

Increased mRNA Expression of α_{2A} -Adrenoceptors, Serotonin Receptors and μ -Opioid Receptors in the Brains of Suicide Victims

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The development of new therapies for the treatment of psychiatric disorders requires an in-depth knowledge of the molecular bases underlying these pathologies, which remain largely unknown. Alterations in adrenoceptors, serotonin receptors, and other G protein-coupled receptors (GPCRs) have been associated with suicide and depression. However, to date, there is little information about mRNA expression of the GPCRs in the frontal cortex of suicide victims. Our goal was to study the expression in the brain of these receptors. For this purpose, we measured mRNA levels by RT-PCR. We found that the expressions of α_{2A} -adrenoceptors, 5-HT_{1A}, 5-HT_{2A} serotonin receptors, and μ -opioid receptors were elevated in the post-mortem brains of these suicide victims with respect to matched controls. Moreover, in the case of α_{2A} -adrenoceptors (the only for which these data were available), a significant correlation was observed between the level of mRNA and protein quantified in the brain of the same subjects, indicating that protein synthesis of this receptor was not influenced by post-translational regulatory mechanisms. In addition, the degree of adrenoceptor and 5-HT receptor expressions appeared to be correlated in the brains of suicide victims and control subjects. Alterations in the expression of adrenoceptors, serotonin, and opioid receptors indicate that these signaling proteins might be related to the etiopathology of suicidal and depressive behaviors. Alternatively, such changes may represent adaptive mechanisms to compensate for other as yet unknown alterations. The results also suggest that these receptors could share common regulatory mechanisms.

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INTRODUCTION

Molecular and functional alterations of neurotransmitter receptors and their norepinephrine and/or serotonin (5-HT) signaling pathways are believed to play a direct role in the pathogenesis of suicide and depression (Mann, 1999; Ressler and Nemeroff, 1999). On the other hand, a wide variety of G protein-coupled receptors (GPCRs; eg α_2 -adrenoceptors, 5-HT receptors, μ -opioid receptors, GABA_B receptors, cholinergic muscarinic M₂/M₄ receptors, etc.) serve as auto- or heteroreceptors, and control neurotrans-

mitter release (Schlicker and Göthert, 1998; Starke, 1981). There is evidence that the density of some of these GPCR proteins, such as α_2 -adrenoceptors and 5-HT receptors, is altered in the brains of suicide victims and in the platelets of depressed patients (Callado *et al*, 1998; De Paermentier *et al*, 1997; García-Sevilla *et al*, 1986, 1999; González *et al*, 1994; Meana *et al*, 1992; Meana and García-Sevilla, 1987; Mendelson, 2000; Ordway *et al*, 1994; Pandey *et al*, 1995; Sargent *et al*, 2000; Stockmeier *et al*, 1998). These alterations have been correlated with impaired neurotransmitter release that results from α_2 -adrenoceptor supersensitivity and/or serotonin receptor dysfunction. Thus, stimulation of presynaptic α_2 - and 5-HT-autoreceptors induce a decrease in the synthesis and release of norepinephrine and 5-HT (Esteban *et al*, 1999). Further evidence of the involvement of α_2 -adrenoceptors and 5-HT receptors in the pathophysiology of suicide and depression is that chronic antidepressant administration reduces the density and activity of these receptors in rat brain (Esteban *et al*, 1999; Invernizzi *et al*, 2001; Smith *et al*, 1981; Sibug *et al*, 1998; Subhash and Jagadeesh, 1997), and in the platelets of depressed subjects (García-Sevilla *et al*, 1986; Stahl, 1994; Sargent *et al*, 1997). Based on these

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observations, it has been suggested that patients with mood disorders might suffer an underlying disturbance of noradrenergic and/or serotonergic function. In this context, it has been shown that the main metabolite in the production of 5-HT (5-hydroxyindolacetic acid) is slightly decreased in the cerebrospinal fluid of suicide victims (Nordstrom *et al*, 1994; Roy *et al*, 1986). Finally, μ -opioid receptors have been implicated in the behavioral alterations associated with stress. Indeed, its mRNA expression is augmented in the brain of rats submitted to stress (Yamamoto *et al*, 2003) and its protein levels were increased in the post-mortem brains of suicides (Gross-Isseroff *et al*, 1990; Gabilondo *et al*, 1995).

