and memory of rats, and on different neurochemical parameters of the cholinergic system (by measuring MR density and activity and AChE activity) in the basal forebrain. In addition, the effects of the treatment on the galaninergic system were analyzed (GAL receptor density and endogenous GAL immunoreactivity).

EXPERIMENTAL PROCEDURES

Animals

Male Sprague-Dawley rats (250-275 g) were divided into 4 groups. The first group (untreated) was comprised of unmanipulated rats. A transcranial cannula was implanted in each animal from the other groups following the same stereotaxic coordinates. One of these groups was not treated (sham), but the animals were handled daily in a similar way to the other operated rats. A third group of rats was treated with artificial cerebro-spinal fluid (aCSF), the vehicle in which the neuropeptide GAL was dissolved for the fourth group of treatment. Animal care and all experiments were carried out according to guidelines approved by the Ethical Committee of the University of the Basque Country (UPV/EHU) following the European Communities Council Directive (86/609/EEC) of 24 November 1986 and the recommendations from the Directive (207/526/EEC).

Materials

[³H]-N-methyl-scopolamine ([³H]-NMS) (70-87 Ci/mmol), [³H]-pirenzepine (86 Ci/mmol), [³H]-oxotremorine (75.8 Ci/mmol), [¹²⁵I]-galanin (2200 Ci/mmol) and guanosine 5'-(γ-[³⁵S]thio)triphosphate ([³⁵S]GTPγS) (1250 Ci/mmol) were purchased from Perkin Elmer (Waltham, MA, USA). Rat GAL was obtained from Bachem (Weil am Rhein, Germany), carbachol, atropine, GTPγS, sodium citrate, tetraisopropyl pyrophosphoramide (Iso-OMPA) and the Tris-maleate buffer were purchased from Sigma (St Louis, MO, USA);

cupric sulphate and potassium ferricyanide from Merck and acetylthiocholine iodide from Fluka Analytical. The following primary antibody was used: rabbit polyclonal antiserum for galanin (Rabbit anti-GAL, developed by Dr. Elvar Theodorsson, Linköping, Sweden), followed by rhodamine-conjugated goat anti-rabbit immunoglobulin (Jackson Immunoresearch, Baltimore, PA, USA). All other chemicals were obtained from standard sources and were of the highest purity commercially available.

Surgery and treatments

Surgery was performed under chloral hydrate anaesthesia (400 mg/kg; i.p.) to compare the results with a previous study (Barreda-Gómez et al., 2005). The cannula were implanted following the stereotaxic coordinates in the left lateral ventricle: -1 mm anteroposterior and -1.5 mm laterodorsal to Bregma and 3.5 mm ventral from the top of skull. The guide cannulas were fixed to the skull by dental cement and the animals were allowed to recover for a six-day period prior to the daily infusion of GAL (1.5 nmol), which was dissolved in artificial cerebrospinal fluid (aCSF) (148 mM NaCl, 2.7 mM KCl, 0.85 $MgCl_2 \cdot 6H_2O$, 1.2 mM $CaCl_2 \cdot 2H_2O$; pH 7.4 adjusted with 1 mM K_2HPO_4), or aCSF (2 μl/1 min) for a further six day period. The sham group of rats was similarly treated, but did not receive aCSF. After surgery the rats received one dose (2.25 mg/kg; i.m.) of oxytretracycline. The injection was administered by using a microinjection pump (CMA/102, Sweden) connected to a Hamilton syringe. During the infusion the rats were slightly restrained by hand and, once finished, the injection cannula was left inside the guide cannula for 60 sec in order to prevent backflow. Finally a dummy cannula was inserted into the guide. After the rats had completed the passive avoidance test, there was a 24 h galanin wash-out period and then they were anaesthetized, decapitated and their brains removed and either frozen at -80ºC (for the experiments of autoradiography; n = 7) or fixed with 4% paraformaldehyde in a phosphate buffer (PBS 0.1 M; pH 7.4) containing

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0.3% picric acid (for the immunohistochemical experiments and the histochemistry of acetylcholinesterase reaction; n = 7).

Passive Avoidance test

The apparatus consisted of two compartments of steel-rod grid floor connected via a guillotine door. One of the compartments was white and open-topped (44 x 42 x 45 cm), and the other was black, smaller (15 x 15 x 29 cm) and closed. The experimental sessions were conducted between 11:00 and 16:00 h and at least 1 h after the administration (i.c.v). On the day of the training trial, the rat was placed in the first compartment and after 30 sec the guillotine door automatically opened. The time that the rat took to enter the dark compartment (latency time) was measured with a stopwatch. When the rat had entered the dark compartment, the sliding door was closed and a foot shock (0.4 mA for 2 sec) was delivered through the grid floor. After 24 h, the retention trial was performed in the same manner but without foot shock. The rat was again placed in the white compartment as in the training trial; the guillotine door opened 30 sec later and remained opened for 300 sec. The latency to enter the dark compartment was recorded (step-through latency, STL or retention latency). If a rat did not enter the dark compartment, a retention latency of 300 sec was assigned.

