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Functional autoradiography and gene expression analysis applied to the characterization of the α_2-adrenergic system in the chicken brain

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ABSTRACT

Here we report a functional autoradiographic study of [^{35}S]GTPγS binding induced by α_2-adrenoceptor activation in chicken brain tissue sections using both 10^{-4} M UK 14304 (bromoxidine or brimonidine) and 10^{-6} M epinephrine as α_2-adrenoceptor agonists. Assays were performed using two different incubation buffers: glycylglycine or Tris–HCl. Changes in the [^{35}S]GTPγS basal binding values were detected, and different [^{35}S]GTPγS specific binding values were also obtained depending on the buffer used for each drug. The best results were obtained with epinephrine in Tris–HCl, with slightly higher stimulation values than the observed with UK 14304 in glycylglycine buffer. The effect of the addition of adenosine deaminase to the incubation buffer was also tested. This effect decreasing basal binding in chicken was very small when compared to mammals, according with differences found in adenosine 1 receptor expression levels. Structures presenting α_2-adrenoceptor-mediated $G_{i/o}$ protein stimulation fitted with areas previously described as enriched in α_2-adrenoceptors in chicken brain, and their homologous areas in mammals. These data confirm the specificity of the results and reinforce the implication of the α_2-adrenoceptors in the function of these brain nuclei. On the other hand, the expression level of the different α_2-adrenoceptor subtypes was tested with real-time PCR. Contrasting with the α_2-adrenoceptor subtype distribution previously described with radioligand competition assays, where α_{2A} was the predominant α_2-adrenoceptor subtype ($\geq$75%); in the present work, the ratio of α_{2A}:$\alpha_{2B/C}$ gene expression was lower than expected both in telencephalon, tectum opticum, and cerebellum.

1. Introduction

Avian biological diversity and its complex social behaviour make an excellent model for studying neuronal functions such as seasonal neurogenesis, apoptosis, learning, neuronal proliferation and plasticity (Bottjer and Arnold, 1997; Tramontin and Brenowitz, 2000). In previous studies we determined the pharmacological profile and distribution of the α_2-adrenoceptor subtypes in the chicken central nervous system (CNS), showing that they are highly conserved receptors (Fernandez-Lopez et al., 1990, 1997) and confirming the presence of the three different α_2-adrenoceptor subtypes described for the human brain: α_{2A}-, α_{2B}-, and α_{2C}-adrenoceptors (Diez-Alarcia et al., 2006). Those data also showed a similar pharmacological profile between chicken and rat α_2-adrenoceptors in the brain, and a highly conserved anatomical distribution of these receptors in birds and mammals CNS. Although chicken genes codifying for α_2-adrenoceptors have not yet been sequenced, the comparison of primary and secondary structures of α_2-adrenoceptors cloned for different species of mammals and fish shows a high correlation coefficient (Svensson et al., 1993; Ruuskanen et al., 2005). Now, in an attempt to better characterize the α_2-adrenoceptor system in this species, we setup for the first time the [^{35}S]GTPγS autoradiography for α_2-adrenoceptors in chicken brain tissue sections. Moreover, we also report the expression levels of the three α_2-adrenoceptor subtypes determined by real-time RT-PCR.

As members of the G protein-coupled receptors (GPCRs) family, the α_2-adrenoceptors interaction with heterotrimeric G proteins

Abbreviations: GPCR, G protein-coupled receptor; CNS, central nervous system; [^{35}S]GTPγS, [^{35}S]guanylyl-5′-O-(γ-thio)-triphosphate; ADA, adenosine deaminase; E, epinephrine; ASB, agonist stimulated binding; NSB, non-specific binding; A1R, adenosine A1 receptor.

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constitutes an essential part of their functional activity. When an agonist drug binds to a GPCR, and this complex interacts with a G protein, the exchange of GDP by GTP in the alpha subunit is induced, and the dissociation of the G protein complex in Gα and Gβγ dimer is triggered. Both units are able to initiate different intracellular signalling pathways by activating or inhibiting enzymes such as adenylyl cyclase, phospholipases, ionic channels, etc. The activation of GPCRs can be assessed resorting to different experimental approaches; one of them is the stimulation of the [^{35}S]GTPγS binding assay. This technique is based on the decrease of the Gα subunit affinity for the GDP induced by the interaction of the G protein with an activated receptor, resulting in an increase in the apparent affinity of the alpha subunit for GTP, and its analogues. In this way, the use of [^{35}S]GTPγS, a non-hydrolyzable GTP analogue, allows to measure the efficacy of an agonist in inducing the receptor activation and its coupling to G proteins (Happe et al., 2000).

The quantification of G protein activation induced by the interaction of a given agonist with its receptor was first set-up on tissue membrane homogenates by using [^{35}S]GTPγS binding studies (Hilf et al., 1989; Traynor and Nahorski, 1995; Gonzalez-Maeso et al., 2000). Then, this technique was optimised to identify activated G proteins in brain tissue slices (Sim et al., 1995), allowing a detailed analysis of the anatomic distribution of the [^{35}S]GTPγS binding induced by agonist. However, it must be taken into account that the stimulation of [^{35}S]GTPγS binding is mainly due to $G_{i/o}$ proteins (Carty and Iyengar, 1994; Sim et al., 1997; Waeber and Moskowitz, 1997), which are the most abundant subtype within the CNS (Sternweis and Robishaw, 1984), and different dissociation rates exist between GDP and the distinct Gα subunit subtypes ($G\alpha_i > G\alpha_s$) (Carty and Iyengar, 1994). A noticeable feature of this approach is the heterogeneously distributed signal obtained under basal assay conditions in many CNS regions. Endogenous adenosine, acting via A1 receptors, is the most important identified factor contributing to these [^{35}S]GTPγS basal binding values in the rat brain (Laitinen, 1999; Moore et al., 2000). However, despite elimination of the adenosine signal, several "hot spots" loci (e.g. the hypothalamus) present high local basal G protein activity, and endogenous ligands responsible for this activity are yet to be identified (Laitinen, 1999). One of the aims of this study was to check the contribution of the A1 receptors activation to the basal and stimulated [^{35}S]GTPγS binding in chicken by carrying out functional autoradiographic assays in the presence and in the absence of adenosine deaminase (ADA).

The [^{35}S]GTPγS autoradiographic functional assays have been successfully performed on different receptor systems (Sim et al., 1997; Waeber and Moskowitz, 1997; Newman-Tancredi et al., 2000; Rodriguez-Puertas et al., 2000; Happe et al., 2000; Pilar-Cuellar et al., 2005; Alonso-Ferrero et al., 2006) and the anatomical distribution obtained has always been similar to that observed using quantitative autoradiographic assays (Happe et al., 2001). However, technical difficulties have been found for some receptors systems, mainly in tissues, when taking this pharmacological approach (Sim et al., 1997; Waeber and Moskowitz, 1997). Thus [^{35}S]GTPγS binding induced by α_2-adrenoceptor agonists seems to be easier to detect in cell line studies where α_2-adrenoceptor density is very high (Wise et al., 1997; Pauwels and Tardif, 2002). In contrast, those performed on native tissues, where expression levels are very low, present relatively modest [^{35}S]GTPγS specific binding even in the receptor-enriched regions (Happe et al., 2000, 2001). Considering that agonist-induced [^{35}S]GTPγS binding depends on many factors, such as receptor density, catalytic efficacy of the receptor-G protein complex (Sim et al., 1997), [^{35}S]GTPγS basal binding values (Happe et al., 2001); the tuning and characterization of the specific assay conditions for each different receptor system, and each sample conditions, species, is very important to achieve the best possible results. A further aim of this study is to compare different conditions of incubation to improve the [^{35}S]GTPγS specific binding mediated by α_2-adrenoceptor activation in chicken brain. Thus, in this study we compare different conditions for functional autoradiography assays on the α_2-adrenoceptor agonist stimulated [^{35}S]GTPγS binding in avian brain tissue sections, and we also describe the anatomical distribution of this stimulation using two different agonists: epinephrine (E) and UK 14304 (brimonidine and also bromoxidine). All the assays were carried out simultaneously in rat brain tissue sections to control the smooth running of the experimental conditions.