Most studies of the biochemical alterations of neurotransmitter receptors during major depression have relied upon assays of radioligand binding. However, the functional state of GPCR proteins (which determines radioligand binding) is modulated by several factors, such as coupling to G proteins, desensitization by protein phosphorylation (mediated by kinases such as GRK, PKC, and PKA), sequestration, and internalization (Chuang *et al*, 1996). Therefore, the results of radioligand binding studies in the brain of suicide victims will not necessarily reveal alterations in these and other molecular events. This study was designed to quantify the mRNA levels for α_{2A} -adrenoceptors, 5-HT_{1A}, 5-HT_{2A}, and μ -opioid receptors in the prefrontal cortex of suicide victims since mRNA levels are not modulated by the above phenomena. For this purpose, reverse transcription followed by polymerase chain reaction (RT-PCR) was used under conditions that permitted quantitative assessment of mRNA cellular concentrations in post-mortem human samples. This technique has the advantage over other approaches to evaluate gene expression in that it is more sensitive, quantitative, and overcomes moderate RNA degradation. As a result, we show that there is a significant increase in the expression of the GPCRs transcripts analyzed in post-mortem brains of suicide victims. As these receptors share signaling mechanisms and common regulatory transcription sequences, we also studied the association between the levels of mRNA expression of the same receptors in the same brains. The results suggest that these receptors could be involved in the pathophysiology of depressive/suicidal behavior and their transcription regulation might be controlled by similar (adrenoceptors and 5-HT receptors) or divergent (opioid receptors) mechanisms.

METHODS

Subject Selection and Brains Samples

This study was approved by the Research Ethics Committee at the University of the Balearic Islands. Human brains were obtained at autopsy from the Institute of Anatomical Forensics (Palma de Mallorca, Spain). Specimens of prefrontal cortex (Brodmann's area 9, middle frontal gyrus) were dissected on a glass surface cooled with ice and care was used to include only gray matter (no more than a 1-mm white-matter border was included) in cortical samples. The suicide group consisted of 12 suicide victims (eight men and four females; mean age \pm SEM: 54.1 ± 4.4 years; post-mortem delay, 26.2 ± 2.6 h; Table 1). The cause of death of

the suicide victims, as determined by the medical examiner, was trauma caused by jumping from a height ($n=5$), hanging ($n=3$), self-inflicted gunshot wound ($n=1$), poisoning ($n=1$, carbon monoxide), drug overdose ($n=1$, fluoxetine), and electrocution ($n=1$). Brain pH was not measured, and no neuropathological exams were performed. In this group of suicide victims, a retrospective review of the antemortem medical histories was performed to determine whether the victims had been clinically diagnosed or were under drug treatment for psychiatric disorders. This analysis was conducted after the completion of the assays and was performed without access to the biochemical findings. Most suicide victims used in this study had a pre-mortem diagnosis of major depression (9/12) (Diagnostic and Statistical Manual of Mental Disorders, third edition, revised [DSM-III-R] criteria). A pre-mortem

Table 1 Characteristics of the Individual Subjects Included in This Study

ID	Age (year)	Sex	PMD ^a (h)	Psychiatric diagnosis	Cause of death
<i>Suicides</i>					
S1	30	M	20	MD ^b	Gunshot wound
S2	69	M	28	MD	Jumping from a height
S3	46	F	22	MD	Poisoning
S4	25	M	24	MD	Jumping from a height
S5	64	F	24	MD	Drug overdose
S6	70	F	48	MD	Jumping from a height
S7	51	M	24	MD	Hanging
S8	47	M	11	MD	Electrocution
S9	62	F	26	MD	Jumping from a height
S10	69	M	28	Unknown	Hanging
S11	67	M	36	Unknown	Hanging
S12	50	M	24	Unknown	Jumping from a height
Mean	54.1	4F/8M	26.2		
SEM	4.4		2.6		
<i>Controls</i>					
C1	30	M	30	None	Motor-vehicle accident
C2	70	M	24	None	Myocardial infarction
C3	42	F	44	None	Drowning
C4	26	M	8	None	Motor-vehicle accident
C5	60	F	24	None	Motor-vehicle accident
C6	75	F	36	None	Motor-vehicle accident
C7	50	M	24	None	Drowning
C8	47	M	24	None	Cirrhosis
C9	50	F	24	None	Motor-vehicle accident
C10	65	M	21	None	Drowning
C11	65	M	36	None	Myocardial infarction
Mean	52.7	4F/7M	26.8		
SEM	4.8		2.8		

^aPMD: post-mortem delay.

