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Galanin receptor/Neuropeptide Y receptor interactions in the dorsal raphe nucleus of the rat

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ABSTRACT

The aim of this study was to evaluate by quantitative receptor autoradiography the interactions between Neuropeptide Y Y1 (NPY Y1) and Galanin (GAL) receptors in the dorsal raphe nucleus (DRN) where both GAL receptors and NPY Y1 receptors exist. The ability of the GAL receptor antagonist M35 to block the GAL action was also evaluated. Double immunocytochemical staining of 5-hydroxytryptmine and c-Fos and stereology techniques were used to study the specific cell activation in the DRN after the intracerebroventricular coinjections of GAL and the NPY Y1/Y5 agonist [^{125}I] Leu^{31},Pro^{34}PYY.

GAL (0.3 nM) decreases [^{125}I] Leu^{31},Pro^{34}PYY binding in the DRN by 48% ($p < 0.01$) as shown by quantitative receptor autoradiography. This effect was reversed with the GAL receptor antagonist M35. Intracerebroventricular coinjections of NPY Y1/Y5 agonist and GAL reduced the c-Fos expression in the serotoninergic cells induced by the NPY Y1/Y5 agonist in DRN. These results indicate the existence of antagonistic interactions between GAL receptors and NPY Y1 receptors in the DRN that may be of relevance in mood disorders.

1. Introduction

Galanin (GAL) is a neuropeptide (Tatemoto et al., 1983) widely distributed in the peripheral and central nervous system (CNS) (Jacobowitz et al., 2004). Three galanin receptor (GALR) subtypes (GalR1, GalR2 and GalR3), have been cloned and belong to the G-protein-coupled receptor family (Branchek et al., 2000). These receptors are widely distributed in the brain as demonstrated in ligand binding, immunohistochemical and, in particular, in situ hybridization studies. The activation of GalR1 and GalR3 leads to inhibition of adenylyl cyclase whereas GalR2 can be coupled to phospholipase C and activate protein kinase C in neurons. GAL and its receptor subtypes have been implicated in several physiological functions including energy homeostasis, food intake, central cardiovascular regulation and mood regulation (Fuxe et al., 1998; Kuteeva et al., 2007, 2008; Lang et al., 2007; Razani et al., 2000, 2001).

Neuropeptide Y (NPY) is also a neuropeptide widely distributed in the mammalian brain. The physiological actions of NPY in the CNS are mediated via Y1, Y2, Y4, Y5 and Y6 receptors (Brain and Cox, 2006). All of them belong to the G-protein-coupled receptor family. Central NPY and its receptors, specially Y1 receptors have been related to a variety of physiological processes and several reports indicate that NPY plays a role in the pathophysiology of mood disorders, including depression and anxiety (Caberlotto et al., 1998, 1999; Heilig, 2004; Ishida et al., 2007; Jimenez-Vasquez et al., 2007; Karlsson et al., 2008).

The presence of both GAL and NPY and/or their receptors in many relevant brain regions related to their functions implies that GAL and NPY may complement the physiological actions of one another (Leibowitz et al., 2005). There is evidence of functional GAL/NPY interactions in cardiovascular regulation, energy metabolism and feeding (Diaz-Cabiale et al., 2006; Hohmann et al., 2004; Parrado et al., 2007).

At the cardiovascular level GAL was able to modify the cardiovascular response produced by a NPY Y1/Y5 agonist on mean arterial pressure and heart rate (Diaz-Cabiale et al., 2006). Moreover GAL inhibits the increase in food intake induced by the NPY Y1/Y5 receptor agonist (Parrado et al., 2007). The quantitative

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autoradiographical results suggested that these functional interactions could take place at the receptor level in the nucleus tractus solitarius and in the arcuate nucleus through a GAL receptor/NPY Y1 receptor interaction (Diaz-Cabiale et al., 2006; Parrado et al., 2007). In fact the antagonist M35 blocks the decrease in [^{125}I] Leu^{31},Pro^{34}PYY binding induced by GAL (Diaz-Cabiale et al., 2006; Parrado et al., 2007).

The dorsal raphe nucleus (DRN) is a nucleus implicated in several behaviours such as those related to stress, anxiety and mood regulation. This nucleus contains abundant 5-hydroxytryptamine (5-HT) containing neurons and has been identified as one specific site of action for antidepressant drugs (see Fuxe et al., 2008). The possible interaction of GAL and NPY in this nucleus has been analyzed since several reports indicate that NPY and GAL play a role in the pathophysiology of mood disorders, including depression and anxiety.