Finally, since a major problem in pharmacologically discriminating α_2-adrenoceptor subtypes is the lack of subtype-selective ligands (Sallinen et al., 2007; Gentili et al., 2007), the autoradiographic characterization of the anatomical distribution of the different α_2-adrenoceptor subtypes is very complicated. Instead of this pharmacological approach, we use herein real-time RT-PCR assays in an attempt to describe the distribution of mRNA of the α_{2A}-, α_{2B}-, and α_{2C}-adrenoceptor subtypes in several structures of the chicken brain.

2. Experimental procedures

All the animals used in this work were treated in accordance with the European Communities Council Directive of 1986 November 24th (86/609/EEC).

For autoradiographic studies, five male one-month-old chickens (*Gallus gallus*) and five male Sprague–Dawley rats were used. Animals were killed by decapitation; brains were rapidly removed, frozen in liquid nitrogen, and stored at −80 °C until use. Coronal (chicken) or sagittal (rat) sections, 12 μm thick, obtained with a cryostat were mounted onto gelatin-coated slides and stored at −80 °C. Sections were used with less than two weeks since cut. One adjacent section of each series was collected and stained with cresyl violet to identify brain regions by comparison with chicken (Kuenzel and Masson, 1998) and rat (Paxinos and Watson, 1998) brain atlas. Unless specified, the nomenclature used for the chicken brain nuclei is from (Reiner et al., 2004).

For real-time PCR assays, four male one-month-old chickens and five male Sprague–Dawley rats were used. Animals were killed by decapitation and brains were rapidly removed and dissected. Chicken telencephalon, tectum opticum, wulst (structure including hyperpallium accesorium + intercallatum + densocellulare), and cerebellum were isolated while for rat gene expression assays, only cortex and cerebellum were dissected. All the samples were obtained under dissecting microscope, rapidly frozen in liquid nitrogen and maintained at −80 °C until further processing.

2.1. Functional autoradiography

Two different α_2-adrenoceptor agonists were used to induce the activation of α_2-adrenoceptors in chicken brain tissue sections. We used as a model the protocol described by Sim et al. (1995), and then tried different buffers and incubation conditions described later to improve α_2-adrenoceptor mediated [^{35}S]GTPγS autoradiographic results in mammalian CNS (Happe et al., 2001).

Sections were thawed for 5 min at room temperature (RT) and dipped in pre-incubation buffer (Tris–HCl or glycylglycine 50 mM, $MgCl_2$ 3 mM, EGTA 1 mM, NaCl 100 mM and GDP 2 mM, pH 7.5) for 30 min RT to remove endogenous ligands. Then, sections were incubated in the incubation buffer (Tris–HCl or glycylglycine 50 mM, $MgCl_2$ 3 mM, EGTA 1 mM, NaCl 100 mM, GDP 2 mM and DTT 0.2 mM, pH 7.5) containing the radioligand ([^{35}S]GTPγS 0.1 nM, Perkin Elmer, UK) for 4 h at RT, and finally dipped and washed for 5 min in washing buffer (Tris–HCl or glycylglycine 50 mM, pH 7.5) at 4 °C. Lastly, sections were dipped in distilled water at 4 °C and dried overnight in a cold air stream. Receptor-stimulated [^{35}S]GTPγS binding was determined by inclusion of agonists, epinephrine or UK 14304, at 1 or 100 μM. Basal [^{35}S]GTPγS binding was determined in the absence of agonist, and non-specific [^{35}S]GTPγS binding was determined in the presence of unlabeled GTPγS 10 μM. All drugs and compounds, unless specified, were provided by Sigma–Aldrich, Spain. The addition of the α_2-adrenoceptor antagonist RX 821002 was used to demonstrate the specificity of these agonists induced [^{35}S]GTPγS binding values. Autoradiograms were generated by exposing tissue sections to ^{35}S sensitive films (Hyperfilm, Amersham-Pharmacia, UK) for five days, together with ^{14}C microscales (Amersham-Pharmacia, UK). The autoradiograms were analyzed and quantified using image analysis software (Scion Corp., Maryland, USA). Densitometric readings were converted into values of radioligand bound to tissue and expressed as nCi/g tissue.

Previous studies have revealed that brain sections generate sufficient amounts of adenosine under basal conditions, resulting in tonic and widespread A1 receptor-dependent G protein activity (Laitinen, 1999; Moore et al., 2000). In an attempt to

improve signal to noise ratio, all the previously described incubation conditions were carried out twice, with or without the addition of 100 mU/ml ADA.

2.2. Real-time RT-PCR assays

2.2.1. Obtaining cDNA

Total RNA was extracted using the Tripure™ Isolation Reagent (Roche), according to the instructions of the manufacturer. The contaminating DNA in the RNA samples was removed by incubation with DNase (Sigma) and confirmed by PCR analysis of total RNA samples prior to reverse transcription. The yield of total RNA was determined by measuring the absorbance (260/280 nm) of ethanol-precipitated aliquots of the samples. Total RNA concentrations were determined using a NanoDrop ND-3300 spectrophotometer (NanoDrop Technologies, USA). Isolated RNA was finally frozen at −80 °C until further processing.

Microcapillary gel electrophoresis was used to check RNA integrity and 100 ng of total RNA from all samples studied were analyzed using the Experion RNA HighSens Analysis Kit (Biorad Laboratories, Wilmington, USA) following the manufacturer's instructions. The 28S/18S rRNA ratio was used to assess RNA quality, using the respective areas under the 28S and the 18S peaks (Kerman et al., 2006).

Reverse transcription was performed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The 20 μl transcription mix for each sample contained 0.6 μg total RNA (10 μl), 2 μl 10× Reverse Transcription buffer, 0.8 μl dNTP 25× (100 mM), 2 μl 10× Reverse Transcription Random Primers, 1 μl MultiScribe™ Reverse Transcriptase (50 units/μl) and 4.2 μl DEPC water. Reactions were performed for 10 min at 25 °C, 2 h at 37 °C and terminated with 5 s at 85 °C. Subsequently, the reaction mixture was maintained at −20 °C until used for PCR amplification.