^bMD: major depression.

diagnosis was not available in three subjects. In suicide victims ($n=6$), a toxicological screening (quantitative assays for antidepressant and other psychotropic drugs, and ethanol) was performed on blood, urine, liver, and kidney samples at the Toxicology Unit, Institute of Forensic Medicine, using standard procedures. In this group of suicide victims, ethanol was detected in five suicide subjects and antidepressant drugs (clomipramine, fluoxetine, and mianserin) were detected in four depressed suicides. However, neither the above drugs nor electrocution or carbon monoxide influenced the transcription rate of adrenoceptors, serotonin receptors, or μ -opioid receptors, except alcohol and fluoxetine that induced reductions in 5-HT_{1B} mRNA (Neumaier *et al*, 1996; Vazquez *et al*, 2002). In six suicides no toxicological screening data were available. The control group for this series of suicide victims consisted of similar brain specimens from 11 subjects (seven men and four females; mean \pm SEM: age, 52.7 ± 4.8 years; post-mortem delay, 26.8 ± 2.8 h; Table 1) who died of different accidental or natural causes. None of the control subjects had documented history or family reports of psychiatric disorders or drug abuse. Suicide victims and control subjects were matched according to the following criteria: (1) gender, (2) age, and (3) post-mortem delay. The mean difference in age between suicide and control for individual matches (absolute values) was 2.4 ± 0.8 years, whereas the mean age difference between the groups was 1.4 ± 4.4 years. The mean difference in post-mortem delay between suicide and control subjects for individual matches was 7.6 ± 1.9 h whereas the mean difference between groups was 0.6 ± 2.5 h. In all, 10 control subjects and suicide victims were paired directly, and one control subject was paired with two suicides.

RNA Isolation from Human Brain Tissue

RNA was isolated from human brain samples by the acid guanidinium thiocyanate/phenol/chloroform extraction method (Chomczynski and Sacchi, 1987). Briefly, approximately 500 mg of human brain cortex was homogenized with 10 volumes of TRIzol (Life Technologies, Grand Island, NY, USA) in a blade-type homogenizer (Ultraturrax, Janke

& Kunkel, GMBH & Co KG, Staufen, Germany). After incubating for 5-min at room temperature, 1 ml of chloroform was added to the homogenate, which was agitated vigorously for 15 s and then incubated for 3 min at room temperature. This mixture was centrifuged for 10 min at 12 000 g and 4°C, and the upper (aqueous) phase was recovered and mixed with 2.5 ml of isopropyl alcohol, before incubating for 10 min at room temperature. This mixture was centrifuged for 10 min at 12 000 g and 4°C, the supernatant was removed, and the RNA pellet was washed with 5 ml of 75% ethanol before centrifuging for 5 min at 7500 g and 4°C. The DNA residue was then digested with RNase-free DNase (bovine pancreas, Boehringer) following the directions provided by the manufacturer. After phenol extraction and ethanol precipitation, the final pellet was resuspended in 100 μ l of RNase-free water and stored at -80°C . The RNA concentration in these samples was usually between 1.5 and 3 $\mu\text{g}/\mu\text{l}$ (as determined by absorption at 260 nm). Finally, the RNA samples were analyzed by agarose gel electrophoresis to ensure that no degradation had occurred (data not shown).

Reverse Transcription and PCR Amplification

Reverse transcription and PCR amplifications were performed with the GeneAmp[®] RNA PCR kit (Perkin-Elmer, Barcelona, Spain). First, cDNAs were synthesized by reverse transcription from 0.5 μg of total RNA. This reaction was performed in PCR buffer (10 mM Tris-HCl, 50 mM KCl, pH 8.3), containing 5 mM MgCl₂, 1 mM of each dNTP, 20 U of RNase inhibitor, 50 U of Moloney murine leukemia virus reverse transcriptase, and 2.5 μM of random hexamers in a final volume of 20 μl . The mixture was incubated for 5 min at 25°C, 15 min at 42°C, 5 min at 98°C, and 5 min at 10°C.