Immunofluorescence assays

After fixing, the brains were kept for 12-14 h in a cryoprotective solution of 20% sucrose in phosphate buffer (PBS). Afterwards, they were rapidly frozen in isopentane at -80°C, and subsequently kept at this temperature until they were cut into sections (14 μm) at -20 to -25°C using a Micron HM 550 cryotome. The sections were then washed in 0.1 M PBS and then preincubated in Normal Goat Serum 4% for 2 h. The tissues were incubated with the primary antibody for 24 h at 4°C and the samples were washed with PBS at room

temperature. Following, the tissue was incubated with goat anti-rabbit labelled by rhodamine for 30 min at 37°C. The samples were washed with PBS (3 x 10 min) to reduce non-specific binding of the antibody. A Zeiss Axioskop 2 plus fluorescent light microscope was used for the observation and digital photography of the labelled slices.

Acetylcholinesterase activity assay

The staining procedure was carried out as described by Tago et al. (1986) with slight modifications. Briefly, brain sections (14 μm) were thawed and dried for 10 min and were then washed by two 10 min immersions in 0.1 M Tris-maleate buffer solution (pH 6). After washing, the incubation process took place in a solution consisting of the following components in this order (per 100 ml): 65 ml of 0.1 M Tris-maleate (pH 6), 5 ml of 0.5 mM $NaH_2C_6H_5O_7$, 10 ml of 3 mM $CuSO_4$, 10 ml of 0.1 mM Iso-OMPA (which inhibits the non-specific cholinesterase activity), 10 ml of 5 mM $K_3Fe(CN)_6$ and finally, 74 mg of acetylthiocholine iodide used as a substrate for the enzyme that catalyzes the AChE reaction. A Zeiss Axioskop 2 plus fluorescent light microscope was used for observation and photography.

Receptor and G protein autoradiography

In preparation for the receptor and G protein autoradiography, coronal tissue sections (20 μm) were obtained and mounted on gelatine-coated slides. Total MR density was labelled using the protocol described by Cortés (Cortés et al., 1984), with some modifications (Rodríguez-Puertas et al., 1997b). Coronal tissue sections were air-dried for 15 min at room temperature and were then incubated in 50 mM phosphate buffer (HNa_2PO4, H_2NaPO_4; pH 7.3) for the same time. A second incubation was performed using the same buffer for 60 min at 30ºC in the presence of [^{3}H]-NMS (1.5 nM). The

concentrations of all the radioligands used for autoradiography were similar to those used in previous studies in CNS. The concentrations used avoid non-specific labelling of other receptor subtypes. After incubation, the slides were washed twice in the buffer solution at 4ºC for 5 min and then dipped in MilliQ water at 4ºC. Finally they were dried under a cold airflow and exposed to a Kodak Biomax MR film for 30 days.

Autoradiography for the evaluation of M_1 receptor density was carried out following the protocol described by Quirion et al. (1989). Briefly, the sections were air-dried for 10 min at room temperature, preincubated in Krebs buffer (NaCl 11 mM; KCl 5 mM; $CaCl_2$ 2.5 mM; $MgSO_4 \cdot 7H_2O$ 1.18 mM; $NaHCO_3$ 25 mM; glucose 5.5 mM; KH_2PO_4 1.18 mM; pH 7.4) for the same time, incubated for 60 min with $[^3H]$-pirenzepine (3 nM) in the presence of oxotremorine (100 nM) to block M_4 binding, and finally washed twice in Tris-HCl buffer (pH 7.4) at 4ºC. Slides were dried under a cold airflow and exposed to a Kodak Biomax MR film for 52 days.

The labelling of M_2 receptors was determined following the experimental procedure already described (Spencer et al., 1986; Vilaró et al., 1992). The slides, in duplicate, were firstly preincubated in Hepes buffer (pH 7.4) for 10 min at room temperature. The incubation was carried out in the same buffer adding $[^3H]$-oxotremorine (2 nM) in the presence of pirenzepine (300 nM) to block the residual labelling of the radioligand to the M_4 subtype. After incubation, the slides were washed three times for 2 min in the same buffer at 4ºC, dried under a cold airflow and exposed to a Kodak Biomax MR film for 5 weeks.

Non-specific binding was determined for MR subtypes in the presence of atropine (10 μM) in consecutive tissue sections.

The functional coupling of MR to $G_{i/o}$ proteins was performed by incubating slides including the hippocampus with $[^{35}S]GTP\gamma S$ (0.04 nM) for 2 h at 30ºC in the presence of

carbachol (100 μM) (Rodríguez-Puertas et al., 2000; Barreda-Gómez et al., 2005). Tissue sections were previously air-dried for 15 min and incubated for 20 min at room temperature in Tris-HCl buffer (Tris-HCl 50 mM, MgCl$_2$ 3 mM, EGTA 0.2 mM, NaCl 100 mM, adenosine deaminase 3 mU/ml, GDP 2 mM, DTT 1 mM). After the incubation, the slides were washed (50 mM Tris-HCl, 15 min, 4ºC), dried and exposed to a Kodak Biomax MR film for 2 days. Non-specific binding was determined with GTPγS (10 μM) and the net stimulation induced by carbachol was evaluated (calculated as the [^{35}S]GTPγS binding in the presence of carbachol in nCi/g t.e. minus the [^{35}S]GTPγS basal binding).