2.2.2. Quantitative real-time PCR

Real-time PCR quantification of α_2-adreneceptor subtypes and A1 receptor mRNA levels was performed in triplicate using gene-specific primers and SYBR® Green. Oligonucleotide primers were designed using Primer Express software (Applied Biosystems, Foster City, CA). The primer sequences and the GenBank accession numbers are given in the following table:

Gene	Forward primer	Reverse primer	Reference sequence
Alpha2Aadr chicken	5′-CCTGGTCTACGTGCGCATCT	5′-CGCTCTGGCCTTTTGTTCAG	XM_426537
Alpha2Badr chicken	5′-AAGGCGTGGTGCGAGATCT	5′-ACGATGGAGGAGGTGCAGAA	AM 87205
Alpha2Cadr chicken	5′-CGCAGGCGGTGGAGTATAAC	5′-GGTGACAATGATGGCCTTGAT	XM_426355
5-HT1A chicken	5′-GGCATCATTATGGGCACCTT	5′-CAGGACCAACGCCACGAT	XM_429136
Ada A1 chick	5′-AGTATTACGGGAAGGAGCTGAAGA	5′-TGAGCGCAAAGAGGAAGAGAA	NM_204316
Alpha2Aadr rat	5′-GGTGGTGATCGGCGTGTT	5′-CGACCGCTATGAGCGTGTAG	NM_012739
Ada A1 rat	5′-GGCAACTCCGCCATGAAC	5′-AAGGTGACCCGGAACTTGTG	NM_017155
18S rRNA	5′-GATTAAGTCCCTGCCCTTTGTA	5′-GATCCGAGGGCCTCACTAAAC	V01270

Real-time PCR was performed on an ABI PRISM 7000 real-time thermal cycler using the SYBR Green PCR Master Mix kit (Applied Biosystems, Foster City, CA) and the following thermal cycler settings: 1 cycle of 2 min at 50 °C, 1 cycle of 10 min at 95 °C, 40 cycles of 15 s at 95 °C and 1 min at 60 °C. The reaction mixture (20 μl) consisted of 2 μl cDNA aliquot (3 ng), 300 nM of each primer and 10 μl of SYBR® Green PCR Master Mix containing AmpliTaq Gold DNA polymerase. As an internal control for normalization, PCR reactions were performed concurrently with the amplification of a reference gene, 18S ribosomal RNA.

To evaluate the efficiency of the amplification for all primers, a standard curve was constructed using the threshold cycle (Ct) versus 10-fold dilutions (log [cDNA]). In all cases the primer efficiency was close to 100%. The specificity of the reaction was given by the detection of the temperature of melting (Tm) of the amplification products immediately after the last reaction cycle. Results were analyzed with the melting curve analysis software provided with the ABI PRISM 7700 sequence detector (Applied Biosystems).

Cycle thresholds for both genes were selected immediately above the baseline and within the linear range on log scaling. Increases in the amount of SYBR Green reporter dye fluorescence during the amplification process were analyzed with Sequence Detector Software (SDS version 1.6 Applied Biosystems). The relative changes in the mRNA expression (normalized mRNA levels) were determined by the equation: fold change = $2^{-\Delta Ct}$, ΔCt = (Ct target − Ct rRNA 18S) (Livak and Schmittgen, 2001), where the Ct value is the cycle number at which the fluorescence signal crosses the threshold. All results are expressed as mean ± SEM of the fold-change.

3. Results

3.1. Functional autoradiography

3.1.1. Non-specific binding (NSB)

Autoradiograms obtained from tissues incubated in the presence of 10 μM of GTPγS (non-specific binding) exhibited low [^{35}S]GTPγS binding for all the buffers used, with similar binding values in the different structures studied. Higher NSB values (about 20%) were observed in glycylglycine than in Tris–HCl buffer, and these differences were statistically significant in several of the studied structures (Tables 1 and 2). The addition of ADA to the different buffers did not significantly modified the levels of NSB, but higher NSB values (about 23%) were observed in glycylglycine + ADA buffer than in Tris–HCl + ADA buffer (Supplementary material, Tables S1 and S2).

3.1.2. Basal binding

Basal binding condition data presented heterogeneity throughout the different structures studied in any of the different buffers; however the average of basal binding values of all structures studied did not present significant differences among buffers. In general terms, high, intermediate, or low basal binding values were obtained for the same structure independently of the buffer used. In this sense, nucleus paramedianus internus thalami, nidopallium, nucleus habenularis lateralis/medialis, and lateral septal nucleus were always the structures with higher basal binding values (Supplementary material, Table S3).

Several structures showed significant differences in basal binding when comparing incubations in different media, particularly the entopallial nucleus and entopallial belt where the use of glycylglycine or Tris–HCl buffer results in outstanding changes in the autoradiogram densities (Fig. 1).

The incubation in glycylglycine + ADA resulted in decreased basal binding values in most of telencephalic structures studied and cerebellum, while an increase or no change in basal binding values was observed in most of diencephalic and brain stem structures. In contrast, the incubation in Tris–HCl + ADA resulted in decreases or no changes in basal binding values in all the structures studied with respect to those observed following the incubation in Tris–HCl (Supplementary material, Table S3).

3.1.3. Agonist stimulated binding

Values of basal binding, NSB, and agonist stimulated binding (both using either epinephrine or UK 14304 as agonists) obtained throughout the chicken brain in glycylglycine buffer and Tris–HCl buffer are showed in Tables 1 and 2, respectively. Values observed in the presence of ADA are shown in supplementary material (Tables S1 and S2).

When UK 14304 was added to glycylglycine buffer, some of the structures studied presented significant increases in agonist stimulated binding values with respect to basal binding values; however the incubation in the presence of this agonist in glycylglycine + ADA buffer induced low or no increases in the binding values in most of these structures. Under both conditions, a 10^{-4} M concentration of UK 14304 resulted in lower agonist stimulated binding values than those observed using 10^{-6} M.

The presence of the agonist UK 14304 in Tris–HCl buffer induced no stimulation of the [^{35}S]GTPγS binding and even small decreases in the agonist stimulated binding values with respect to basal binding values were obtained. As in glycylglycine buffer incubation in the presence of 10^{-4} M UK 14304 resulted in lower

Table 1
[^{35}S]GTPγS binding values (nCi/g tissue, mean ± SEM, $n = 5$) obtained from the different studied nuclei of the chicken brain using glycylglycine as incubation buffer. BB: basal binding and NSB: non-specific binding. The [^{35}S]GTPγS stimulated binding was obtained in the presence of the α_2-adrenoceptors agonist epinephrine 10^{-4} M, and UK 14304 10^{-6} M. RX 821002: values of [^{35}S]GTPγS binding obtained when RX 821002 10^{-5} M was added to the incubation in the presence of UK 14304 10^{-6} M. (1) The qualitative classification of specific [^{3}H]RX 821002 binding values for each structure referred to the structure with maximal binding value (MBV) (lateral septal nucleus (SL)) are indicated in the right column [0–25% (+), 25–50% (++), 50–75% (+++), 75–100% (++++)] (Diez-Alarcia et al., 2006).

