

GALANIN (1-15)-FLUOXETINE INTERACTION IN THE NOVEL OBJECT RECOGNITION TEST. INVOLVEMENT OF 5-HT1A RECEPTORS IN THE PREFRONTAL CORTEX OF THE RATS

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Abstract

Galanin (1-15) [GAL(1-15)] participates in mood regulation and depression. GAL(1-15) is also able to enhance the antidepressant effects induced by Fluoxetine (FLX) in the forced swimming test through interaction between GALR1-GALR2 and 5-HT1A receptors that induced changes in the binding characteristics and mRNA of the 5-HT1AR in the hippocampus.

Since the medial prefrontal cortex (mPFC) is a core region for the interaction between emotional processing and cognition with a high density of 5-HT1AR and GALR1 and GALR2, we have analyzed the binding characteristics and mRNA levels of 5-HT1AR in the mPFC after GAL(1-15)-FLX administration in the rats. GAL(1-15) increased the K_d and the B_{max} of the 5HT1AR agonist binding in the mPFC as well as the mRNA levels of 5-HT1AR in mPFC. Moreover, GAL(1-15) reversed the effects of memory impairment induced by FLX(10mg/Kg) in the Novel Object Recognition task. GALR2 was involved in these effects, since the specific GALR2 antagonist M871 blocked GAL(1-15) mediated actions at behavioral level. On the contrary GAL(1-15) did not reverse the effect of FLX in the Object Location Memory task.

In conclusion, our results describe an interactions between GAL(1-15) and FLX in the mPFC involving interactions at the 5-HT1AR receptor level in the plasma membrane with changes at the transcriptional level with implications also at functional level. The GALR1-GALR2-5-HT1A heteroreceptor could be postulated to be used to reverse some of the adverse effects of FLX on memory processes.

1. Introduction

Galanin (GAL) is a neuropeptide (Tatemoto et al., 1983) widely distributed in neurons within the Central Nervous System (CNS) (Jacobowitz et al., 2004). Three GAL receptor (GALR1-3 receptors) subtypes with high affinity for GAL have been cloned (Branchek et al., 2000; Mitsukawa et al., 2008). GALR1 and GALR2 receptors are found in many regions of the CNS as demonstrated with *in situ* hybridization, radioligand binding and immunohistochemical studies (Jacobowitz et al., 2004). GALR1 and GALR3 mainly activate inhibitory G proteins Gi/Go, while GALR2 mainly couples to Gq/G11 to mediate excitatory signaling (Branchek et al., 2000; Wang et al., 1997).

These three GALR are involved in a number of central functions modulating neuroendocrine, pain control, cardiovascular, addiction and food intake (Diaz-Cabiale et al., 2010; Mitsukawa et al., 2008; Picciotto, 2010) GAL also participates in mood regulation and depression (Bellido et al., 2002; Juhasz et al., 2014; Weiss et al., 1998). The activation of GALR1 and GALR3 results in a depression like behavior while stimulation of GALR2 leads to anti-depressant-like effects (Bartfai et al., 2004; Kuteeva et al., 2008; Lu et al., 2005). Moreover, GAL modulates 5-HT_{1A} receptor (5-HT_{1A}R) function at autoreceptor and postsynaptic level in the brain (Fuxe et al., 1988; Hedlund and Fuxe, 1996; Kehr et al., 2002; Misane et al., 1998; Razani et al., 2000; Razani et al., 2001). Recent results indicate that this interaction could be in part due to the existence of GALR1-5-HT_{1A}R heteroreceptor complexes in discrete brain regions (Borroto-Escuela et al., 2010; Fuxe et al., 2012). It has been suggested that in depression, the GALR1-5-HT_{1A}R heteroreceptor complexes is dysfunctional which may lead to disturbances in meso-limbic 5-HT neurotransmission (Borroto-Escuela et al., 2017; Borroto-Escuela et al., 2018).

Not only GAL but also the N-terminal fragments like GAL(1-15) are active in the central nervous system (CNS) (Diaz-Cabiale et al., 2007; Diaz-Cabiale et al.,

2010; Diaz-Cabiale et al., 2005; Flores-Burgess et al., 2017; Millon et al., 2017; Millon et al., 2016; Millon et al., 2015). Recently we described that GAL(1-15) induces strong depression-related and anxiogenic-like effects in rats and these effects were significantly stronger than the ones induced by GAL (Millon et al., 2015). The receptor for GAL(1-15) is the GALR1-GALR2 isoreceptor dimer (Borroto-Escuela et al., 2014; Millon et al., 2015) for which GAL(1-15) has a high affinity. The strong depression-like and anxiogenic-like effects of GAL(1-15) disappeared in the siRNA GALR1 and siRNA GALR2 receptor knockdown rats (Millon et al., 2015) and were related to the disappearance of the *in situ* PLA signals and, thus, to the disappearance of the GALR1-GALR2 isoreceptor complexes (Millon et al., 2015).

GAL(1-15) is also able to enhance the antidepressant effects induced by the 5-HT_{1A}R agonist 8-OH-DPAT in the forced swimming test (FST) (Millon et al., 2016), effect that was again significantly stronger than the ones induced by GAL. Importantly, the mechanism underlying this action involved interactions at the receptor level in the plasma membrane with changes also at the transcriptional level. GAL(1-15) increased the K_d and B_{max} values and the mRNA levels of the postjunctional 5-HT_{1A}R in the dorsal hippocampus but not of the 5-HT_{1A} autoreceptors in the DR (Millon et al., 2016). The 5-HT_{1A} autoreceptor complexes appear to be differentially regulated since a reduction of mRNA levels in the DR was observed. The putative existence of a trimeric GALR1-GALR2-5-HT_{1A}R heteroreceptor complex was therefore postulated which can help explain this interesting enhancement by GAL(1-15) of the antidepressant effects of 8-OH-DPAT (Borroto-Escuela et al., 2018; Millon et al., 2016) and underlie as a mechanism of action independent from GAL.

In line with these results, GAL(1-15) and not GAL enhanced the antidepressant effects of the SSRI fluoxetine (FLX) using the FST (Flores-Burgess et al., 2017). In the combined treatment with GAL(1-15) and FLX, the modulation of the 5-HT_{1A}R agonist binding sites in the dentate gyrus was also different from the

modulation found with GAL(1-15). The modulation by combined treatment with FLX and GAL(1-15) produced an increased affinity of the 5-HT_{1A}R agonist binding sites and a reduction of their B_{max} values in the dentate gyrus (Flores-Burgess et al., 2017), in contrast to the reduced affinity and increased B_{max} levels found in this region with GAL(1-15) (Millon et al., 2016). However, the increase in 5-HT_{1A}R mRNA levels in the hippocampus was still obtained after the combined treatment with GAL(1-15) and FLX, as found after treatment with GAL(1-15) alone (Flores-Burgess et al., 2017).

These results illustrate that GAL(1-15) can produce differential changes in mood from depressive to antidepressant actions, being dependent on how the GALR come together with the 5-HT_{1A}R. The molecular complexes are likely highly dynamic and vary from one brain region to another.

The serotonergic system is also implicated in the neurobiological control of learning and memory (Meneses, 2007). One of the serotonergic receptors more extensively studied in behavioural learning paradigms is the 5-HT_{1A}R (Meneses et al., 2007; Ogren et al., 2008). For example, in the Novel Object Recognition task, the systemic administration of the 5-HT_{1A}R specific agonist 8-OH-DPAT in rats impaired the recognition of the new object, and the antagonist WAY100635 blocked these deficits (Meneses et al., 2007; Pitsikas et al., 2003).