The primers for PCR amplifications were chosen from coding regions of each gene that exhibited poor homology to related GPCRs or other known human genes, and they were designed to produce amplicons of different sizes (Table 2 and Figure 1). Considering these restrictions, the length and GC content of forward and reverse primers was maintained as close as possible within each primer set. PCR amplifications were carried out in PCR buffer containing

Table 2 Primer Sequences for PCR Amplification of α_{2a} -Adrenoceptor, 5-HT_{1A}, 5-HT_{2A}, μ -Opioid Receptor, and β -Actin cDNAs

Template	Sequence	L	%GC	Product size (bp)
α_{2a} -Adrenoceptor	Forward: 5' GCAGAGGCCGAACCGCTGCCCCACCCAGCTC 3'	30	73	103
	Reverse: 5' AGCCGGCCGAGCCGCGCTGCGAGAT 3'	25	76	
5-HT _{1A} receptor	Forward: 5' ACCCGGAAGACCGCTCGGACCCCGACGCA 3'	30	73	219
	Reverse: 5' CTGCGGGGCGGGAGATGCTCCATGGCGGGT 3'	30	73	
5-HT _{2A} receptor	Forward: 5' CAGGACGATTCTGAAGGTCTTTAAGGAGGGG 3'	30	53	180
	Reverse: 5' CCGTGTGCCAAGATCACTTACACAAAGT 3'	30	47	
μ -Opioid receptor	Forward: 5' CCAGAACTACGTTCCAGACTGTT 3'	24	46	168
	Reverse: 5' AGTGGAGTTTTGTTGCTCAAT 3'	21	38	
β -Actin	Forward: 5' ATCGTGGGCCGCCCTAGGCA 3'	20	70	240
	Reverse: 5' GGGGAGACTTGGGATTCCGGT 3'	21	62	

The length (L) and GC content (%GC) of each primer, as well as the length of the PCR product (base pairs) for each set of primers, are indicated. For other details see text.

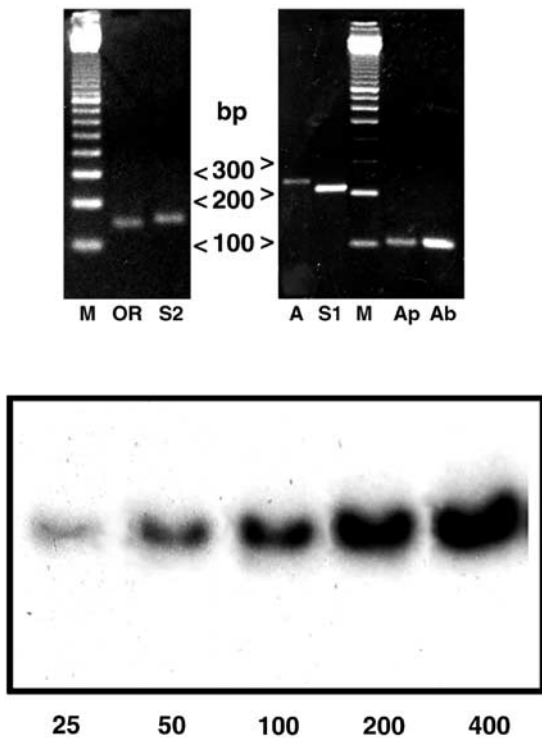


Figure 1 Upper panel: Agarose gel electrophoresis of RT-PCR amplification products of the human brain α_2A -adrenoceptor (Ab), 5-HT $_{1A}$ (S1), 5-HT $_{2A}$ (S2), and μ -opioid (OR) receptors, and β -actin (A). The human platelet α_2A -adrenoceptor (Ap) was also amplified as a positive control since this is the only α_2 -adrenoceptor subtype expressed in platelets. Negative controls (no RNA or cDNA and no reverse transcriptase) did not yield PCR products (data not shown). A 100-base pair ladder (M) was used to determine product sizes. In addition to the correct size, PCR products were sequenced to confirm their identity. For other details see the Subjects and Methods section. Lower panel: RT-PCR amplifications corresponding to a standard curve for the α_2A -adrenoceptor. The amount of total RNA and the corresponding IOD values for the bands shown were: 25 ng and 2628 IOD units; 50 ng and 8754 IOD units; 100 ng and 20894 IOD units; 200 ng and 42115 units; and 400 ng and 73726 IOD units.