The density of galanin receptors was measured by autoradiography [^{125}I]-galanin (0.1 nM). Autoradiographical analysis of galanin receptors was performed following the protocols described by Melander (Melander et al., 1988) and Köhler (Köhler et al., 1989), with some modifications (Rodriguez-Puertas et al., 1997a). Tissue sections were air-dried for 120 min at room temperature and were then preincubated in a Hepes 20 mM buffer with CaCl$_2$ 2.3 mM, MgCl$_2$ 1 mM, NaCl 136 mM, KCl 2.6 mM; glucose 5.5 mM, BSA 0.1% and bacitracine 0.05 %; pH 7.4. The incubation was carried out in the same buffer adding [^{125}I]-galanin (0.1 nM) for 1 h at room temperature. After incubation, the slides were washed four times for 2 min in the same buffer at 4ºC, and then dipped in deionized cold water. Finally, tissues were dried under a cold airflow. Non-specific binding was assessed in the presence of 1 µM of rat galanin in a consecutive tissue section.

Sections were exposed to a Kodak Biomax MR film for 4 weeks at -20ºC together with ^{14}C standards. These are useful for the calibration of optical densities produced by any ^{125}I labeled radioligand (Baskin and Wimpy, 1989).

The labelling was quantified by transforming the different grey densities to nCi/g or fmol/mg of tissue equivalent. The autoradiographic results were calculated as maximal density of receptors (B$_{max}$), using previously reported Kd for each of the radioligands used.

Therefore, the results are interpreted as modulation of B_{max}, however one cannot rule out the possibility of a modulation of the affinity.

Data analysis and Statistics

Behavioural data are presented as box plots in which the ends indicate the 25% and 75% interquartile ranges and the line in the box denotes the median. The non-parametric Kruskal-Wallis analysis of variance and the Mann-Whitney *U*-test, as a post-hoc, were used to compare the latency times in the passive avoidance test. Behavioural data were also analysed by using the Fisher´s exact test. In addition, passive avoidance retention latency was also represented as Kaplan-Meier survival curves and compared by Log-Rank (Mantel-Cox). The statistical analysis evaluates the probability of the animals to reach the 300 sec cut off time and the survival curve shows the retention time of each rat.

The autoradiography results were expressed as mean ± SEM. After testing the normal distribution of data and the homogeneity of variances, the statistical analyses were carried out by using either one-way analysis of variance (ANOVA) and Bonferroni´s test, as a post-hoc, to examine differences between groups, or the Kruskal-Wallis analysis followed by Dunn´s test for multiple comparisons.

For the evaluation of ventricular size, the areas of three different slices at three coronal levels were measured (corresponding to three different Bregma coordinates; 0.48, -1.2 and -2.76 mm). The percentage of the ventricular size in the total area of each coronal section was calculated and the results were expressed as the mean size of the three obtained values.

Cholinergic cell quantification was carried out in coronal sections of one specific Bregma level (-1.2 mm) corresponding to the anatomical location of the nbM. AChE positive cells were estimated by quantifying them in five sections (14 μm) of the basal forebrain area. The values corresponding to the number of cells included in one mm^3 were

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estimated. Statistical evaluations of the changes in the size of lateral ventricles and in the number of positive AChE cells were performed by ANOVA followed by Bonferroni´s multiple comparison test.

All the analyses were performed using GraphPad Prism (v 5.0) or SPSS (v 9.0). The criterion for statistical significance was $p \leq 0.05$ in the evaluations.

RESULTS

Effects of Galanin treatment on passive avoidance response

There were no differences in the latency times of the four groups of animals in the training trials (untreated, sham-operated, aCSF-treated and galanin-treated; data not shown). Regarding the passive avoidance response in the retention trial, the recorded step-though latency times (STL) were shorter for the group that had received aCSF in comparison with both the group of untreated rats and the group that had been cannulated and handled daily, but had not received any treatment (sham-operated) (222 ± 28 *vs* 300 sec, maximal time recorded; $p < 0.05$; Kruskal-Wallis followed by Mann-Whitney *U*-test). However, treatment with GAL prevented the reduction in STL (222 ± 28 *vs* 291 ± 9 sec; $p < 0.05$; Kruskal-Wallis followed by Mann-Whitney *U*-test) (Fig. 1, left). The effect of GAL-treatment on the number of positive or negative responses when evaluating the number of rats that enter the dark compartment during the retention trial in the passive avoidance test, was also analysed and the value of Fisher´s test was $p = 0.036$ with a RR = 6 (aCSF vs GAL-treatment). In addition, retention latency was also represented as Kaplan-Meier survival curves and compared by Mantel-Cox log-rank test with a $p = 0.030$ (Fig. 1, right).

Ventricular size, AChE activity and immunofluorescence

There was an increase in the mean size of the ipsilateral ventricle areas both in the group treated with aCSF (1.31 ± 0.17 %) and in that treated with GAL (1.36 ± 0.16 %) in comparison with the untreated group (0.9 ± 0.09 %).

The percentage of AChE positive cells in the area of nbM was found decreased in the aCSF and the GAL group when compared to both untreated and sham-operated animals (decreases of 35% and 30% for the aCSF and the GAL group, respectively) (Fig. 2). Furthermore, we observed a reduced arborization of the AChE positive cells in the aCSF-treated animals. But the cytoarchitecture of the surviving AChE positive cells in the rats treated with the neuropeptide was similar to that of untreated and sham groups (Fig. 2).