The role of medial prefrontal cortex (mPFC) in single-item recognition memory is not fully elucidated. Initial studies showed that large aspiration lesions of the mPFC produced delay dependent impairments in delayed non-matching to sample tasks (Ragozzino et al., 2002; Warburton and Brown, 2015), suggesting an involvement for mPFC in this process (Morici et al., 2015), but excitotoxic lesion studies indicate that the mPFC is not involved in the NOR task based on single-item recognition memory. (Warburton and Brown, 2015). However, other studies using more

functional approaches, based mainly in the activation of immediate early genes (IEGs), have showed that the mPFC increases the expression of c-Fos (Rinaldi et al., 2010; Castilla-Ortega et al., 2012) and Zif-268 (Freitas Barbosa et al., 2013) in response to the test phase in a NOR task, supporting that this region is involved at least in the retrieval of this recognition memory

Since the mPFC has been recently identified as a core region for the interaction between emotional processing and cognition (Ray and Zald, 2012) with a particular a high density of 5-HT1AR and GALR1 and GALR2 (Jacobowitz et al., 2004; Pompeiano et al., 1992, 1994), we have analyzed the binding characteristics and mRNA levels of 5-HT1AR in the mPFC after GAL(1-15)-FLX administration. Moreover, we have studied the effect of GAL(1-15) on FLX-mediated behavioural responses using two memory tasks where FLX effect has been previously described (Ampuero et al., 2013; Castane et al., 2015), the Novel Object Recognition (NOR) and the Object Location Memory (OLM). **In both tasks FLX treatment did not affect learning or short-term memory but specifically impaired long-term memories since animals did not explore the new objects in the test session performed 24 h post-training either in the OLM or in the NOR tasks (Ampuero et al., 2013; Castane et al., 2015).** In addition, we tested the involvement of GALR2 in the GAL(1-15) effect with the selective GALR2 antagonist M871.

2. Material and Methods

2.1. Animals

Male Sprague Dawley rats (body weight 225–250g) were obtained from CRIFFA, Barcelona and maintained in a 12h dark/light cycle under controlled conditions of humidity (55-60%) and temperature (22±2°C). The animals had free access to food pellets and tap water. Behavioral tests were performed during the light phase of the cycle. All Experimental procedures were approved

by the Institutional Animal Ethics Committee of the University of Málaga, Spain.

2.2. Stereotaxic surgery

This protocol has been described previously (Diaz-Cabiale et al., 2011). Briefly, the rats were anesthetized intraperitoneally with equitesin (3.3ml/kg body weight). A chronic 22-gauge stainless-steel guide cannula was stereotaxically implanted into the right lateral cerebral ventricle (+1.4mm lateral, -1mm posterior to bregma, and 3.6mm below the surface of the skull) (Paxinos, 1986). Animals were individually housed after surgery and had a recovery period of 7 days prior to behavioural test.

2.3. Administration of substances and drugs

In the experimental groups we used the following drugs: Fluoxetine hydrochloride (Sigma-Aldrich, San Luis, EEUU) and GAL(1-15) and the GALR2 antagonist M871 (TOCRIS, Bristol, Reino Unido). Intracerebroventricular (icv) administration of substances was performed for one minute in a volume of 5µl using a 26-gauge stainless-steel injection cannula connected to a Hamilton syringe via PE-10 tubing **with special care that during the process the dilutions were administered fluently and without overflowing. All rats that received the i.c.v. injections were assumed to have proper cannula placement and none were excluded.** The solutions were prepared using artificial cerebrospinal fluid (aCSF). For subcutaneous (sc) administration of Fluoxetine (FLX), solutions was prepared using distillate water for improving the solubility in a volume of 2ml/Kg (Lopez-Rubalcava and Lucki, 2000).

2.4. Double Immunofluorescence

The procedures have been previously used (Millon et al., 2015). Primary antibody rabbit anti-GAL2 receptor (Alomone Lab, 1/250) was incubated for 12 hours at 4°C and detected with the red secondary antibody mouse anti-rabbit DyLight 549 (Jackson inmunoResearch Laboratories, 1/100). Goat anti-GAL1 receptor (Santa Cruz Biotechnology INC, EEUU, 1:250) was incubated 12 hours

at 4°C and detected with the secondary antibody rabbit biotinylated anti-goat (Vector Labs Inc.) and Alexa Fluor 488-conjugated Streptavidin (Jackson Laboratories ImmunoResearch, 1:1000). Sections were mounted on slides with fluorescent mounting medium (Dako) and visualized using a Leica SP8 confocal microscope. The double immunolabeling was performed in the tissue of rats without treatment.

2.5. Autoradiography and in situ hybridization histochemistry

The procedure to perform receptor autoradiography and in situ hybridization histochemistry was previously described (Flores-Burgess et al., 2017; Millon et al., 2016; Saenz del Burgo et al., 2013). 6 rats per group received three sc injections of FLX or vehicle at 23, 5 and **1.25 h** prior to the sacrifice, and GAL(1-15) or aCSF was administered by a single injection via icv 30 minutes before sacrifice. **In these experiments we have used groups of animals that did not perform object recognition tasks. We have used the doses and injection schedule based on our previous work where we observed that the coadministration of FLX and GAL(1-15) modified the binding characteristics and mRNA expression of 5-HT1AR in the dentate gyrus** (Flores-Burgess et al., 2017; Millon et al., 2016; Millon et al., 2015).

The pattern of FLX injection was shown to produce effects in the FST similar to those obtained after chronic treatment and to those leading to its clinical effects in humans (Detke et al., 1997; Estrada-Camarena et al., 2003; Estrada-Camarena et al., 2006). Their brains were rapidly removed and frozen in -40°C isopentane and stored at -80°C until its use. Coronal sections (14µm thick) were cut in a cryostat at bregma levels, according to the atlas of (Paxinos, 1986) (medial prefrontal cortex (mPFC): 3.50mm to 2.70mm), thaw-mounted on gelatin-coated slides stored at -20°C until further processing.

2.5.1. Quantitative autoradiography

Saturation experiments were performed using [3H]-8-OH-DPAT (specific activity: 142Ci/mmol, Perkin Elmer, Waltham, Massachusetts) in

concentrations ranging from 0.24 to 10.8 nM. In each saturation experiment, two groups of ten sections were prepared in order to perform the total binding and the non-specific binding. Nonspecific binding was defined as the binding of [3H]-8-OH-DPAT in the presence of 10⁻⁵M of 5-HT (Sigma Aldrich, St. Louis, CA). The brain sections were preincubated for 30 minutes at room temperature in 50mM Tris-HCl buffer (pH 7.6), containing 4mM CaCl₂, 0.01% ascorbic acid and 10mM pargyline. The sections were then incubated for 60 minutes at room temperature with [3H]-8-OH-DPAT in the same solution as above. Thereafter, the sections were washed twice for 5 minutes in ice cold buffer and rinsed briefly in ice-cold distilled water before being dried under a stream of cold air. Dried sections were exposed to [3H]- Kodak Biomax-MR (Sigma Aldrich, St. Louis, CA) for 6 weeks along with [3H]-radiolabeled standards plastic strips (Amersham, UK).

2.5.2. *In situ* hybridization histochemistry

For *in situ* hybridization studies, frozen tissue sections were air-dried at room temperature and fixed in 4% paraformaldehyde during 20 minutes and washed in phosphate-buffered saline (PBS 1X: 8mM Na₂HPO₄, 1.4mM KH₂PO₄, 136mM NaCl, 2.6mM KCl) for 5 min in 3X PBS at room temperature and twice for 5 min each in 1X PBS, then were incubated in a freshly prepared solution of predigested pronase (Calbiochem, San Diego, USA) at a final concentration of 24U/ml in 50mM Tris-HCl, pH 7.5, 5mM EDTA. The enzymatic activity was stopped by immersion for 30s in 2mg/mL glycine in 1X PBS. Tissues were finally rinsed in 1X PBS and dehydrated through a graded series of ethanol. The oligonucleotides used were complementary to the following base sequences and were custom-synthesized by Isogen Bioscience (Maarsden, The Netherlands): 5HT_{1A}, 1-48, 763-810 and 1219-1266 [Gen Bank acc. No NM_012585.1]. The oligonucleotides were labeled at their 3'-end by using [33P]dATP (3000Ci/mmol) and terminal deoxynucleotidyl-transferase (TdT, Oncogene Research Products, San Diego, CA, USA), and purified using MicroSpin G-50 columns (Amersham, UK). For hybridization,

radioactively labeled probes were diluted to a final concentration of 206cpm/mL in a solution containing 50% formamide, 4X SSC (1X SSC: 150mM NaCl, 15mM sodium citrate), 1X Denhardt's solution (0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin), 10% dextran sulfate, 1% sarkosyl, 20mM phosphate buffer pH 7.0, 250µg/mL yeast tRNA and 500µg/mL salmon sperm DNA. Tissues were covered with 80µL of the hybridization solution and overlaid with Nescofilm (Bando Chemical Inc., Kobe, Japan) coverslips to prevent evaporation. Sections were incubated in humid boxes overnight at 42°C and then washed 4 times (45min each) in 600mM NaCl, 10mM Tris-HCl, pH 7.5, 1mM EDTA at 60°C and once in the same buffer at room temperature for 30 min. Hybridized sections were exposed to Kodak BIOMAX-MR film (Sigma Aldrich, St. Louis, CA) for 10 days at -70°C with intensifying screens.