	(1)	BB	NSB	E10^{-4} M	UK 14304 10^{-6} M	RX 821002
		Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM
Telencephalon						
Hyperpallium accesorium (HA)	++	193 ± 11	80 ± 3	227 ± 14	255 ± 12*	189 ± 11
Hyperpallium intercalatum (HI)	+++	208 ± 21	87 ± 4	250 ± 18	258 ± 10	189 ± 9
Hyperpallium densocellularum (HD)	++	205 ± 17	99 ± 2	250 ± 11	274 ± 17*	207 ± 16
Nidopallium (N)	++	233 ± 22	94 ± 0	277 ± 29	278 ± 25	226 ± 23
Entopallial nucleus of the entopallium (E)	+	222 ± 8	140 ± 11	252 ± 24	289 ± 14	288 ± 15
Entopallial nucleus belt (Ebelt)	+	308 ± 35	171 ± 4	328 ± 31	317 ± 12	325 ± 11
Lateral striatum (LSt)	++	224 ± 1	95 ± 8	224 ± 25	230 ± 29	189 ± 27
Medial striatum (MSt)	++	185 ± 12	74 ± 3	201 ± 24	228 ± 24	167 ± 21
Globus pallidus (GP)	+++	204 ± 16	82 ± 0	216 ± 13	235 ± 17	215 ± 16
Lateral septal nucleus (SL)	MBV	230 ± 12	69 ± 3	214 ± 14	207 ± 28	108 ± 23
Medial septal nucleus (SM)	+++	179 ± 5	72 ± 4	179 ± 4	187 ± 19	99 ± 19
Bed nucleus of the stria terminalis (BSt)	+++	166 ± 10	60 ± 2	314 ± 19*	225 ± 31	157 ± 29
Nucleus supraopticus (SO)	++++	140 ± 15	76 ± 2	272 ± 19*	265 ± 45	160 ± 28
Nucleus prepticus (PO)	+++	244 ± 9	76 ± 6	304 ± 19	270 ± 15	254 ± 13
Diencephalon						
Nucleus habenularis lateralis/medialis (HL/HM)	+++	206 ± 12	92 ± 8	235 ± 6	238 ± 9	154 ± 7
Dorsal nucleus of the thalami (NDT)		198 ± 18	75 ± 4	182 ± 10	179 ± 17	106 ± 13
Nucleus paramedianus internus thalami (PMI)	++++	166 ± 3	66 ± 0	220 ± 2*	185 ± 16	136 ± 15
Regio lateralis hypothalami (LHy)	+++	166 ± 16	65 ± 4	262 ± 16*	248 ± 20*	232 ± 18
Mesencephalon						
Stratum griseum et fibrosum superficiale (SGFS 1)	++	224 ± 2	94 ± 4	246 ± 16	224 ± 9	178 ± 6
Stratum griseum et fibrosum superficiale (SGFS II)	++	139 ± 3	103 ± 5	196 ± 21*	186 ± 15*	154 ± 13
Stratum griseum et fibrosum superficiale (SGFSIII)	++	214 ± 6	91 ± 3	177 ± 31	179 ± 22	124 ± 21
Stratum griseum periventriculare (SGP)	+++	135 ± 5	87 ± 20	143 ± 23	178 ± 0*	101 ± 5
Nucleus mesencephalicus lateralis, pars dorsalis (MLd)	+	117 ± 8	139 ± 4	242 ± 16	256 ± 7	154 ± 11
Substantia grisea centralis (GCt)	+++	192 ± 18	81 ± 6	200 ± 14	168 ± 13	137 ± 9
Nucleus of Edinger–Westphal (EW)	+++	144 ± 9	99 ± 3	191 ± 6*	173 ± 24	114 ± 22
Nucleus interpeduncularis (IP)	++++	131 ± 5	83 ± 3	342 ± 75*	241 ± 27*	219 ± 23
Pons						
Locus ceruleus (LoC)	+	159 ± 5	80 ± 1	155 ± 25	191 ± 22	105 ± 19
Locus subceruleus (SC)	++	163 ± 7	87 ± 1	182 ± 14	170 ± 28	106 ± 26
Cerebellum						
Granular layer	+	172 ± 3	90 ± 8	209 ± 17	185 ± 18	104 ± 15
Molecular layer	+	193 ± 8	75 ± 0	166 ± 41	143 ± 6*	124 ± 4

* When the incubation in the presence of the agonist induced a statistically significant increment of the binding over the basal binding values (Student's t test; $p < 0.05$).

agonist stimulated binding values than those observed using 10^{-6} M. Incubations with UK 14304 in Tris–HCl + ADA also resulted in slight decreases in agonist stimulated binding values with respect to basal binding values, and similar binding values were obtained for both 10^{-4} M and 10^{-6} M UK 14304 concentrations. Interestingly, bed nucleus of the stria terminalis, nucleus supraopticus, region lateralis hypothalami and nucleus interpeduncularis presented agonist stimulated values significantly higher than basal binding values in both conditions.

Stimulation with epinephrine in Tris–HCl buffer resulted in agonist stimulated binding value increases with respect to basal binding values in most of the structures studied, however these agonist stimulated binding values were lower or even similar to basal binding values, when ADA was added to the incubation in the presence of epinephrine in Tris–HCl buffer. As in the case of UK 14304, bed nucleus of the stria terminalis, nucleus supraopticus, regio lateralis hypothalami, and nucleus interpeduncularis presented net stimulation values in both conditions. Concentration of 10^{-4} M epinephrine resulted in similar or higher agonist stimulated binding values than those observed using 10^{-6} M in both conditions in most of the structures studied.

When epinephrine was used in glycylglycine buffer we also observed net stimulation in several of the structures studied. The addition of ADA to the buffer resulted in a decrease in the number of structures showing stimulation of [^{35}S]GTPγS binding. Concentration of 10^{-4} M epinephrine resulted in similar or higher net stimulation values than those observed using 10^{-6} M in most of structures when glycylglycine buffer was used. After addition of ADA the incubation in the presence of 10^{-4} M epinephrine resulted in similar net stimulation in the telencephalon, while for several structures in the brain stem lower net stimulation was obtained.

3.1.4. Inhibition of the agonist stimulated binding

The presence of the selective α_2-adrenoceptor antagonist 10 μM RX 821002 did not modify [^{35}S]GTPγS binding in any brain region when tested alone. However, in the presence of this concentration of RX 821002, the UK 14304-induced net stimulation was inhibited in almost all the structures studied (Tables 1 and 2).

3.1.5. RAT

Simultaneously, autoradiographic studies of α_2-adrenoceptor agonist mediated stimulation of [^{35}S]GTPγS were carried out for rat brain tissue sections, to control if different assays conditions were properly working. Similar results to those previously described in the literature were obtained (Newman-Tancredi et al., 2000; Happe et al., 2000). [^{35}S]GTPγS binding values observed after the incubation in the presence of UK 14304 and epinephrine obtained for the different rat brain structures studied

Table 2
[^{35}S]GTPγS binding values (nCi/g tissue, mean ± SEM, $n = 5$) obtained from the different studied nuclei of the chicken brain using Tris–HCl as incubation buffer. BB: basal binding and NSB: non-specific binding. The [^{35}S]GTPγS stimulated binding was obtained in the presence of the α_2-adrenoceptors agonist epinephrine 10^{-4} M, and UK 14304 10^{-6} M. RX 821002: values of [^{35}S]GTPγS binding obtained when RX 821002 10^{-5} M was added to the incubation in the presence of UK 14304 10^{-6} M. (1) The qualitative classification of specific [^{3}H]RX 821002 binding values for each structure referred to the structure with maximal binding value (MBV) (lateral septal nucleus (SL)) are indicated in the right column [0–25% (+), 25–50% (++), 50–75% (+++), 75–100% (++++)] (Diez-Alarcia et al., 2006).