On the other hand, the immunohistochemical assays for endogenous GAL in nbM, showed a slight increase in galaninergic fibres of rats treated with aCSF and in GAL-treated animals, which was slightly higher than that found in the aCSF group (Fig. 3, top row). In cortical areas the immunofluorescence measurements for GAL were analysed in the area damaged by the injection tract. The immunohistochemistry for GAL around the injection tract showed immunoreactivity of fibres and cells in response to the injury during the injection. However, no GAL immunoreactivity was observed in the group treated with the neuropeptide itself (Fig. 3, bottom row).

Effects of Galanin treatment on [^{35}S]GTPγS binding in the hippocampus

The galanin treatment reduced the [^{35}S]GTPγS basal binding in the CA3 radiatum layer of ventral hippocampus (Untreated: 864 ± 43; aCSF: 1010 ± 43; GAL: 681 ± 62 nCi/g t.e., aCSF vs GAL $p \leq 0.01$). On the other hand, the stimulation of [^{35}S]GTPγS binding evoked by carbachol (M_2/M_4) minus the [^{35}S]GTPγS basal binding, i.e. the net binding, showed slight increases in carbachol net stimulation in the dorsal hippocampus (Table 1, Fig. 4).

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Effects of Galanin treatment on muscarinic receptor density

The group of rats that were treated with the vehicle (aCSF) for 6 days showed an increase in the density of MR when labelled with the tritiated antagonist, [3H]-NMS, in the nucleus of the lateral olfactory tract when compared to untreated rats. However, in the GAL treated group there was a similar density to that of the untreated group (Table 2). The group of rats that had received GAL also showed a decrease in [3H]-NMS binding in the substantia nigra, facial nucleus and striatum, but small increases were observed in some hippocampal areas when compared to those of the untreated group (Table 2 and Fig. 5).

The density of M_1 receptors, measured by [3H]-pirenzepine binding, in aCSF treated animals was significantly lower than that observed in untreated rats at the glomerular and plexiform external layers of the olfactory bulb, some amygdaloid nuclei (medial and cortical), and in CA3 oriens and pyramidal layers of the dorsal hippocampus (Table 3). However, the decreases were not detected in the same areas of the GAL treated group, with the exception of the cortical amygdaloid nucleus. There were no changes of M_1 densities in CA3 of ventral hippocampus. Furthermore, GAL administration led to a slight increase in M_1 receptor density in the lateral olfactory tract nucleus (Table 3 and Fig. 5).

The administration of the vehicle, aCSF, decreased [3H]-oxotremorine binding in the anterior part of the paraventricular thalamic nucleus and induced slight reductions in the CA1 pyramidal and subiculum of ventral hippocampus. M_2 density was increased in the superficial grey layer of the superior colliculus (Table 4). Changes in M_2 receptor density were prevented in these regions by the treatment with GAL (Table 4). Moreover, GAL treatment produced an enhancement of [3H]-oxotremorine binding sites in CA3 pyramidal layer and subiculum of the ventral hippocampus and in layers I and II of spinal cord, but a decrease in the medial amygdaloid nucleus (Table 4 and Fig. 5).

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Effects of galanin treatment on galanin receptor density

The [^{125}I]-galanin binding sites were quantified in coronal sections by autoradiography, obtaining very low densities. One of the brain areas with highest densities was the ventral subiculum (1.45 ± 0.17 fmol/mg t.e., untreated group), where aCSF-treated group of rats experimented a significant up-regulation of the [^{125}I]-galanin binding sites (2.40 ± 0.25; p ≤ 0.01). On the contrary, the galanin-treated animals had a density of GAL receptor comparable to the untreated group (1.60 ± 0.01; p ≤ 0.01). A slight increase was also observed in the entorhinal cortex (Untreated: 1.48 ± 0.14 vs aCSF: 1.76 ± 0.31; p = 0.06; GAL: 1.45 ± 0.22).

DISCUSSION

The present study shows that a six-day subchronic treatment of GAL is able to prevent some learning deficits probably by modulating the activity of some GPCR and specifically, the density and/or activity of muscarinic receptors. The i.c.v. administration of the vehicle, aCSF, during six days induced cognitive deficits in rats. The increase in intracranial pressure, as was seen by the enlargement of the ventricles, could explain these cognitive deficits. The rat model of kaolin-induced hydrocephalus also presents impairment of memory and learning that may be attributable to both the degeneration of septo-hippocampal cholinergic neurons, caused by ventricular enlargement, and the decrease in cholinergic activity (Tashiro et al., 1997; Shim et al., 2003). In this context, the increase in GAL concentration in the CSF of hydrocephalus patients is reduced when intracranial pressure is lowered after shunt surgery, and this is accompanied by a recovery of cognitive impairments (Mataró et al., 2003). Furthermore, the aCSF treatment causes a loss of AChE positive neurons in basal forebrain that GAL treatment was not able to prevent but maintains the dendritic arborization of the surviving neurons.