2.5.3. Image analysis

Measurements using the ImageJ system (NIH, USA) were made in the distinctly labelled region in the mPFC using a square as a sampling field (0.3 mm²). One observation per region and rat was obtained, since the average of the measurements was calculated. [3H]-radiolabeled standards plastic strips (Amersham, UK) were used to convert the grey values into fmol/mg protein values in the receptor binding quantitative autoradiography. The semiquantitative evaluations of the *in situ* hybridization autoradiograms were performed based on the optical density (O.D.).

2.6. Object Location Memory (OLM) and Novel Object Recognition (NOR) task

OLM and NOR were performed in an open field test apparatus (100x100 cm) under dim light. The task procedures consist of three phases: habituation, training, and test (Ampuero et al., 2013) (Fig. 1). The animals were handled 2 days before the habituation phase. In the habituation phase, animals were allowed to explore the open field 10 minutes. 24 h later, in the test phase, two identical objects (two plastic boats 20cm high and filled with water) were placed

in two opposite corners of the apparatus 10 cm from the side wall and the animals were allowed to explore during 3 minutes. The day after we performed the test phase. For the NOR task we change one object to other differently shaped and colored, the objects were of similar in weight and size (Fig 1A). In the OLM we use the same objects employed in the training phase changing the location of one of them (Fig 1B). Animals were allowed to explore 3 minutes in the test phase. Exploration was defined as sniffing or touching the object with the nose and/or forepaws. Turning around or sitting on the object was not considered as exploratory behavior. The time spent by the rats exploring each object, familiar (F) or new (N), in the test phase was recorded by a video camera and **was analyzed blind to the treatment**. A discrimination index (DI) was calculated as: $DI=(N-F)/(N+F)$, this ratio represent the difference in exploration time expressed as a proportion of the total time spent exploring the two objects (Ennaceur and Delacour, 1988). We also analyzed the locomotor activity using the video-tracking software EthovisionXT (Noldus, Wageningen, Nederland). Between trials, the role of familiar or new object and the relative position were counterbalanced and randomly permuted also the open field and the objects were cleaned thoroughly in every phase (Akirav and Maroun, 2006; Ampuero et al., 2013). The pharmacological treatments were administered between the training and test phases.

2.6.1. Behavioural assessment

Different groups of rats were assessed in the NOR and OLM. In the first set of experiment in order to evaluate the interaction of FLX with GAL(1-15) in the NOR task, groups of rats received before the test phase three injections (23, 5 and 1 h) of sc FLX(10mg/kg) or vehicle and a single icv injection of a threshold dose of GAL(1-15)(1nmol) or aCSF 15 minutes before the test. We also determined the involvement of GALR2 in the effect of GAL(1-15) on FLXmediated action in rats that received three injections of sc FLX(10mg/kg), a single icv injection of GAL(1-15)(1nmol) and the GALR2 antagonist M871 (3nmol) icv in combination. In the second set of experiments we evaluate the

interaction of FLX with GAL(1-15) in the OLM task, groups of rats received three injections of FLX(10mg/Kg) alone or in combination with GAL(1-15)(1nmol).

2.7. Statistical analysis

Data are presented as the mean \pm SEM and samples number (n) are indicated in figure legends. All data were analyzed using GraphPad PRISM 6.0 (GraphPad Software, La Jolla, CA). One-way analysis of variance (ANOVA) followed by Newman-Keuls comparison post hoc-test was performed. Differences were considered significant at $p < 0.05$ (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). The data from the saturation experiments were analyzed by nonlinear regression analysis for the determination of the dissociation constant (Kd) and the total number of agonist binding sites (Bmax) using GraphPad PRISM 4.0.

3. Results

3.1. Neurochemical effects

3.1.2. 5-HT_{1A}R agonist radioligand binding. Saturation curves.

An extensive colocalization of GALR1 and GALR2 immunoreactivities was observed in the nerve cells of the mPFC (Fig. 2).

In the autoradiographic experiments, the coadministration of the three injections of sc FLX(10mg/kg) and a single icv injection of GAL(1-15)(1nmol) produced a significant increase in the Kd value (Fig. 3A; $F_{3,20}=14.36$, $p < 0.001$; post hoc $p < 0.01$) and in the Bmax value (Fig. 3B; $F_{3,18}=5.57$, $p < 0.01$; post hoc $p < 0.05$) of the agonist radioligand [³H]-8-OH-DPAT compared with FLX(10mg/Kg) in the mPFC. Representative autoradiograms with a low radioligand concentration (1nM) illustrate the decrease of labelling (increase in Kd values) in the mPFC 30min after the coadministration of the three injections of sc FLX(10mg/kg) and a single icv injection of GAL(1-15)(1nmol) (Fig. 3C).

3.1.3. 5-HT_{1A}R mRNA levels

In the *In situ* hybridization experiments, the coadministration of the three sc injections of FLX(10mg/kg) and a single icv injection of GAL(1-15)(1nmol) produced a significant increase in the 5-HT_{1A}R mRNA levels in the mPFC (Fig. 4A; $F_{3,19}=6.418$, $p<0.01$; post hoc $p<0.01$) compared with FLX(10mg/Kg) group. Representative autoradiograms illustrate the increase of 5-HT_{1A}R mRNA levels in the mPFC, after the coadministration of the three sc injections of FLX (10mg/kg) and a single icv injection of GAL(1-15)(1nmol) (Fig. 4B).

3.2. Behavioral effects

3.2.1. NOR

The overall one way ANOVA revealed a significant effect between treatments in DI in the NOR task (Fig. 5; $F_{3,18}=6.393$, $p<0.01$).

The coadministration of the three sc injections of FLX(10mg/kg) and a single icv injection of GAL(1-15)(1nmol) reversed the effects of memory impairment induced by FLX(10mg/Kg) alone (Fig. 5; post hoc $p<0.05$) in the NOR showing the same DI values as the control group.

In agreement with other studies where FLX induces an impairment memory effects in the NOR when tested at 24h post training phase (Ampuero et al., 2013; Castane et al., 2015), our pattern of administration of FLX with three injections given sc at 10mg/kg between the training and test phase also induced the same effects in this task (Fig. 5; post hoc $p<0.05$).

In the same experiment we tested the involvement of the GALR2 in the GAL(1-15)-FLX(10mg/kg) interaction with the selective GALR2 antagonist M871. M871(3nmol) blocked the effects of GAL(1-15) over FLX in the NOR task since the coadministration of the three sc injections of FLX(10mg/kg) and a single icv injection of GAL(1-15)(1nmol) in combination with M871(3nmol) induced a significant reduction of the DI (Fig. 5; post hoc $p<0.05$) compared with the group of animals treated with FLX(10mg/Kg) plus GAL(1-15)(1nmol).

No pharmacological treatment used in the NOR task in this experiment significantly modified the analyzed parameters of locomotor activity (Table1A; $F_{2,14}=0.19$; $p=0.828$) and the total time of exploration (Table1A; $F_{2,14}=2.19$; $p=0.148$) in the test phase.

In the training phase, the total object exploration time confirmed that there was no difference in the baseline exploration levels between the groups (Table 2).