	(1)	BB	NSB	E10^{-4} M	UK 14304 10^{-6} M	RX 821002
		Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM
Telencephalon						
Hyperpallium accesorium (HA)	++	208 ± 6	59 ± 1	292 ± 33	162 ± 9	113 ± 11
Hyperpallium intercalatum (HI)	+++	204 ± 9	64 ± 0	332 ± 9*	160 ± 4*	141 ± 7
Hyperpallium densocellularum (HD)	++	194 ± 10	67 ± 5	389 ± 29*	187 ± 18	146 ± 12
Nidopallium (N)	++	244 ± 5	62 ± 1	331 ± 40	203 ± 25	149 ± 21
Entopallial nucleus of the entopallium (E)	+	188 ± 4	95 ± 0	171 ± 24	112 ± 17	181 ± 13
Entopallial nucleus belt (Ebelt)	+	144 ± 2	116 ± 8	174 ± 23	109 ± 10	130 ± 11
Lateral striatum (LSt)	++	194 ± 10	73 ± 8	250 ± 36	128 ± 11*	122 ± 9
Medial striatum (MSt)	++	150 ± 12	58 ± 3	195 ± 22	128 ± 1*	129 ± 6
Globus pallidus (GP)	+++	201 ± 3	57 ± 5	267 ± 11*	184 ± 6	214 ± 14
Lateral septal nucleus (SL)	MBV	258 ± 10	56 ± 4	225 ± 19	129 ± 10*	107 ± 9
Medial septal nucleus (SM)	+++	189 ± 10	62 ± 4	177 ± 9	118 ± 4*	99 ± 8
Bed nucleus of the stria terminalis (BSt)	+++	170 ± 17	51 ± 2	300 ± 36*	220 ± 13*	282 ± 23
Nucleus supraopticus (SO)	++++	144 ± 10	69 ± 3	308 ± 35*	246 ± 9*	162 ± 15
Nucleus prepticus (PO)	+++	263 ± 9	45 ± 0	356 ± 37	254 ± 21	180 ± 21
Diencephalon						
Nucleus habenularis lateralis/medialis (HL/HM)	+++	179 ± 12	88 ± 18	207 ± 9	163 ± 6	160 ± 5
Dorsal nucleus of the thalami (NDT)		157 ± 7	56 ± 2	141 ± 9	117 ± 5	101 ± 8
Nucleus paramedianus internus thalami (PMI)	++++	178 ± 15	51 ± 1	192 ± 4	136 ± 11	120 ± 13
Regio lateralis hypothalami (LHy)	+++	171 ± 5	55 ± 2	296 ± 9*	235 ± 20	219 ± 22
Mesencephalon						
Stratum griseum et fibrosum superficiale (SGFS 1)	++	259 ± 19	62 ± 6	257 ± 24	155 ± 3*	150 ± 5
Stratum griseum et fibrosum superficiale (SGFS II)	++	127 ± 28	79 ± 8	183 ± 22	131 ± 10	101 ± 9
Stratum griseum et fibrosum superficiale (SGFSIII)	++	208 ± 22	75 ± 10	149 ± 14	113 ± 3*	98 ± 7
Stratum griseum periventriculare (SGP)	+++	136 ± 10	77 ± 12	138 ± 28	83 ± 8*	89 ± 8
Nucleus mesencephalicus lateralis, pars dorsalis (MLd)	+	108 ± 9	97 ± 3	148 ± 5	134 ± 4	134 ± 11
Substantia grisea centralis (GCt)	+++	159 ± 9	70 ± 0	196 ± 5*	148 ± 7	141 ± 9
Nucleus of Edinger–Westphal (EW)	+++	112 ± 7	75 ± 4	147 ± 4*	125 ± 12	112 ± 7
Nucleus interpeduncularis (IP)	++++	119 ± 13	58 ± 2	281 ± 11*	188 ± 36	202 ± 18
Pons						
Locus ceruleus (LoC)	+	152 ± 14	67 ± 1	126 ± 8*	130 ± 7	117 ± 12
Locus subceruleus (SC)	++	149 ± 25	77 ± 7	144 ± 3	99 ± 5*	104 ± 15
Cerebellum						
Granular layer	+	199 ± 7	78 ± 10	240 ± 8*	121 ± 15*	153 ± 13
Molecular layer	+	221 ± 23	64 ± 8	146 ± 7*	197 ± 14	84 ± 3

* When the incubation in the presence of the agonist induced a statistically significant increment of the binding over the basal binding values (Student's t test; $p < 0.05$).

were always higher than those obtained for the chicken brain. In rat, in contrast to that observed for chicken, the use of adenosine deaminase exerted a remarkable effect over our basal binding values (between 10 and 30%). This effect of ADA was observed both in incubations with Tris–HCl and glycylglycine buffer. A summary of these results obtained for rat brain are shown in supplementary material (Table S4).

3.2. Real-time RT-PCR assays

Adenosine A1 receptor gene mRNA levels were quantified in four different regions of the chicken brain, and compared with those from rat cortex and cerebellum (Fig. 2). Striking differences were found in A1R mRNA levels between chicken and rat brain. For example, the A1R expression levels observed in cerebellum were significantly higher in chicken than in rat brain, while it was more than twofold higher in rat cortex than in chicken telencephalon.

The mRNA levels obtained for the genes codifying for the different α_2-adrenoceptor subtypes in chicken brain are shown in Fig. 3. There are strong differences in the α_2-adrenoceptor subtypes gene expression levels between the different structures analyzed. While a predominance of the expression of the α_{2A}-adrenoceptor subtype was expected in base of preliminary radioligand binding assays in mammalian and avian brain, a surprising high density of the $\alpha_{2B/C}$-adrenoceptor subtypes mRNA expression was observed. The ratio of α_{2A}:α_{2B} gene expression is close to 1 in all chick brain structures studied, while levels of α_{2C}:α_{2A} is close to 1 in telencephalon, about 2 in tectum opticum, and up to 7 in cerebellum.

4. Discussion

From a methodological view the use of functional autoradiography in chicken brain presents outstanding differences when compared to the use of this technique in rat. To set up the best experimental conditions for this functional assay some modifications of the protocols described in the literature for mammals (Sim et al., 1995, 1997; Waeber and Moskowitz, 1997; Happe et al., 1999, 2001) were tested. Thus, chicken brain tissue sections were incubated in two different buffers, Tris–HCl and glycylclycine; in the presence or in the absence of different α_2-adrenoceptor agonists; as well as in the presence or the absence of adenosine deaminase. The two agonist used, epinephrine and the synthetic drug UK 14304 (bromoxidine), have been described as full agonists for the α_2-adrenoceptor stimulation (Happe et al., 2000), and, as previously demonstrated in our laboratory, both drugs have similar affinities for chicken and rat α_2-adrenoceptors (Diez-Alarcia et al., 2006).