Based on these studies the aim of this study was to evaluate by quantitative receptor autoradiography the interactions between NPY Y1 and GALR in the DRN where both GALR and NPY Y1 receptors exist (Branchek et al., 2000; Lang et al., 2007; Parker and Herzog, 1999). The occipital cortex was included in the study to analyze the specificity of the interaction. The ability of the GAL receptor antagonist M35 (Bartfai et al., 1992) to block the GAL action was also evaluated. Double immunocytochemical staining of 5-hydroxytryptmine (5-HT) and c-Fos (c-Fos IR) and stereology techniques were used to study the specific cell activation in the DRN after the intracerebroventricular coinjections of GAL and the NPY Y1/Y5 agonist.

2. Materials and methods

2.1. Animals

Male Sprague Dawley rats (body weight 225–250 g) were obtained from Criffa (Barcelona, Spain) and they were maintained in humidity and temperature controlled (20–22 °C) room under a 12:12-h light/dark cycle (lights on 08:00 and off 20:00 h). Four rats were housed per cage. The animals had free access to food pellets and tap water. All animal experimentation was conducted in accordance with the University of Málaga Guidelines for the Care and Use of Laboratory Animals.

2.2. Quantitative receptor autoradiography

The procedure for receptor autoradiography has been described elsewhere (Dumont et al., 1993). Briefly, the rats were killed by decapitation and the brains were rapidly removed from the skull and frozen under a CO_2 stream, and then kept at −80 °C until sectioned in a Leitz cryostat. Coronal sections were cut at 10 μm (Bregma levels DR: −7.3 mm to −8.3 mm) (Paxinos, 1986) and thaw-mounted on gelatin-coated slides and immediately processed for binding studies.

The effects of GAL (NeoMPS, Strasbourg, France) on NPY Y1 receptor binding were studied in two sets of experiments. In the first set of experiments four groups of animals ($n = 6$) were used to study the NPY Y1/Y5 agonist binding in the presence of GAL at different concentrations (0.1, 0.3, 1, 3 nM). Each concentration tested had its own paired control (same rat) of the same group of animal.

In the second set of experiments the effects of GAL (0.3 nM) on NPY Y1/Y5 agonist binding were evaluated in the presence of M35 (10 nM) (Endoh et al., 2008), a GAL receptor antagonist (Bartfai et al., 1992). Six animals were used in this experiment and each animal received in vitro the four different treatments (control, GAL, M35 and GAL + M35).

Briefly, the sections were preincubated for 1 h at room temperature in a Krebs–Ringer phosphate buffer (KRP) at pH 7.4 and then incubated for 2 h in a fresh preparation of KRP buffer supplemented with 0.1% BSA, 0.05% bacitracin, 25 pM NPY Y1/Y5 agonist [^{125}I] Leu^{31},Pro^{34}PYY (Perkin–Elmer, USA) (Dumont et al., 1993) in the presence or absence of GAL at different concentrations. Non-specific binding was defined as the binding in the presence of NPY 1 μM. At the end of the incubation period, sections were washed four times (2 min each) in ice-cold KRP buffer, dipped in deionised water to remove salts, and rapidly dried under a stream of cold air. Sections were then placed in X-ray cassettes and apposed against Hyperfilms for 6 days together with ^{125}I microscales (Amersham International) as reference standards.

2.3. Computer-assisted image analysis

The [^{125}I] Leu^{31},Pro^{34}PYY binding sites of the dorsal raphe nuclei were analyzed from the receptor autoradiograms using the NIH image analysis system. Measurements in autoradiograms were made in the ventral part of the midline area of the DR using a square as a sampling field (0.09 mm^2)(Razani et al., 2000). In the occipital cortex measurements were made bilaterally using a square as a sampling field (0.09 mm^2). One observation per region and rat was obtained since the average of the measurements in the occipital cortex was calculated. Prefabricated ^{125}I-labelled polymer strips (Amersham Microscale, Amersham, Little Chalfont, UK) were used to convert the grey values into fmol/mg protein values (Diaz-Cabiale et al., 2006).

2.4. Intracerebroventricular injections

This protocol has been used previously (Parrado et al., 2007). Briefly, the rats were anesthetized intraperitoneally with sodium pentobarbital (60 mg/kg body weight), and stereotaxically implanted with a unilateral chronic 22-gauge stainless-steel guide cannula into the right lateral cerebral ventricle using the following coordinates: 1.4 mm lateral and 1 mm posterior to the bregma, and 3.6 mm below the surface of the skull (Paxinos, 1986). This guide cannula was secured to the skull using stainless-steel screws and dental acrylic cement.