The administration of a single icv injection of GAL(1-15)(1nmol) or M871(3nmol) alone did not modified any parameter analyzed in the NOR task: DI (Table 1B; $F_{2,18}=0.097$; $p=0.907$), locomotor activity (Table 1B; $F_{2,18}=0.144$; $p=0.866$), total time of exploration (Table 1B; $F_{2,18}=0.67$; $p=0.524$).

3.2.2. OLM

The coadministration of the three sc injections of FLX(10mg/kg) and a single icv injection of GAL(1-15)(1nmol) in this task potentiate FLX-action in the DI although it did not reach the statistical significance, indicating an opposite effect of GAL(1-15) than in the NOR task.

The sc administration of three injections of FLX(10mg/Kg) between the training and test phase in the OLM task showed a reduction in the DI compared with the control group although it didn't reach the significance (Fig. 6; $F_{2,15}=3.24$, $p=0.0675$).

No pharmacological treatment used in the OLM task significantly modified the analyzed parameters of locomotor activity (Table1; $F_{2,15}=2.03$; $p=0.165$) and the total time of exploration (Table1; $F_{2,15}=0.174$; $p=0.841$) in the test phase.

4. Discussion

In the present work we described for the first time the interactions between GAL(1-15) and FLX in the mPFC involving interactions at the 5-HT_{1A}R level in the plasma membrane with changes also at the transcriptional level. Thus, the coadministration of GAL(1-15) and FLX increased the K_d and the B_{max} of the 5HT_{1A}R agonist binding in the mPFC as well as the mRNA levels of 5-HT_{1A}R in mPFC. Moreover, GAL(1-15) reversed the effects of memory impairment induced by FLX(10mg/Kg) in the Novel Object Recognition. GALR2 was involved in these effects, since the specific GALR2 antagonist M871 blocked GAL(1-15) mediated actions at behavioral level. On the contrary GAL(1-15) did not reverse the effect of FLX in the Object Location Memory.

We have described previously an interaction between GAL(1-15) and FLX; GAL(1-15) enhanced the antidepressant effects of FLX in the forced swim test (Flores-Burgess et al., 2017). The mechanism of this effect was explained on the bases of the existence of a trimeric GALR1-GALR2-5-HT_{1A}R heteroreceptor complex (Borroto-Escuela et al., 2018; Flores-Burgess et al., 2017; Millon et al., 2016) since an increase in the mRNA levels of postjunctional 5-HT_{1A}R in the dorsal hippocampus and a decrease in K_d and B_{max} values, specifically in the dentate gyrus of the hippocampus was observed (Flores-Burgess et al., 2017). Such heteroreceptor complexes could work as integrative nodes in 5-HT neurotransmission and as major targets for the antidepressant effects of GAL(1-15)+FLX at postjunctional level of 5-HT transmission of the raphe-hippocampal neurons (Fuxe et al., 2012; Fuxe et al., 2008; Millon et al., 2016).

Our results in the present paper suggest that the molecular complexes GALR1-GALR2-5-HT_{1A}R are likely highly dynamic and vary from one brain region to another. **In fact, GAL(1-15)+FLX administration induced the opposite effect in the modulation of 5-HT_{1A}R in the mPFC compared to the dentate gyrus** (Flores-Burgess et al., 2017), however the increase in 5-HT_{1A}R mRNA levels in the mPFC was still obtained after the GAL(1-15)+FLX administration. It's

interesting to highlight that the administration of FLX or GAL(1-15) alone did not change the K_d or the B_{max} of 5-HT_{1A}R neither the mRNA levels in the mPFC. This results are in agreement with our previous work (Li et al., 1997; Flores-Burgess et al., 2017) and suggest that GAL(1-15) and FLX interact at membrane level in the mPFC to modify this parameters.

The differential effects observed with the combination of GAL(1-15)+FLX in the modulation of the 5-HT_{1A}R recognition in the mPFC vs the hippocampus may be explained by differences in the composition of the 5-HT_{1A}R heteroreceptor complexes involved in the two regions. This can lead to alterations in the allosteric receptor-receptor interactions and in the signaling of the heteroreceptor complexes. Also the adapter proteins in the heterocomplexes may differ between the two regions with consequences for the allosteric receptor-receptor interactions. It has also been described regional differences in the coupling of 5-HT_{1A}R to G protein. 5-HT_{1A}R interact equally with G_{α_0} and $G_{\alpha_{i3}}$ in the cerebral cortex but mainly with G_{α_0} and weakly with $G_{\alpha_{i3}}$ in the hippocampus (Mannoury la Cour et al., 2006). Such differences may contribute to the opposite effect in the affinity and B_{max} of the 5-HT_{1A}R binding sites in the mPFC in contrast to the hippocampus (Borrito-Escuela et al., 2018; Flores-Burgess et al., 2017).

Since FLX induced increases in extracellular levels of 5-HT likely increasing 5-HT signaling not only over the 5-HT_{1A}R receptor, but also over a number of other 5-HT receptor subtypes, the participation of other 5-HT receptor subtypes with high density in the mPFC such as 5-HT_{2A} (Castane et al., 2015) in the interaction remains to be tested.

At behavioural level, the interaction between GAL(1-15)+FLX was demonstrated in the NOR task. The NOR task was developed to provide a valid measure of memory function based upon spontaneous exploratory activity in the rat (Ennaceur et al., 1997). Object recognition can be assessed from the

preference normal rats display for investigating novel rather than familiar complex objects **having been characterized as declarative rather than non-declarative memory task** (Ennaceur et al., 1997).

The role of mPFC in single-item recognition memory is not fully elucidated. Initial studies showed that large aspiration lesions of the mPFC produced delay dependent impairments in delayed non-matching to sample tasks (Ragozzino et al., 2002; Warburton and Brown, 2015), suggesting an involvement for mPFC in this process (Morici et al., 2015), but excitotoxic lesion studies indicate that the mPFC is not involved in the NOR task based on single-item recognition memory. (Warburton and Brown, 2015). However, other studies using more functional approaches, based mainly in the activation of immediate early genes (IEGs), have showed that the mPFC increases the expression of c-Fos (Rinaldi et al., 2010; Castilla-Ortega et al., 2012) and Zif-268 (Freitas Barbosa et al., 2013) in response to the test phase in a NOR task, supporting that this region is involved at least in the retrieval of this recognition memory. Moreover, other studies using intra-mPFC infusions of D1, D2 and D3 agonists and antagonist, were able to affect the long-term memory consolidation in a NOR task (Papp et al., 2017). All these results suggest that mPFC is involved in single-item recognition memory (NOR), and that its main role seems to be associated with both, memory retrieval and long-term memory consolidation (Frankland and Botempi 2005; Leon et al., 2010).

With the experimental design used in our study, where FLX was administered 23, 5 and 1 h and GAL(1-15) 15 min before memory retrieval phase in the NOR, it is clear that GAL(1-15) was able to counteract the impairment induced by FLX in this task, affecting the retrieval of a consolidated memory. In addition, this effect cannot be explained by the modulation of other memory phases as the acquisition or the retention. Interestingly, several results based in the use of IEGs, support the

involvement of the mPFC in the retrieval phase in this task (Rinaldi et al., 2010; Castilla-Ortega et al., 2012; Freitas Barbosa et al., 2013), suggesting that likely GAL(1-15) is acting through the mPFC in order to modulates memory retrieval.

The fact that GAL(1-15) reversed the effects of memory impairment induced by FLX(10mg/Kg) in the NOR task could be **associated with** the proposed trimeric GALR1-GALR2-5-HT1AR heteroreceptor in the mPFC. Since GAL(1-15) acts through a GALR1-GALR2 heteroreceptor complexes that inhibits the Gq/G11-mediated signaling of the GALR2 protomer and switches it towards Gi/o-mediated signaling enhancing Gi/o-mediated signaling of GALR1 in the GALR1-GALR2 heterodimer (Borroto-Escuela et al., 2014; Millon et al., 2015), the mechanism for the GALR1-GALR2-5-HT1AR heteroreceptor complex in the mPFC could be explain on the bases that this enhancement of Gi/o-mediated signal will impair 5-HT1AR receptor signaling in the trimer complex explaining that the coadministration of GAL(1-15)+FLX reversed FLX-mediated effects. GALR2 is involved in the GAL(1-15) effects since the GALR2 antagonist M871 blocked the effects induced by GAL(1-15) on FLX-mediated effects.