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The i.c.v. administration of GAL prevented the impairment of memory provoked by the administration of aCSF in rats as recorded using the passive avoidance test, which differs from the previously described impairment in the processes of acquisition and retention in experimental animals after the administration of this neuropeptide. Ukai and co-workers described a decrease in the step-down latency of passive avoidance when intermediate doses of GAL were administered before the training trial in mice (Ukai et al., 1995). This effect of using intermediate doses was also found when learning capacity and memory retention were analysed in the Morris water maze task (Ogren et al., 1996). In the aforementioned studies high doses of the neuropeptide did not affect these cognitive processes, but a low dose (1 nmol/rat) improved learning and retention when it was administered in the ventral hippocampus (Ogren et al., 1996). Moreover, the enhancement of acetylcholine release in the ventral hippocampus after the injection of GAL in the medial-septal region also produced an improvement in learning capacity (Elvander et al., 2004). GAL seems to have different effects depending on the dose and the region where it is injected, and according to our results and previous data, low doses of GAL could be beneficial due to the fact that they prevent the impairment of cognition and protect against the consequences of different brain injuries.

GAL treatment also down-regulated the [^{35}S]GTPγS basal binding in the hippocampus to control physiological levels. We also measured a similar effect in diverse thalamic nuclei following the same treatment (Barreda-Gómez et al., 2005). Other authors, using diverse experimental models, have described the neuroprotective effects of the neuropeptide. GAL reduces the toxicity of beta-amyloid (βA) in vitro in cell cultures containing human cortical neurons, cultured rat hippocampal neurons or basal forebrain cholinergic neurons (Cui et al., 2010; Cheng and Yu, 2010; Ding et al., 2006). GAL levels are increased in vivo after cortical lesions (Cortés et al., 1990; Mufson et al., 2005). Different studies have described an up-regulation in GAL gene expression, galanin concentration or GAL receptor densities

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in diverse neurodegenerative diseases such as AD or Parkinson's disease (Chan-Palay, 1988; Beal et al., 1990; Mufson et al., 1993; Rodríguez-Puertas et al., 1997a; Mufson et al., 2000). Furthermore, the administration of GAL is able to attenuate spatial memory impairment and βA levels in a rat model of AD (Li et al., 2013).

In accordance with these studies, the increase of galaninergic immunoreactivity observed in the basal forebrain of both groups of rats, the one treated only with the vehicle and the other with GAL, suggests a neuroprotective effect on cholinergic cells. We also observed an increase in positive galaninergic cells and fibres in the cerebral cortex adjacent to the site where the cannula was implanted in the aCSF group, but not in the GAL-treated group. The endogenous GAL could act against the effects observed in the aCSF group as a neuroprotective response, but the exogenous GAL could take over this role. However, the immunoreactivity detected in the nbM could be the sum of the endogenous response to injury plus the effects mediated by the exogenous neuropeptide.

Jhamandas´s group observed that GAL had an excitatory effect on basal cholinergic forebrain neurons and suggested that may play a compensatory role in the activity of cholinergic neurons in AD to offset the degeneration involved in the illness (Jhamandas et al., 2002). However, in the present study the administration of GAL did not prevent the decrease in AChE positive neurons in the nucleus basalis of Meynert, but was able to enhance the immunoreactivity for GAL in the aCSF-treated group.

The increase in [^{35}S]GTPγS basal binding was not produced after GAL treatment in CA3 radiatum layer of ventral hippocampus. But GAL receptors activity is increased following the GAL treatment (Barreda-Gómez et al., 2005). Therefore, we explored the possibility that the effects of the neuropeptide on the [^{35}S]GTPγS basal binding would be a consequence of regulations on MR densities and/or activities.

As stated above, different studies have demonstrated that GAL modulates acetylcholine release, the intracellular second messenger activation mediated by MR and the functional coupling of these receptors to $G_{i/o}$ proteins (Fisone et al., 1987; Amoroso et al., 1992; Palazzi et al., 1991; Barreda-Gómez et al., 2005). In relation to its effect on the cholinergic system, the results obtained in the present study show that the administration of GAL tends to increase the functional coupling of MR (induced by carbachol), presumably the M_2/M_4 subtypes, to $G_{i/o}$-proteins in diverse cerebral areas. GAL produced a slight enhancement in the total density of MR, in CA1 and CA3 layers of the ventral hippocampus. The i.c.v administration of GAL in the lateral ventricle immediately above the dorsal hippocampus may be responsible for the limited effects in ventral hippocampus. GAL treatment also prevented the decrease in M_1 receptor density observed in the dorsal hippocampus after aCSF administration. The M_2 receptor density, measured by [^{3}H]-oxotremorine binding, was found increased in diverse subareas of ventral hippocampus of the GAL-treated animals. Carbachol-induced [^{35}S]GTPγS binding, which reflects MR activity, also tends to increase at dorsal hippocampus after GAL treatment.

Regulation of M_2 receptor subtype density and activity could contribute to the reduced impairment of memory observed in the GAL-treated group. This muscarinic receptor subtype is mainly located on GABAergic neurons and the activation of the M_2 receptors could decrease the inhibitory effect of these neurons, improving cognitive processes (Levey et al., 1995; Hajos et al., 1998). In support to this hypothesis, the administration of the GABA$_A$ receptor antagonist, bicuculline, is able to restore short-term potentiation responses and significantly increase the long-term potentiation in hippocampus, recovering the impaired synaptic plasticity observed in M_2 receptor KO mice (Seeger et al., 2004).