The rats were maintained under anaesthesia giving supplementary doses of sodium pentobarbital every 45 min to avoid pain and stress and the injections in the lateral ventricle were performed using a 26-gauge stainless-steel injection cannula connected via a PE-10 tubing to a Hamilton syringe. This protocol has been previously used to analyze the interaction of GALR and NPY Y1 at c-Fos level (Diaz-Cabiale et al., 2006; Parrado et al., 2007).

Solutions were prepared freshly and the peptides were dissolved in artificial cerebrospinal fluid (aCSF, composition is 120 nM NaCl, 20 nM NaH_2CO_3, 2 nM KCl, 0.5 nM KH_2PO_4, 1.2 nM $CaCl_2$, 1.8 nM $MgCl_2$, 0.5 nM Na_2SO_4, and 5.8 nM D-glucose, pH 7.4). The total volume was 9 μl per injection and the infusion time was 1 min. Groups of rats received GAL at a dose of 3 nmol and the NPY Y1/Y5 receptor agonist Leu^{31}, Pro^{34}NPY at 2.5 nmol alone or in combination based on previous work (Parrado et al., 2007).

2.5. c-Fos/serotonin immunohistochemistry

Animals were divided into four experimental groups: (1) aCSF: control group injected with artificial Cerebrospinal fluid ($n = 4$); (2) GAL: group pretreated with Galanin 3 nmol ($n = 4$); (3) Y1: group receiving the NPY Y1/Y5 receptor agonist Leu^{31}-Pro^{34}NPY 2.5 nmol ($n = 4$); (4) GAL + Y1: group administered with both substances ($n = 4$). After the 90 min administration period, the rats were anaesthetized with sodium pentobarbital (Mebumal; 100 mg/kg body weight, i.p.) and intracardially perfused with 200 ml isotonic ice-cold saline followed by 200 ml of fixation fluid (4 °C) during 8 min. The fixative consisted of 4% paraformaldehyde (w/v) in saline 0.1 M sodium PB (PBS) (pH 7.4). The brains were removed, postfixed for 2 h in the same fixative and cryoprotected in sucrose (10% for 24 h followed by 30% for 1 week, at 4 °C). The brainstem was cut on a cryostat in 30 μm coronal sections sampled every six sections with a random start.

The sections were processed free-floating. Endogenous peroxidase activity was removed by incubating the sections and permeabilization with 0.3% H_2O_2 for 20 min. After blocking with 1% normal goat serum (Sigma, Spain) with 0.3% Triton X-100 (10 min, room temperature) the sections were incubated with a rabbit polyclonal antibody (Santa Cruz Biotech. sc-52, USA) raised against the c-Fos protein (1:5000). Overnight incubation at 4 °C was performed in 0.1 M PBS containing 0.3% Triton X-100. The sections were washed 3 times in PBS and incubated with biotinylated specific secondary antibodies (1:200; Vector Labs Inc, Burlingame, CA) for 1 h at room temperature. The immunostaining was performed according to the ABC method using the Vectastain kit (Vector, Burlingame, CA). The chromogen used was 0.03% 3-3′-diaminobenzidine tetrahydrochloride (DAB) (Sigma, Spain) intensified with nickel chloride hexahydrate (Sigma, Spain) 0.04% (w/v) giving a darker black nuclei staining and 0.03% fresh H_2O_2 in 0.1 M Tris–HCl (pH 7.6).

5-HT immunostaining was performed under the same conditions as c-Fos but using 0.9% saline Tris buffer 0.1 M (pH 7.6) instead of saline phosphate buffer. The primary antibody was a monoclonal antibody raised against 5-HT (INCSTAR, USA) (1:50,000). Nickel chloride was not added to the chromogen solution in the second incubation for immunostaining in order to get a brownish reaction. Sections from every animal from each experimental group were processed simultaneously. Omission of the primary antibody resulted in no detectable staining. After mounting the sections on gelatin-chromalum coated slides, the sections were dehydrated and coverslipped with DPX (Panreac, Barcelona, Spain). Thus, every section was numbered according to the rostrocaudal level (Paxinos, 1986).

An Olympus BX51 microscope (Olympus, Denmark) was interfaced with a computer and a colour JVC digital video camera. For stereological analysis, sampling of c-Fos/5-HT (as applicable) positive cells was performed throughout the dorsal raphe nucleus in the rostrocaudal dimension using the optical fractionator. This method combines the optical dissector with a fractionator sampling scheme (Gundersen et al., 1988) to exclude volume divergences. Counterstaining with phase contrast allowed delineation of the DRN area in each section (Paxinos, 1986). Sections were sampled every 150 μm, starting at the ventral part of the dorsal raphe nucleus, (approximately 7.56 mm posterior to Bregma). Sampling ended at approximately 8.40 mm posterior to Bregma at the dorsal part of the dorsal raphe