FLX induces an impairment memory effects in the NOR when tested at 24h post training phase (Ampuero et al., 2013; Castane et al., 2015), without any effect in the spontaneous motor behaviours (Ampuero et al., 2013). Our results with FLX are in agreement with these studies and confirm our pattern of administration of FLX and the behavioural model used.

On the contrary GAL(1-15) did not reverse the effect of FLX in the OLM task. In this task the rats are presented with two identical, familiar objects, one of which is in its previous location while the other is in a new location. Normal rats spontaneously spend more time exploring the object in the novel location (Ennaceur et al., 1997). The OLM task assess spatial memory in a less aversive manner than in the Morris water maze task (Akirav and Maroun, 2006; Ampuero

et al., 2013).

Since the hippocampus is crucial in spatial memory tasks such as the OLM task (Barker and Warburton, 2011), the fact that GAL(1-15) did not reverse FLX action in the OLM task, its in agreement with previous results from our group that demonstrate that the GALR1-GALR2-5-HT1AR heteroreceptor in the hippocampus enhances 5-HT1AR receptor signaling in the complex by, inter alia, reducing 5-HT1AR internalization from the plasma membrane (Borrito-Escuela et al., 2018; Flores-Burgess et al., 2017).

Although several authors described the key role of the mPFC in the associative learning tasks based on the natural exploration as the NOR (Muller and Knigh, 2006; Meneses 2007) and the hippocampus as a crucial region in the OLM task (Barker and Warburton, 2011), we can no exclude the influence of other brain areas related to the regulation of both emotional and cognitive behaviors (Ampuero et al., 2013; Meneses 2007).

In conclusion, our results describes an interactions between GAL(1-15) and FLX in the mPFC involving interactions at the 5-HT1AR level in the plasma membrane with changes also at the transcriptional level and these interactions **may be associated** with the effects at functional level, since GAL(1-15) reversed the effects of memory impairment induced by FLX in the Novel Object Recognition. The target for GAL(1-15) may mainly be GALR1-GALR2-5-HT1AR heteroreceptor and confirmed that these molecular complexes are likely highly dynamic and vary from one brain region to another and can be used to reverse some of the adverse effects of FLX on memory processes.

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Figure Legends

Figure 1: Schematic representation of the procedures followed to perform the behavioural experiments in the NOR (**A**) and OLM (**B**). In both tasks animals had a recovery period of 7 days prior to behavioural test and the animals performed the tasks in three phases, separated 24 hours from each other, where they explore freely during 10min in the habituation phase without objects, 3min in the training phase with two identical objects and finally 3min in the test phase with one object changed in the NOR or one object in other location respect the training phase in the OLM. The pharmacological treatments were

administered to the different groups of animals between the training and testing phases.

Figure 2: Representative microphotography of the colocalization of GALR1 and GALR2 in neurons of the mPFC. Double-immunolabeled for GALR1 (green, rhombus) and GALR2 (red, arrowhead) in mPFC. The colocalization of GALR1 and GALR2 is shown in the merge (orange-yellow, arrows). Escale bar, 50 μm .

Figure 3: Effects on the binding characteristics of 5-HT_{1A}R agonist [³H]-8 OHDPAT in the mPFC after the coadministration of FLX(10mg/kg) or GAL(1-15)(1nmol) alone or in combination. FLX was administered sc 23, 5 and 1,25h before sacrifice and GAL(1-15) or aCSF was injected icv 30min before. Saturation experiments were performed with ten concentrations of [³H]-8 OHDPAT (0.26-10nM) in sections from the mPFC. Non-specific binding was defined as the binding in the presence of 10mM serotonin. The K_d (**A**) and the B_{max} (**B**) values are shown as mean \pm SEM (n=6 per group). In the control group the K_d(nM) value was 0.6 \pm 0.04 and B_{max}(fmol/mg prot) was 654 \pm 15. *p<0.05, **p<0.01, ***p<0.01 versus FLX(10mg/Kg)+GAL(1-15)(1nmol) group and ##p<0.01, ###p<0.001 versus control group according to one way ANOVA followed by Newman-Keuls Multiple Comparison Test. (**C**) Representative autoradiograms from mPFC sections of rat showing a decrease of the affinity of the agonist for 5-HT_{1A}R in the mPFC with a low concentration of the radioligand (1nM).

Figure 4: Effects of the coadministration of FLX(10mg/kg) and GAL(1-15) (1nmol) on 5-HT_{1A}R mRNA levels in the mPFC. FLX was administered sc 23, 5 and 1,25h before sacrifice and GAL(1-15) or aCSF was injected icv 30min before. (**A**) The values of optical density (O.D.), mean \pm SEM (n=6 per group), are expressed as percentages respect of the control values (100%). **p<0.01 versus FLX(10mg/Kg)+GAL(1-15)(1nmol) group and ##p<0.01 versus control group according to one way ANOVA followed by Newman-Keuls Multiple

Comparison Test. **(B)** Representative autoradiograms from mPFC showing the levels of mRNA coding for 5-HT_{1A}R determined by in situ hybridization and the analyzed areas.

Figure 5: Graphic shows the ability of rats to discriminate the novel object at 24h post-training after the administration of FLX(10mg/kg) alone or in combination with a threshold doses of GAL(1-15)(1nmol) or GAL(1-15) plus the GALR2 antagonist M871(3nmol) in the NOR task. FLX or vehicle was administered sc 23, 5 and 1h before the test. aCSF, GAL(1-15) and GAL(1-15)+M871 were injected icv 15 min before the test. Data represents mean \pm SEM of the DI during the 3 min of the test phase (n=5-6 rats per group). *p<0.05 versus FLX(10mg/Kg)+GAL(1-15)(1nmol) group and #p<0.01 versus control group according to one way ANOVA followed by Newman-Keuls Multiple Comparison Test.

Figure 6: Graphic shows the ability of rats to discriminate the relocated object at 24h post-training after the administration of FLX(10mg/kg) alone or in combination with a threshold doses of GAL(1-15)(1nmol) in the OLM task. FLX or vehicle was administered sc 23, 5 and 1h before the test. aCSF, GAL(1-15) were injected icv 15min before the test. Data represents mean \pm SEM of the DI during the 3 min of the test phase (n=6 rats per group). No statistically significant difference was observed between the experimental groups according to one way ANOVA followed by Newman-Keuls Multiple Comparison Test.

Table 1: Table shows **A)** the locomotor activity and total exploration time of the rats in the NOR and OLM tasks after the administration of FLX(10mg/kg) alone or in combination with a threshold dose of GAL(1-15)(1nmol) or GAL(1-15)(1nmol)+M871(3nmol) during the test phase. FLX or vehicle was administered sc 23, 5 and 1h before the test. aCSF or GAL(1-15) were injected icv 15min before the test. **B)** Discrimination index, locomotor activity and total exploration time of the rats in the NOR tasks after the administration of GAL(1-

15)(1nmol) or M871(3nmol) alone during the test phase. Vehicle was administered sc 23, 5 and 1h before the test. aCSF, GAL(1- 15) or M871 were injected icv 15 min before the test. No statistically significant difference was observed vs control group according to one way ANOVA followed by Bonferroni's Multiple Comparison Test.

Table 2: Total object exploration time by groups in the training phase of the NOR and OLM tasks before the administration of the treatments. No statistically significant difference was observed between the experimental groups according to one way ANOVA followed by Newman-Keuls Multiple Comparison Test.