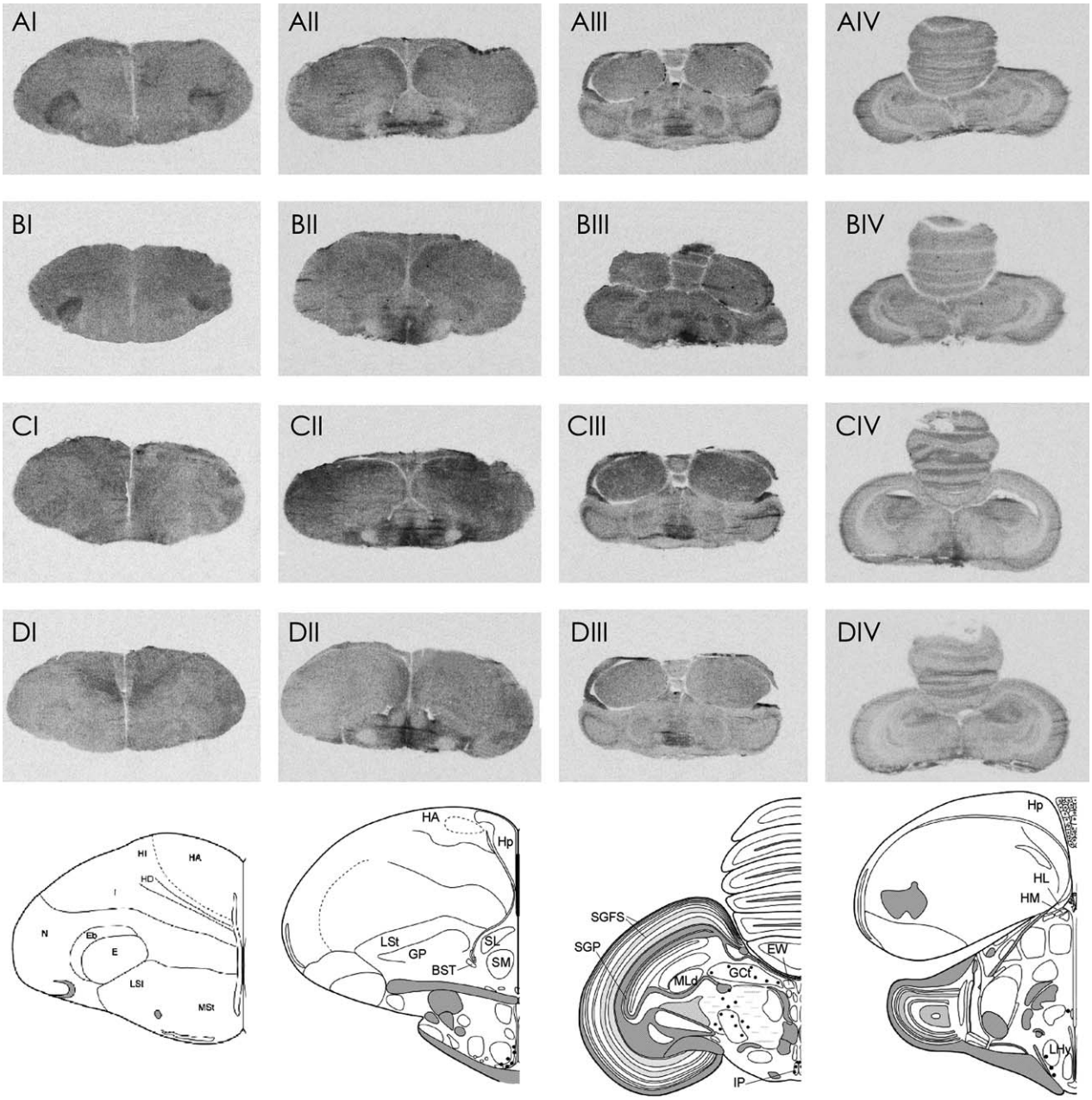

Fig. 1. Representative autoradiographs corresponding to the basal binding incubation condition obtained in the presence of the different incubation buffers tested: glycylglycine (A), glyclyglycine + ADA (B), Tris–HCl (C), and Tris–HCl + ADA (D), through four different transversal levels of the chicken brain (sheets A03.0 (I), A05.8 (II), A08.2 (III) and A10.6 (IV) from the chicken brain atlas at http://avianbrain.org/nomen/Chicken_Atlas.html).

Several considerations on the use of different buffers must be taken into account. Differential effects have been described in the literature for glycylglycine, HEPES and Tris–HCl buffers on [^{35}S]GTPγS binding supporting the idea that buffers can affect receptor conformation, and that buffer effects on basal and non-specific binding are independent. It has been described that Tris–HCl and HEPES buffers may modulate the interaction of receptors with G proteins in the absence of agonist to increase basal activity level and/or to increase the affinity of the unoccupied receptor-G protein complex for [^{35}S]GTPγS (Happe et al., 2001). Consistent with an increase in precoupled receptors due to buffer effects, drugs known to act as inverse agonists on the α_2-adrenoceptors (Murrin et al., 2000) have been shown to reduce apparent basal binding in a region-specific way, and this reduction of basal binding is higher in Tris–HCl and HEPES buffers than in glycylglycine buffer (Happe et al., 2001). Moreover, an increase in the ratio of α_2-adrenoceptor low affinity sites (G protein-uncoupled receptors) with glycylglycine compared to Na_2KHPO_4 buffer has been previously observed (Raymon et al., 1992).

In our assays, we also observed region-specific differences on the basal binding values depending on the incubation buffer used suggesting that GPCRs responsible of these G_i proteins' basal activity are different in chicken telencephalon, where this binding is decreased by addition of ADA to the glycylglycine buffer, and in chicken brain stem, where no changes or even an increase or the basal binding values are observed in this incubation condition. This buffer-specific effect is also detectable in our data from the incubation in the presence of RX 821002, showing, as discussed below, a reduction of the binding values even under basal binding values mainly when the incubation was carried out in Tris–HCl

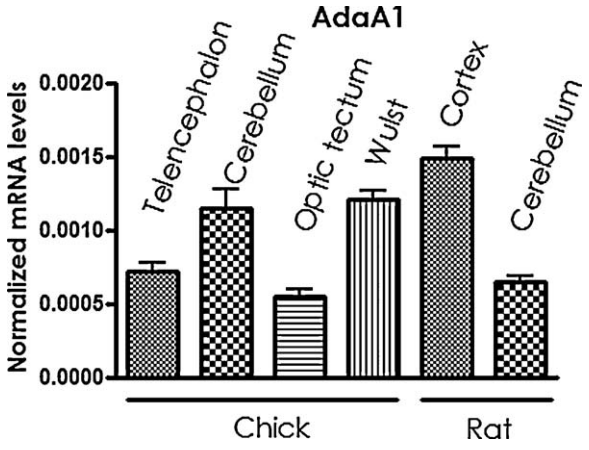

Fig. 2. Normalized expression levels of adenosine A1 receptor obtained for different brain structures of chicken and rat brain. The relative change in the mRNA expression (normalized mRNA levels) was determined by the equation: fold change = $2^{-(Ct}$, (Ct = (Ct target − Ct 18S) (Livak and Schmittgen, 2001). Data are shown as mean ± SEM, n = 3–5. Note: wulst (structure including hyperpallium accesorium + intercallatum + densocellulare).

buffer. In the same way, due to the higher basal binding values observed in Tris–HCl buffer and the modest agonist stimulated [^{35}S]GTPγS binding values obtained for some regions, only some brain areas showed considerable stimulation of the [^{35}S]GTPγS when any of the agonists are used in this incubation buffer. In this regard, the different anatomical distribution and expression levels of the three α_2-adrenoceptor subtypes described in chicken brain could explain the different regional responses observed if the stimulation of each subtype depends on the buffer used. An example could be the higher expression of α_{2C}-adrenoceptor mRNA both in the cerebellum and tectum opticum than in the telencephalon found in our assays. In summary, the three-state model predicts that the same receptor may exhibit different patterns of agonist and inverse agonist activity when different response measurements are made, when different assay systems are used, and when the level of constitutive receptor activity varies (Leff et al., 1997).