GAL administration also increased M_2/M_4 coupling to $G_{i/o}$ proteins in the anterior and medial thalamic nuclei (Barreda-Gómez et al., 2005). A similar enhancement of M_2

receptors has been observed in the anterior thalamus during learning (Vogt et al., 1991). The increase in the activity of these receptors could contribute to restore the memory deficits observed in aCSF-treated animals in the passive avoidance test. The administration of the neuropeptide protected M_1 and M_2 receptor densities in the medial amygdaloid area. However, in this area there was no reduction of carbachol-induced [^{35}S]GTPγS stimulation in the aCSF treated group, when compared to the untreated group, but a significant increase was observed in the GAL treated group (Barreda-Gómez et al., 2005). Therefore, GAL signalling contributes to the preservation of memory and modulates MR densities and activity. The M_1 preservation at dorsal hippocampus, and M_2 subtype at ventral hippocampus, together with the increase in M_2/M_4 activity could be some of the neurochemical mechanisms underlying in the maintenance of learning and memory. Globally the present results suggest that GAL could act on the cholinergic system as a neuroprotective molecule. Further experiments administering MR agonist or antagonist to galanin-treated rats would confirm the contribution of the neuropeptide in cognitive processes mediated by MR signalling.

Regarding the results obtained for GAL receptor densities measured by [^{125}I]-galanin binding, indicate that they are up-regulated after the aCSF administration in subiculum and entorhinal cortex, but the subchronic GAL-treatment is sufficient to maintain the GAL receptors densities at physiological levels, probably by a down-regulation mechanism. Similar up-regulations of GAL receptors have been observed in neurodegenerative processes in the hippocampus including the AD (Rodríguez-Puertas et al., 1997a).

CONCLUSIONS

The experimental evidences show that a subchronic GAL treatment with low doses could protect against learning capacity impairments caused by injuries produced after ventricular pressure increases. The neurochemical mechanisms involve the modulation of

both MR densities and activities. The data support the neuroprotective functions of the neuropeptide in cholinergic pathways controlling learning and memory from the basal forebrain to subcortical projection areas. The findings of the present study reveal that a subchronic administration of GAL could maintain the signalling of the surviving cholinergic cells at the basal forebrain. In this context, it has been observed that neuropeptides become active when the nervous system is damaged through injury or challenged by stress. The actions of GAL could be explained as a response to injury as indicated by the increase of galaninergic cells and fibres in the tract produced by the cannula, specifically in the cortex.

There is an increase in the relative ventricular size and a loss of AChE positive neurons in both, aCSF and GAL-treated groups. However, the modulation of MR densities induced by the administration of aCSF were prevented by GAL, and this fact together with the previously reported compensation of GAL increasing the MR activity, could constitute the neurochemical basis for the improvement in STL in the passive avoidance test observed after GAL-treatment.

The present study supports the neuroprotective actions of GAL on the basal forebrain cholinergic system in the control of memory and learning. The study of MR and GAL receptors activity in these brain pathways during the Alzheimer's disease progression would contribute to elucidate the potential of the galaninergic system modulation as a therapeutic strategy.

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Table 1. Stimulation of [^{35}S]GTPγS binding by carbachol (100 μM) in coronal sections of untreated, aCSF and GAL-treated rats

Brain region	Net binding induced by carbacol (M$_2$/M$_4$ receptors) (nCi/g t.e.)		
	Untreated	aCSF	Galanin
Dorsal hippocampus			
CA1 oriens	169 ± 85	151 ± 111	455 ± 43
CA1 pyramidal	158 ± 105	103 ± 105	426 ± 70
CA1 radiatum	75 ± 109	118 ± 92	408 ± 61
CA3 oriens	177 ± 78	147 ± 81	315 ± 65
CA3 pyramidal	297 ± 51	223 ± 95	341 ± 77
CA3 radiatum	188 ± 68	198 ± 76	356 ± 75
Polymorph layer of dentate gyrus	199 ± 86	150 ± 78	230 ± 108
Granular layer of dentate gyrus	70 ± 63	112 ± 93	333 ± 58
Molecular layer of dentate gyrus	148 ± 104	120 ± 96	359 ± 37

Data are mean ± S.E.M. values of 5-7 animals
ANOVA followed by Bonferroni's test or Kruskal-Wallis followed by Dunn's test.

Table 2. [^{3}H]-NMS binding to coronal sections of untreated, aCSF and GAL-treated rats

Brain region	[^{3}H]-NMS binding (B$_{max}$= fmol/mg t.e.)		
	Untreated	aCSF	Galanin
Lateral olfactory tract nucleus	345 ± 13	393 ± 11 *a	359 ± 10
Striatum	420 ± 26	393 ± 23	328 ± 20 *b
Medial amygdaloid n., posterior part	170 ± 7	158 ± 5	173 ± 3
Ventral hippocampus			
CA1 oriens	254 ± 9	269 ± 18	289 ± 7
CA1 pyramidal	286 ± 10	302 ± 15	315 ± 11
CA1 radiatum	310 ± 10	293 ± 16	323 ± 11
CA1 lacunosum molecular	237 ± 12	232 ± 12	246 ± 14
CA3 oriens	285 ± 5	272 ± 16	301 ± 9
CA3 pyramidal	233 ± 5	226 ± 13	249 ± 5
CA3 radiatum	285 ± 9	279 ± 19	311 ± 10
Subiculum	287 ± 8	282 ± 11	301 ± 9
Thalamic nucleus			
Centromedial	271 ± 7	251 ± 6	274 ± 7
Dorsomedial	170 ± 6	163 ± 3	175 ± 5
Substantia nigra			
pars compacta	81 ± 2	83 ± 3	76 ± 1 *b
pars reticulata	68 ± 1	69 ± 2	63 ± 1 *c
Facial nucleus	123 ± 14	133 ± 18	72 ± 4*b,*c

Data are mean ± S.E.M. values of 5-7 animals
[a] aCSF *vs* untreated, [b] galanin *vs* untreated, [c] galanin *vs* aCSF (*p < 0.05); ANOVA followed by Bonferroni's test or Kruskal-Wallis followed by Dunn's test.