Table 1

A		Exploration Time (s)	Locomotor activity (cm)
NOR	Control	23.4±2.2	2550±343
	FLX(10mg/Kg)	24.3±2.4	2870±251
	FLX(10mg/Kg)+ GAL(1-15)(1nmol)	18.3±1.9	2662±264
	FLX(10mg/Kg)+ GAL(1-15)(1nmol)+ M871(3nmol)	15.5	2030±107
OLM	Control	20.8±5	2471±335
	FLX(10mg/Kg)	23.78±1.7	2707±104
	FLX(10mg/Kg)+ GAL(1-15)(1nmol)	23.47±3.8	1985±276

B		Exploration Time (s)	Locomotor activity (cm)	DI (discrimination index)
NOR	Control	23.6±2.9	2463±233	0.30±0.04
	GAL(1-15)(1nmol)	22.9±2.9	2663±333	0.31±0.07
	M871(3nmol)	27.2±1.3	2599±290	0.27±0.06

Table 2: Total object exploration time by groups in the training phase.

OLM			NOR			
Control	FLX(10mg/Kg)	FLX(10mg/Kg)+ GAL(1-15)(1nmol)	Control	FLX(10mg/Kg)	FLX(10mg/Kg)+ GAL(1-15)(1nmol)	FLX(10mg/Kg)+ GAL(1-15)(1nmol) + M871(3nmol)
22.1±5.4	29.5±5.1	23.7±3.2	31.2±4.5	34.2±3.7	25.6±3.9	25.3±3.3