A specific effect of the buffers was also observed in entopallium and entopallial belt where the incubation in glycylglycine buffer resulted in high [^{35}S]GTPγS binding values both in basal binding and agonist stimulated binding conditions (for both α_2-adrenoceptor agonists used) while these structures did not show any labelling using Tris–HCl as incubation buffer. Moreover, quantitative autoradiographic assays with the [^{3}H]RX 821002 as radioligand (Diez-Alarcia et al., 2006) did not show α_2-adrenoceptor labelling for any of these structures; and the presence of ADA in the glycylglycine buffer did not result in any significant effect on basal or stimulated [^{35}S]GTPγS binding. Therefore, the [^{35}S]GTPγS binding observed in these structures must be induced by the use of glycylglycine buffer and its possible effect over GPCRs activation state rather than by the α_2-adrenoceptor or adenosine receptor specific activation. These data highlight the importance of a careful choice of the buffer-agonist combination.

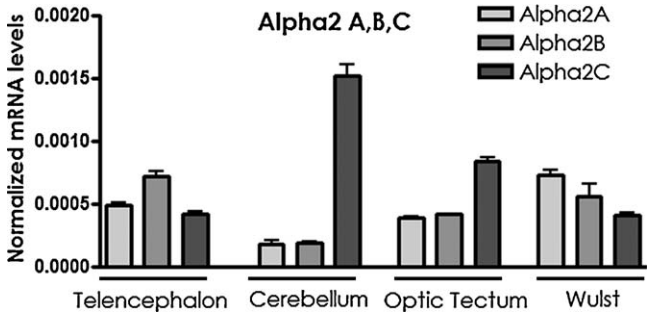

Fig. 3. Normalized expression levels of the three different α_2-adrenoceptor subytpes obtained for different chicken brain structures. The relative change in the mRNA expression (normalized mRNA levels) was determined by the equation: fold change = $2^{-(Ct}$, (Ct = (Ct target − Ct 18S) (Livak and Schmittgen, 2001). Data are shown as mean ± SEM, n = 3–5. Note: wulst (structure including hyperpallium accesorium + intercallatum + densocellulare).

The effect of different neurotransmitter systems in functional autoradiography results has been described in mammals, where the action of the endogenously synthesized adenosine through the adenosine A1 receptors has been proved to induce high [^{35}S]GTPγS basal binding values in some brain regions (Parkinson and Fredholm, 1992). This undesirable effect can be reduced by the use of adenosine deaminase (ADA) or A1 receptor specific antagonists (Laitinen, 1999; Moore et al., 2000), however the use of ADA does not always solve this problem (Laitinen, 1999). In our experiments, the addition of ADA to the incubation buffer was used in an attempt to describe the presence of an adenosine component of the [^{35}S]GTPγS basal binding, through the chicken brain non-described until now. The previously mentioned buffer-specific effect over basal activation state of GPCRs is extensible to A1R, because a higher effect of ADA was observed when the Tris–HCl buffer was used. Anyway, the effect of ADA on [^{35}S]GTPγS basal binding in chicken brain slices was very poor in contrast to that observed in our rat control assays, and that described for mammals functional autoradiography (Moore et al., 2000) suggesting a lower contribution of the adenosine component to basal binding values for the chicken brain. Our mRNA expression data showed lower levels of A1R mRNA in chicken than in rat tissue, especially in telencephalon (Fig. 2). These low expression levels of A1R mRNA could result in low density of A1R protein, and could explain the smaller effect of ADA on chicken [^{35}S]GTPγS basal binding. Moreover, some of these chicken brain structures where the effect of ADA is higher (e.g. cerebellum) were those showing the highest A1R mRNA expression levels which could determine a higher A1R density in these areas.

Regarding the specificity of the response observed in this study, we should notice that a similar pharmacological profile has been previously described for α_2-adrenoceptors both in chicken and rat brain (Diez-Alarcia et al., 2006). In the study here presented we measured the [^{35}S]GTPγS binding to compare the activation of α_2-adrenoceptors present in the chicken brain by both epinephrine and the synthetic agonist UK 14304, as previously described for rat CNS (Sim et al., 1995, 1997; Waeber and Moskowitz, 1997; Happe et al., 1999, 2001), since this ligands have been described as full agonists for the α_2-adrenoceptor stimulation (Happe et al., 2000). In our assays, 10 μM α_2-adrenoceptor antagonist RX 821002 proved to be effective to reduce the agonist-induced [^{35}S]GTPγS binding both in chicken and rat brain slices, thus demonstrating the specificity of the α_2-adrenoceptor stimulation in agreement with previous reports on mammalian CNS (Happe et al., 2000). Moreover, although only a few structures were strongly labelled, this [^{35}S]GTPγS binding stimulation induced by epinephrine and/or UK 14304 correlates with the previously reported density of α_2-adrenoceptors throughout the chicken brain using quantitative receptor autoradiography (Diez-Alarcia et al., 2006), giving additional evidence that the here reported stimulation data are mediated by α_2-adrenoceptor activation. This correlation is specially noticeable in structures such as the nucleus interpeduncularis, the bed nucleus of the stria terminalis (BSt), and the nucleus supraopticus which present both the highest [^{35}S]GTPγS binding values in functional autoradiography and the highest densities in quantitative autoradiographic assays using the radioligand [^{3}H]RX 821002 (Diez-Alarcia et al., 2006). In agreement, these structures in mammals have been reported to present

a high number of α_2-adrenoceptors using autoradiographic assays (Unnerstall et al., 1984; Happe et al., 2004), and to receive noradrenergic innervation (Armstrong, 1986; Battisti et al., 1987; Moons et al., 1995; Smith and Aston-Jones, 2008). A strong stimulation of the [^{35}S]GTPγS binding following epinephrine stimulation has been also described for the BSt in the rat brain (Happe et al., 2000). All these data support the idea that α_2-adrenoceptors play an important role in these structures.

Our data show that some structures (see Table 1) present lower [^{35}S]GTPγS binding values in the presence of RX 821002 than those observed in the basal binding. Some of these differences could be explained by the ability of RX 821002 to act as an inverse agonist on α_2-adrenoceptors in some tissues (Erdbrugger et al., 1997; Murrin et al., 2000; Ge et al., 2003), as previously described for other α_2-adrenoceptor inverse agonists (Murrin et al., 2000). Besides, RX 821002 has been described to show a slightly higher affinity for the α_{2A}-adrenoceptor subtype (Uhlen and Wikberg, 1991; Renouard et al., 1994). Although little is known about α_2-adrenoceptor subtype distribution through the chicken brain, this slight preference for α_{2A}-adrenoceptors could also explain the nuclei-specific effects observed for RX 821002. Anyway, it should be noticed that the low signal obtained in several nuclei makes some of the changes observed under the different incubation conditions not reliable. Moreover, it should also be noticed that the α_2-adrenoceptor agonist UK 14304 presents some affinity for 5-HT1A receptors in mammals (Newman-Tancredi et al., 1998). However, since pharmacology, density and anatomical distribution of the serotonergic receptor system, as well as the 5-HT1A receptor affinity for UK 14304 have not been studied in the chicken brain, the UK-14304 effect on 5-HT or other GPCRs modulating G_i protein activity cannot be confirmed or discarded in our assays.