Table 3. [^{3}H]-pirenzepine binding to coronal sections of untreated, aCSF and GAL-treated rats

Brain region	[^{3}H]-pirenzepine binding (B_{max}= fmol/mg t.e.)		
	Untreated	aCSF	Galanin
Lateral olfactory tract nucleus	309 ± 14	302 ± 31	413 ± 36
Glomerular and plexiform external layers of olfactory bulb	114 ± 9	67 ± 12 *a	100 ± 12
Amygdala			
Medial amygdaloid n., posterior part	130 ± 13	87 ± 9 *a	115 ± 7
Posteromedial cortical amygdaloid n.	257 ± 19	191 ± 15 *a	190 ± 8 *b
Dorsal hippocampus			
CA3 oriens	243 ± 11	176 ± 16 **a	208 ± 13
CA3 pyramidal	191 ± 9	143 ± 18 *a	173 ± 9
CA3 radiatum	254 ± 15	201 ± 18	233 ± 13
Polymorphic layer of dentate gyrus	197 ± 12	165 ± 7	201 ± 7
Molecular layer of dentate gyrus	479 ± 31	387 ± 20	442 ± 33
Ventral hippocampus			
CA3 oriens	260 ± 17	239 ± 11	229 ± 4
CA3 pyramidal	236 ± 14	210 ± 8	206 ± 2
CA3 radiatum	302 ± 23	267 ± 18	264 ± 15
Subiculum	279 ± 21	255 ± 15	243 ± 8

Data are mean ± S.E.M. values of 5-7 animals
[a] aCSF *vs* untreated, [b] galanin *vs* untreated, [c] galanin *vs* aCSF (*p < 0.05,**p < 0.01); ANOVA followed by Bonferroni's test or Kruskal-Wallis followed by Dunn's test.

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Table 4. [³H]-oxotremorine binding to coronal sections of untreated, aCSF and GAL-treated rats

Brain region	[³H]-oxotremorine binding (B_{max} = fmol/mg t.e.)		
	Untreated	**aCSF**	**Galanin**
Medial septal and vertical limb of the diagonal band	612 ± 23	690 ± 25	722 ± 37
Entorhinal cortex	149 ± 13	143 ± 8	166 ± 3
Piriform cortex	200 ± 21	163 ± 6	152 ± 9
Amygdala			
Medial amygdaloid n., posterior part	210 ± 14	160 ± 8	135 ± 16 **b
Posteromedial cortical amygdaloid n.	124 ± 15	72 ± 12	98 ± 14
Ventral hippocampus			
CA1 pyramidal	238 ± 19	213 ± 15	242 ± 8
CA3 pyramidal	216 ± 18	221 ± 18	292 ± 9 *b, *c
Subiculum	219 ± 28	136 ± 22	263 ± 13 **c
Thalamic nucleus			
Centromedial	670 ± 32	596 ± 41	582 ± 24
Laterodorsal, dorsomedial and ventrolateral parts	344 ± 19	311 ± 20	377 ± 21
Paraventricular, anterior part	504 ± 24	385 ± 34 *a	496 ± 12 *c
Superficial gray layer of the superior colliculus	1146 ± 38	1332 ± 46 *a	1237 ± 38
External cortex of the inferior colliculus	544 ± 9	506 ± 55	570 ± 14
Substantia nigra, pars reticulata	61 ± 5	51 ± 7	48 ± 13
Prepositus hypoglossal nucleus	382 ± 29	299 ± 11	338 ± 38
Spinal cord, layers I-II	478 ± 56	625 ± 34	649 ± 33 *b

Data are mean ± S.E.M. values of 5-7 animals
[a] aCSF *vs* untreated, [b] galanin *vs* untreated, [c] galanin *vs* aCSF (*p < 0.05,**p < 0.01); ANOVA followed by Bonferroni's test or Kruskal-Wallis followed by Dunn's test.

FIGURE LEGENDS

Fig.1: (Left) Effects of GAL administration (i.c.v.) on the passive avoidance test. GAL treatment improves learning deficits induced by the administration of aCSF. The last dose of GAL was administered 1 hour before the training trial. [a] aCSF *vs* sham-operated and untreated groups of rats and [b] GAL *vs* aCSF; *p < 0.05; Kruskal-Wallis followed by the Mann-Whitney *U*-test. (Right) Retention latency represented as Kaplan-Meier survival curves for the four groups and compared by Mantel-Cox log-rank test with a p= 0.030. The number of positive or negative responses of rats that enter the dark compartment during the retention trial in the passive avoidance test, was also analysed and the value of Fisher´s test was p = 0.036 with a RR = 6 (aCSF vs GAL-treatment). Cut-off time (300 sec).