Figure 1

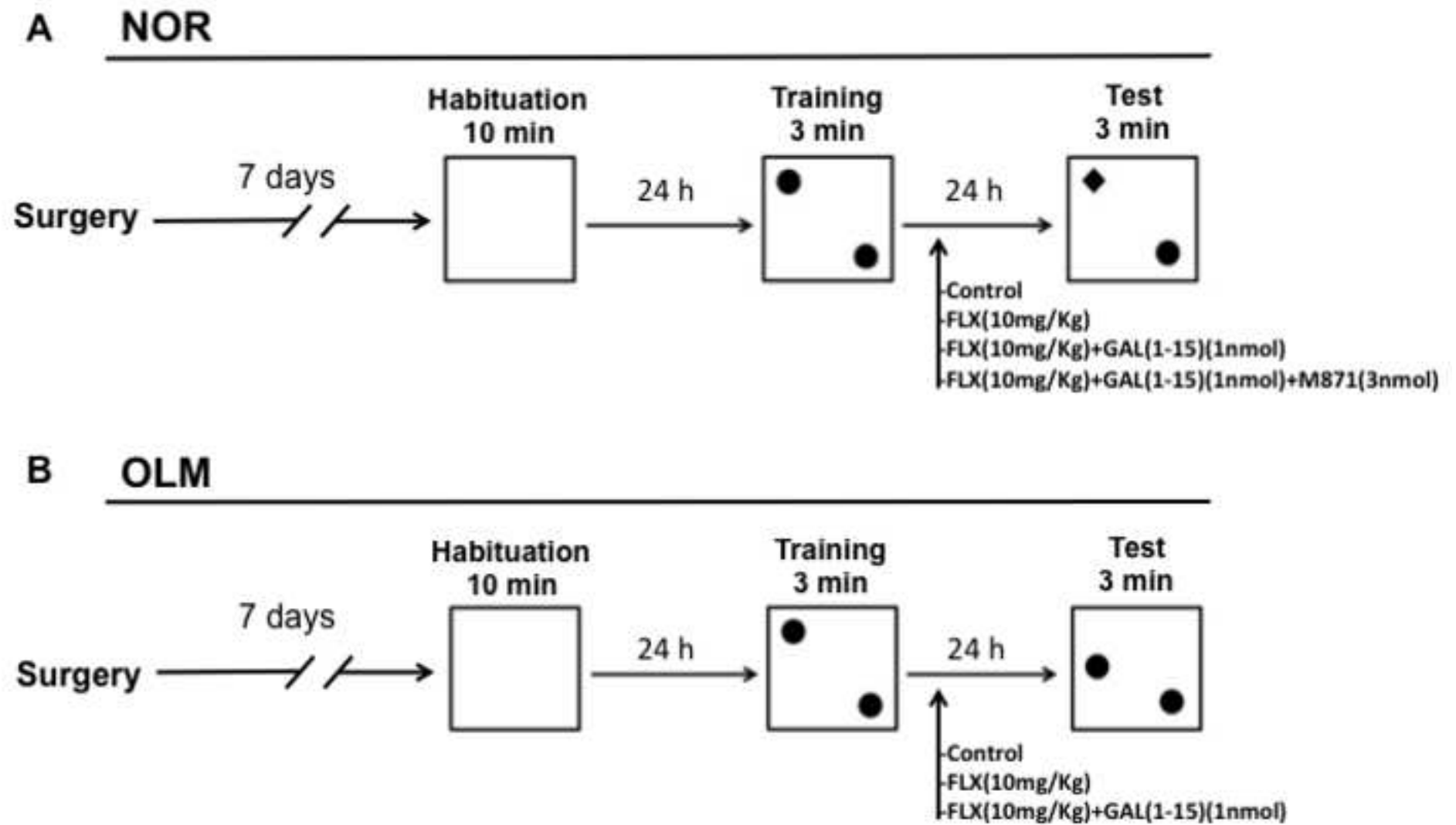


Figure 2

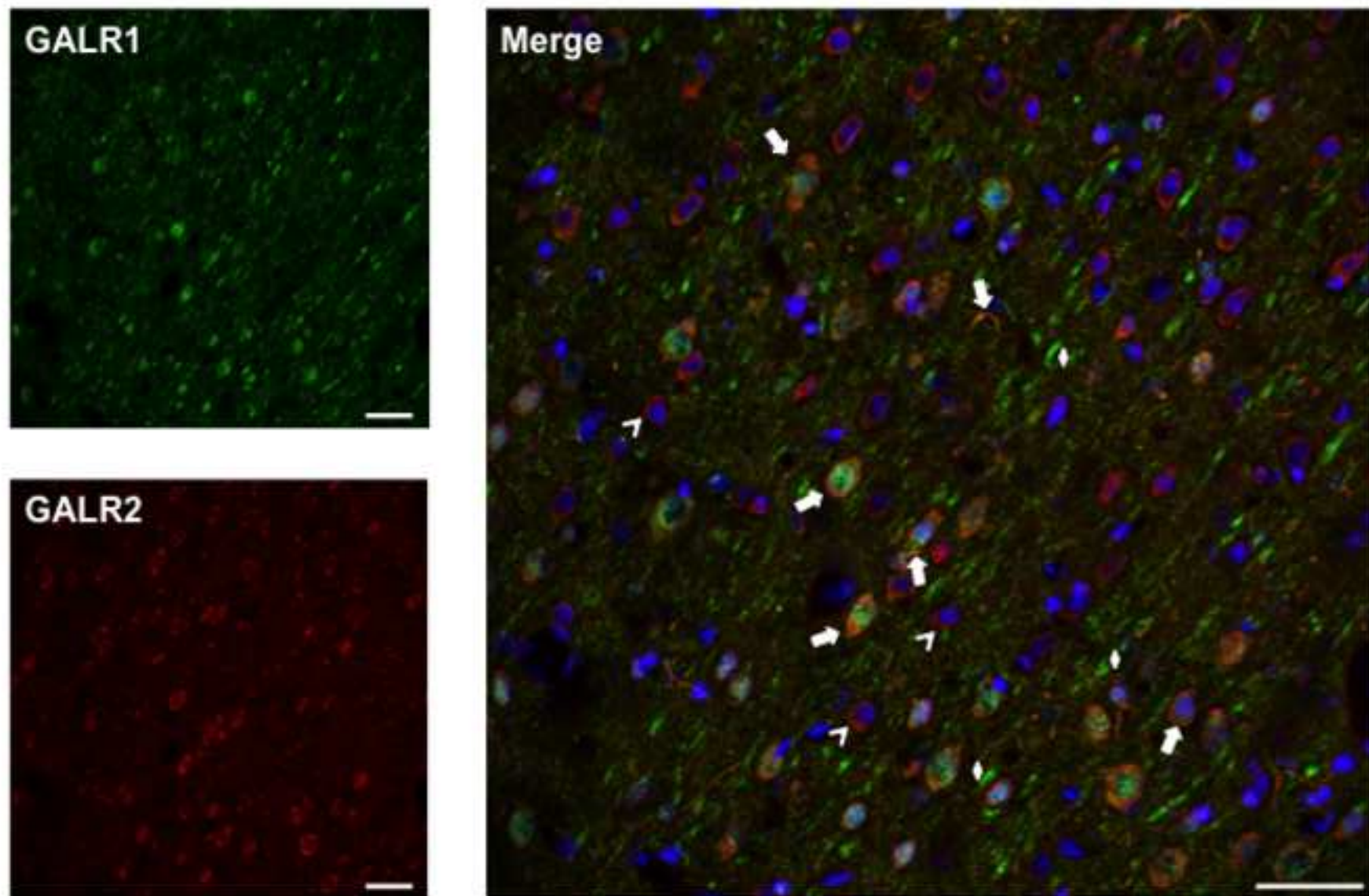
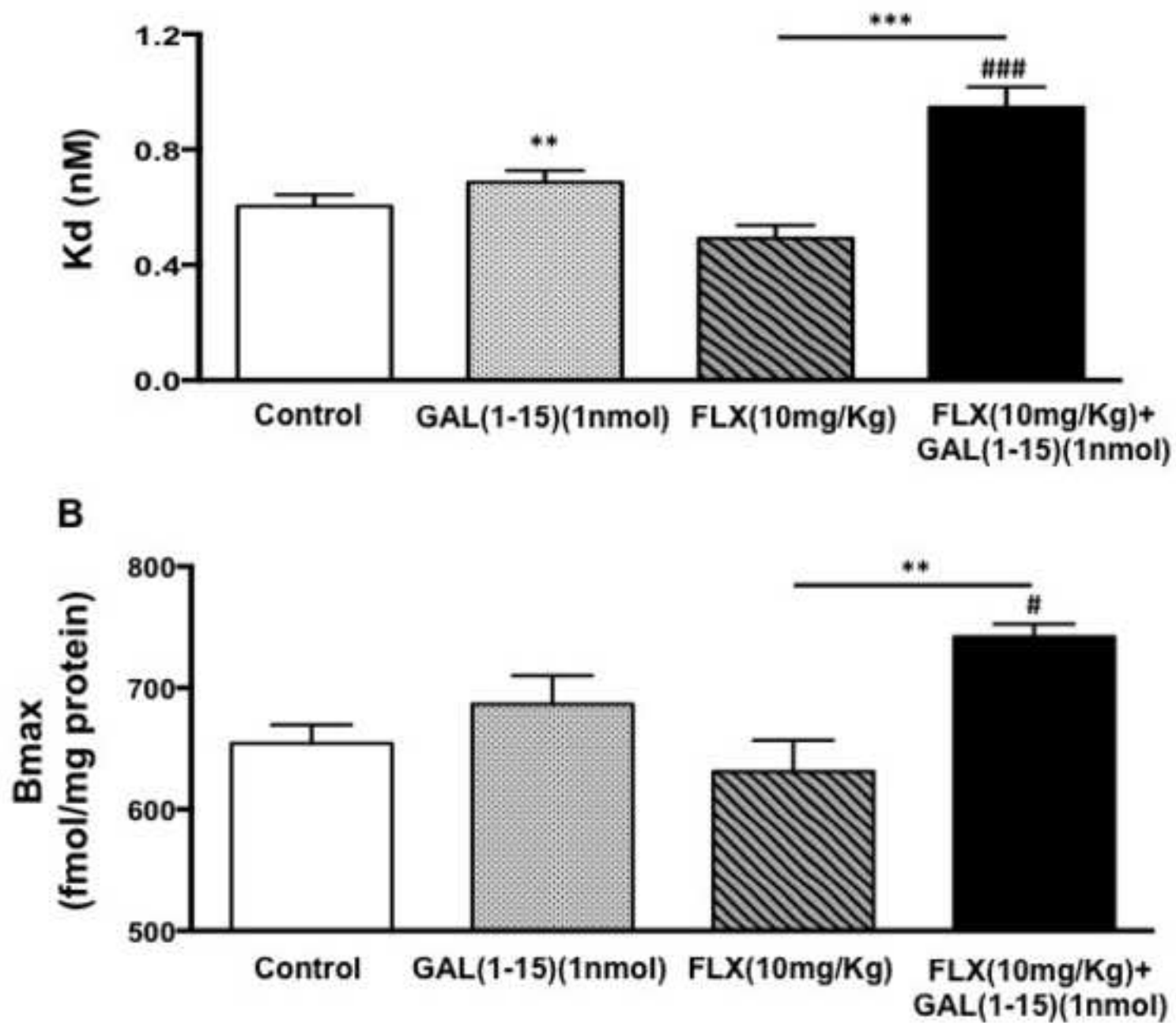