Values for mRNAs do not always mirror real protein levels, and in this sense our α_2-adrenoceptor subtype mRNAs values present some discrepancy with the α_2-adrenoceptor subtype distribution previously described by means of pharmacological tools (Diez-Alarcia et al., 2006). Thus, pharmacological characterization of α_2-adrenoceptor subtypes observed in the chicken brain using radioligand binding techniques showed a predominance of α_{2A}-adrenoceptor subtype (about 75%), and a low $\alpha_{2B/C}$-adrenoceptor component (about 25%) which is only detected in the telencephalon. These data were in agreement with the CNS distribution of the mammalian α_2-adrenoceptor subtypes (Sastre and Garcia-Sevilla, 1994; Grijalba et al., 1996). The α_{2A}-adrenoceptor mRNA density observed in the present work throughout the different studied structures from the chicken brain is comparable to α_{2A}-adrenoceptor expression levels found in parallel assays carried out with rat brain tissue. However, our real-time RT-PCR data contrast with the low presence of α_{2C}-adrenoceptors described on the light of radioligand techniques (Diez-Alarcia et al., 2006), and show high levels of α_{2C}-adrenoceptor subtype mRNA in all the chicken brain structures studied. In this way, the ratio α_{2C}:α_{2A}-adrenoceptor subtype is close to 1 in telencephalon, about 2 in tectum opticum, and about 7 in cerebellum. In the same way, while α_{2B}-adrenoceptor mRNAs in mammals have been reported to be restricted to thalamus and cerebellum (Scheinin et al., 1994), similar amounts of α_{2A} and α_{2B}-adrenoceptor subtypes mRNA levels appear in our RT-PCR studies on the chicken brain (α_{2A}:α_{2B} ratio close to 1 in all the structures studied). Poor subtype specificity of α_2-adrenoceptor available ligands (Sallinen et al., 2007; Gentili et al., 2007), or a post-transcriptional regulation of α_2-adrenoceptor mRNAs, could explain these discrepancies between the pharmacological and real-time PCR studies for α_2-adrenoceptors.

A number of anatomical aspects have to be considered from the results of this study. We should first indicate that in the locus coeruleus, the main noradrenergic structure in the CNS, the moderate α_2-adrenoceptor labelling previously observed by means of quantitative autoradiography (Diez-Alarcia et al., 2006) correlates with the moderate agonist stimulated binding values in our functional autoradiography assays. The correlation between the anatomical distribution obtained by means of quantitative and functional autoradiography is outstanding in structures such as the nucleus interpeduncularis, the bed nucleus of the stria terminalis (BSt), and the nucleus supraopticus which present both the highest [^{35}S]GTPγS binding values in functional autoradiography and the highest densities in quantitative autoradiographic assays using the radioligand [^{3}H]RX 821002 (Diez-Alarcia et al., 2006). A strong stimulation of the [^{35}S]GTPγS binding following stimulation with epinephrine has been described for the BSt in the rat brain (Happe et al., 2000). In agreement, these structures have been reported to receive noradrenergic innervation (Armstrong, 1986; Battisti et al., 1987; Moons et al., 1995; Smith and Aston-Jones, 2008), and to present a high number of α_2-adrenoceptors using autoradiographic assays (Unnerstall et al., 1984; Happe et al., 2004) in mammals.

The distribution of the α_2-adrenoceptors functional binding through the chicken brain confirms their functional presence in areas related to the visual system described for other avian species such as the pigeon, quail or duck (Ball et al., 1989; Fernandez-Lopez et al., 1990, 1997; Muller and Gerstberger, 1992) using other methodological approaches. The α_2-adrenoceptors activation can be observed in hyperpallia and tectum opticum, both related to avian visual pathways (Korzeniewska and Gunturkun, 1990); in the interpeduncular nucleus, related to brightness discrimination retention (Hemmendinger and Moore, 1984); and in the Edinger–Westphal nucleus (also known as the accessory oculomotor nucleus), that is the accessory parasympathetic cranial nerve nucleus of the oculomotor nerve. Therefore, the present data provide additional support to the involvement of the α_2-adrenoceptor system in the acquisition and processing of visual information (Korzeniewska and Gunturkun, 1990; Fernandez-Lopez et al., 1997; Happe et al., 2004). On the other hand, BSt is related with responses to acute and chronic stress (Choi et al., 2008), and fear response in mammals (Fendt et al., 2005), while nucleus supraopticus is related with emotional behaviours and social recognition (Yoshida et al., 2009), again functions with which α_2-adrenoceptors have classically been related (Ruffolo et al., 1993, 1995; Murrin et al., 2000). High α_2-adrenoceptor activity was also detected in hypothalamic structures of the chicken CNS, as described in birds and mammals (Unnerstall et al., 1984; Bylund, 1988; Dermon and Kouvelas, 1989; Happe et al., 2004), supporting the participation of α_2-adrenoceptors in neuroendocrine functions such as body temperature regulation (Myers and Lee, 1984; Boulant, 2000) or sexual dimorphism (Fernandez-Lopez et al., 1997).

Controversial data have been found in the septal nuclei. High levels of α_2-adrenoceptors have been previously described in these nuclei by means of quantitative autoradiography. However, a lack of adrenergic agonist-induced stimulation of [^{35}S]GTPγS binding values, or even a reduction or the basal binding has been observed in our functional autoradiography assays. One of the factors suggested to explain this controversy is the ratio receptors/G proteins on the hypothesis that the higher the concentration of available G proteins, the more frequently receptor and G protein interact. This hypothesis is supported by studies on cannabinoid receptors showing high amplification factors in the lower receptor density regions in contrast with the low amplification factors observed in high density regions (Breivogel et al., 1997). Other possible factors reported have to do with coupling of α_2-adrenoceptors to different types of G proteins (Eason et al., 1992; Chabre et al., 1994; Wise et al., 1997; Jasper et al., 1998) or differences in the G proteins coupling between the different α_2-

adrenoceptor subtypes (Eason et al., 1992). In any case, discrepancies between receptor density and functionality are often reported in the literature. Thus, predictions about the magnitude of a drug effect in a specific brain region must be made not only on the basis of receptor binding analysis but also considering the degree of activation of intracellular signal transduction mechanisms by those receptors (Breivogel et al., 1997).

In summary, our data show that functional autoradiography is a useful technique for characterizing the agonist-induced activity of α_2-adrenoceptors in chicken brain. The best results were obtained when the incubation was carried out with epinephrine in Tris–HCl, while slightly lower stimulation values where observed with the agonist UK 14304 in glycylglycine buffer. On the other hand, when the gene expression levels of the different α_2-adrenoceptor subtypes were tested, an unexpected relevant expression level of the $\alpha_{2B/C}$-adrenoceptor component has been observed throughout the chicken brain. Further investigation about the α_2-adrenoceptor subtypes density and distribution would be necessary to clarify the discrepancies between radioligand binding and gene expression assays.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchemneu.2009.09.002.

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