Fig. 2: (A) Quantification of the number of AChE positive cells in the nucleus basalis of Meynert of untreated, sham-operated, aCSF and GAL-treated groups of rats. Data are expressed as the mean of the number of cells per mm^3 (aCSF vs sham-operated, *p < 0.05; ANOVA followed by Bonferroni´s test). (B, C, D and E) Representative images of AChE staining pattern in coronal sections including the nucleus basalis of Meynert in the contralateral side of the administration site. Untreated rat (B), sham-operated (C), aCSF-treated (D) and GAL-treated (E). Note the different morphology of cells in the aCSF group (D), which were smaller and with less dendritic arborization than the cells of untreated (B), sham-operated (C) and GAL-treated groups (E). Scale bar = 150 μm.

Fig. 3: Immunohistochemical labelling of GAL in the basal forebrain of the hemisphere where the cannula was introduced (top row). Note the increase in positive fibres for GAL in both, the representative section from a rat treated with aCSF and from those treated with

GAL. Scale bar = 100 μm. Immunofluorescence micrographs in the cortical area where the cannula was implanted (bottom row). Immunoreactivity for GAL was observed in cells throughout the insertion tract of the cannula in animals treated with aCSF. However, in the animals treated with GAL, only a few labelled fibres were observed. Scale bar = 50 μm.

Fig. 4: Left, representative autoradiograms showing the [^{35}S]GTPγS basal binding (A, B, C) and in the presence of carbachol (D, E, F) in the dorsal hippocampus of an untreated rat (A, D), aCSF-treated rat (B, E) and GAL-treated rat (C, F). Note that GAL treatment increases the activity mediated by M_2/M_4 MR in the oriens and radiatum layers of the CA1 area. Scale bar = 1 mm. Right, histogram showing the increase in the [^{35}S]GTPγS binding induced by carbachol (100µM) in granular layer of dentate gyrus of GAL-treated rats.

Fig. 5: Left column, autoradiographic images of [^{3}H]-NMS binding from an untreated (A), an aCSF-treated (B) and a GAL-treated (C) rat. GAL treatment slightly increases muscarinic receptor density in some hippocampal areas (CA1 *oriens* of ventral hippocampus: CA1*or*). Center column, [^{3}H]-pirenzepine binding in a representative coronal section of dorsal hippocampus from an untreated (D), an aCSF-treated (E) and a GAL-treated (F) rat. Note that GAL treatment prevented the reduction in M_1 density observed in the aCSF treated rat. CA3 py: CA3 pyramidal layer; DG mol: molecular layer of the dentate gyrus. Right column, histograms showing the [^{3}H]-oxotremorine binding to the *subiculum* and CA3 *pyramidal* layer of the ventral hippocampus from untreated, aCSF and GAL-treated rats. [b] GAL *vs* untreated; [c] GAL *vs* aCSF; *p < 0.05; **p < 0.01; ANOVA followed by Bonferroni´s test.

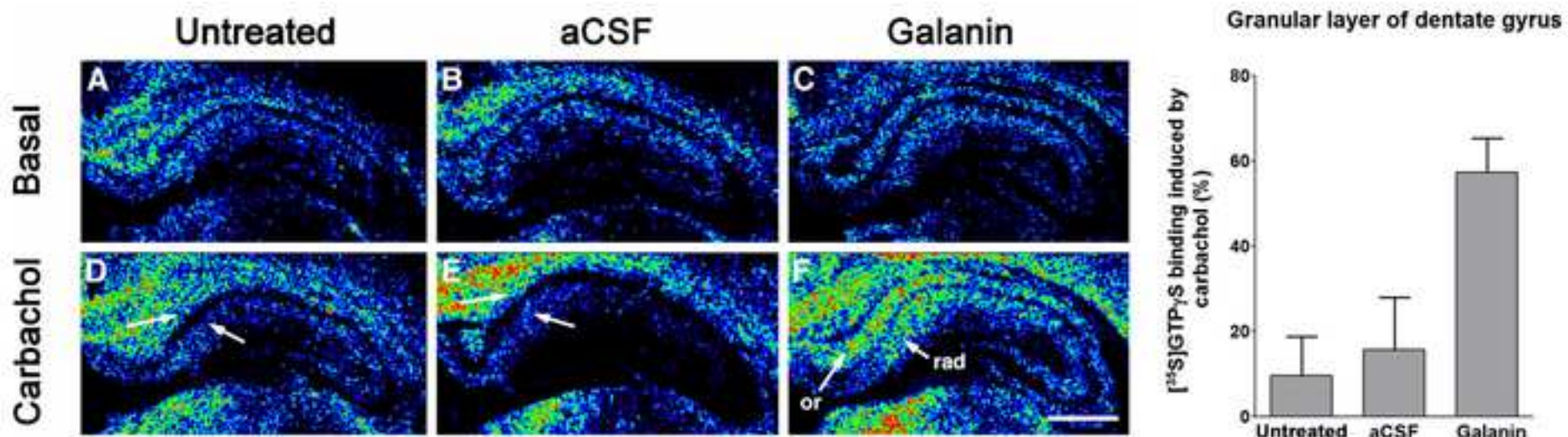**Figure 2**
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| Untreated | Sham | aCSF | Galanin |

Figure 4

[³H]-NMS   [³H]-pirenzepine   [³H]-oxotremorine