Figure 3 A



C

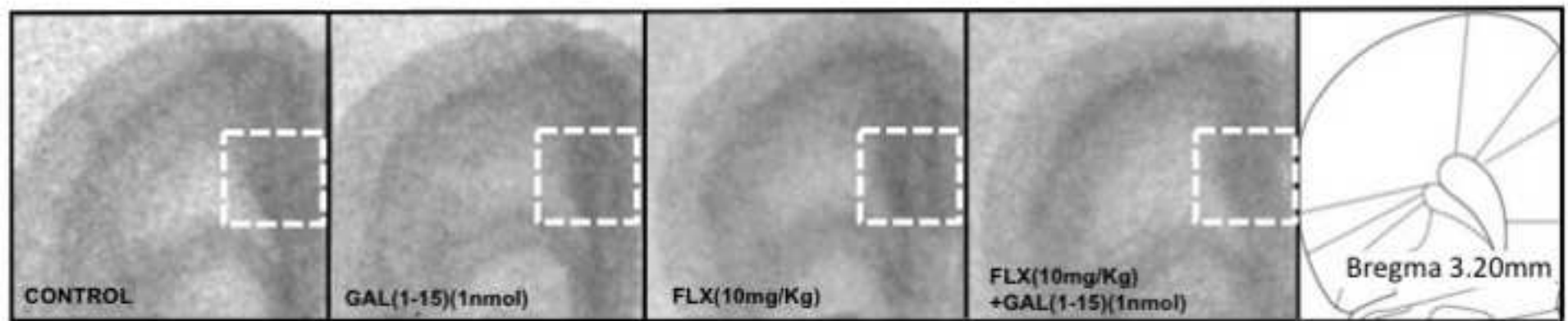


Figure 4

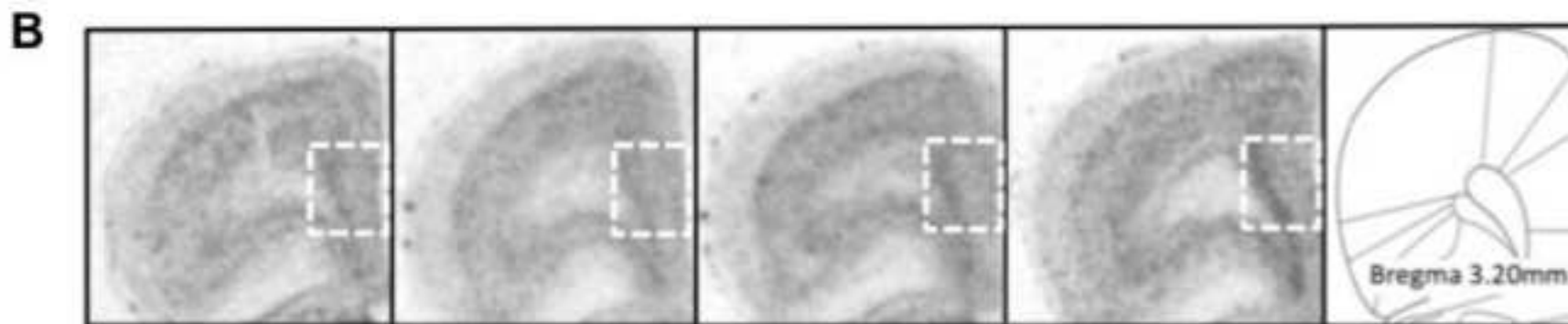
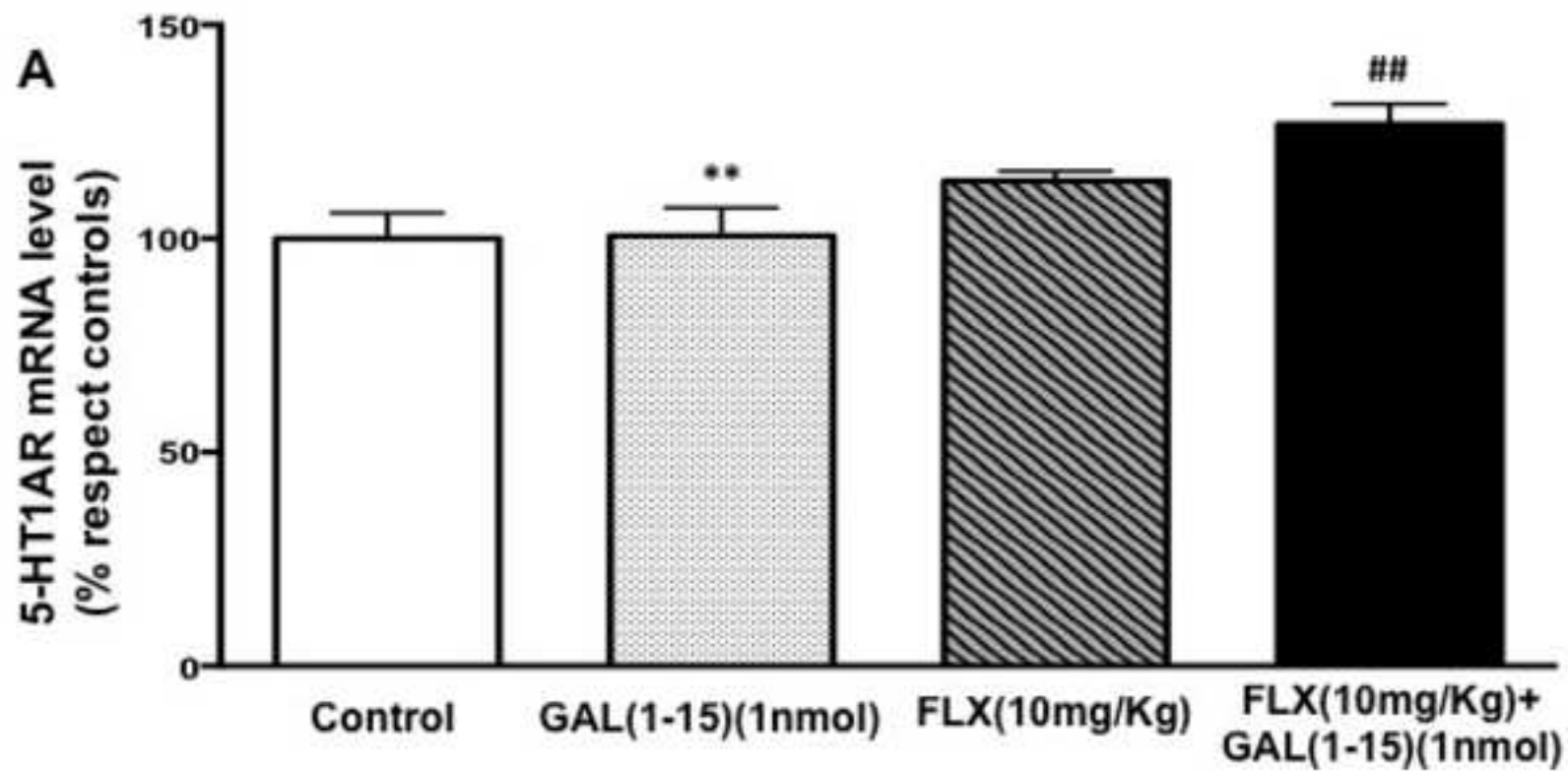


Figure 5

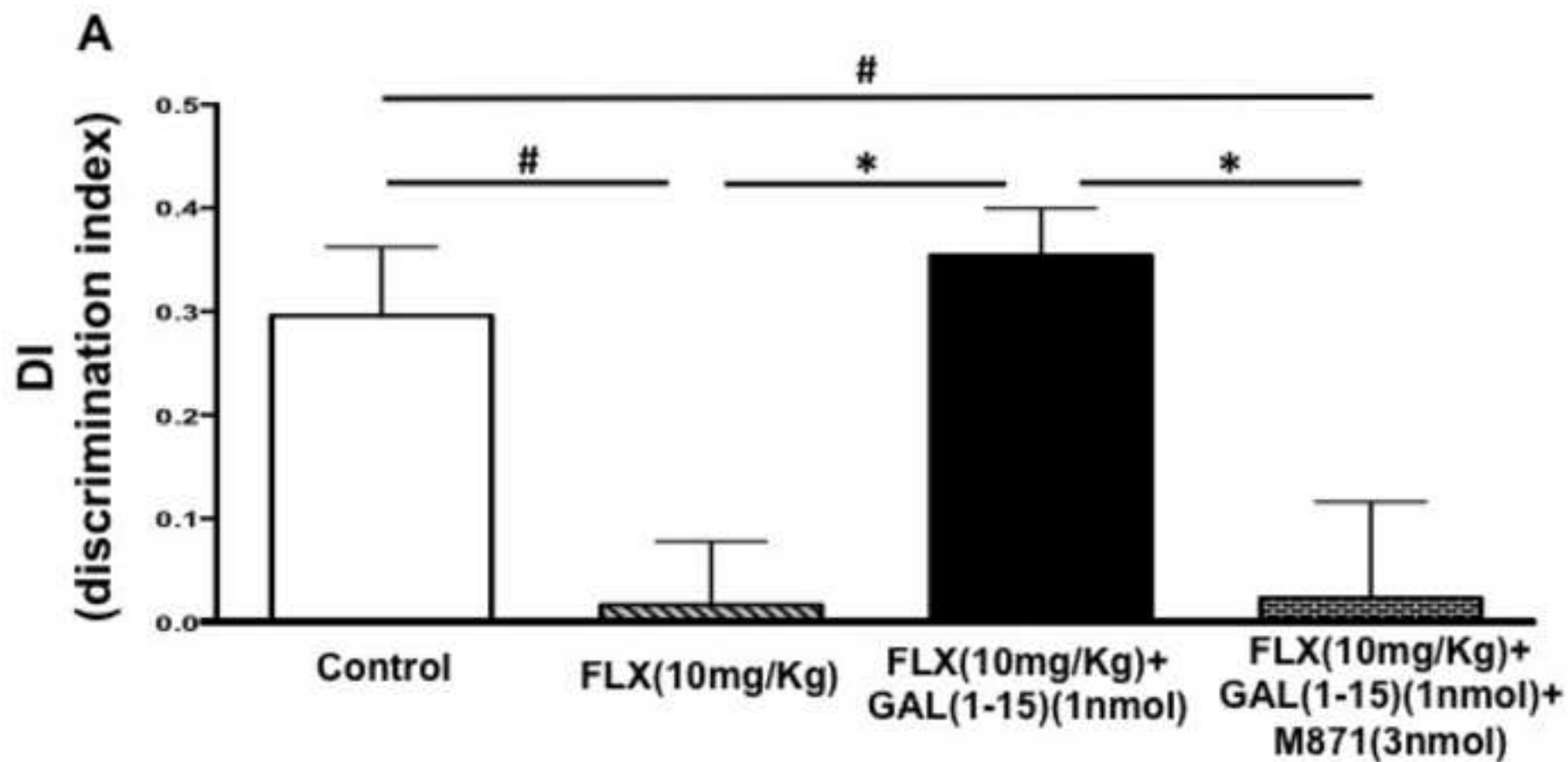


Figure 6