2 mM MgCl $_2$, 1 mM of each dNTP, 1 nmol of [32 P]dCTP (3 μ l of a 1:10 dilution of 3000 Ci/mmol and 10 mCi/ml, Amersham Pharmacia Biotech, Barcelona, Spain), 2.5 U of Ampli Taq DNA polymerase, and 0.15 μ M of each (forward and reverse) primer, in a total volume of 100 μ l. Other experimental conditions (volume of reverse transcription reaction used for PCRs, annealing temperatures, and number of cycles) were determined empirically for each mRNA species. The conditions were optimized to obtain a linear relation between the initial amount of RNA and the integrated optical density (IOD) of the signal obtained for each product (see below). For α_2A -adrenoceptors, 5-HT $_{1A}$, 5-HT $_{2A}$, and μ -opioid receptors, 4 μ l of reverse-transcription reaction medium was used for PCR amplifications (corresponding to 100 ng of total RNA in the reverse transcription reaction). For β -actin 0.3 μ l (3 μ l from a 1:10 dilution, corresponding to 7.5 ng of total RNA) was used. For quantitative evaluations, standard curves of five PCR reactions containing various amounts of cDNA template from one control subject were prepared. For standard

curves to quantify α_2A -adrenoceptors, 5-HT $_{1A}$, 5-HT $_{2A}$, and μ -opioid receptors, 1–16 μ l of reverse transcription reaction medium was used (25, 50, 100, 200, and 400 ng of total RNA). For standard curves of β -actin 0.1–1 μ l (1–10 μ l from a 1:10 dilution, corresponding to 2.5, 5, 7.5, 15, and 25 ng of total RNA) was used. Before each reaction, the cDNA templates (from the reverse transcription reaction) were diluted in water to a final volume of 20 μ l, heated to 80°C, and added to tubes containing the other PCR reagents (80 μ l), previously incubated for 2 min at 80°C to avoid nonspecific annealing ('hot start'). Then, following a 2-min denaturalization step at 94°C, thermal cycling commenced. The thermal cycles consisted of two or three steps at the following temperatures and times: 94°C for 1 min, 62°C for 1 min, and 72°C for 20 s (30 cycles) for α_2A -adrenoceptors and 5-HT $_{1A}$ receptors; 94°C for 1 min, 51°C for 1 min, and 72°C for 30 s (31 cycles) for μ -opioid receptors and β -actin; 94°C for 1 min and 65°C for 1 min (30 cycles) for 5-HT $_{2A}$ receptors. After thermal cycling, the samples were maintained at 72°C for 7 min and the PCR products were analyzed as indicated below.

Detection and Quantification of mRNA Expressions

Preliminary series of RT-PCRs were carried out to ascertain the nature of the amplified PCR products. For these series, the methods used were the same as above, except that the number of thermal cycles was 35–36, instead of 30–31, and [32 P]dCTP was not included in the reaction mix. After PCR, 10 μ l of reaction mixture was combined with 2 μ l of electrophoresis loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol) and separated on 2% agarose gels in TAE buffer (40 mM Tris-Acetate, 1 mM EDTA) containing SYBR Green I (1:10 000 dilution; Molecular Probes, Barcelona, Spain). Under these experimental conditions, each RT-PCR produced a unique band of the expected size (Figure 1, Table 2). These PCR products were further analyzed by DNA sequencing of both the sense and antisense DNA strands, which confirmed the origin of the amplified DNA.

PCR reactions in which the amplified products were labelled with [32 P]dCTP (since only 30–31 cycles were used to fall into the linear part of the PCR-kinetics sigmoid curve) produced signals detectable by autoradiography, whose intensities were proportional to the initial RNA concentration used for the RT-PCR (Figure 1). To detect the PCR products, 10 μ l of the PCR mixture was combined with 2 μ l of electrophoresis loading buffer and the products were separated by electrophoresis on 15-well 7% polyacrylamide gels (6 \times 8 cm 2 , 0.75 cm thickness) in TBE buffer (45 mM Tris-Borate, 1 mM EDTA). The evaluation of mRNA expression from each subject and for each receptor was performed in three independent RT-PCR experiments. From each experiment, four test samples and four control samples were loaded along with the standards (five samples of known concentration) on the same gel, for a total of 12 measurements per subject and mRNA species. After electrophoresis, gels were dried, covered with a plastic wrapping, and exposed to autoradiographic film (Hyperfilm, Amersham) with an intensifying screen at –80°C for between 15 min and 4 h. The IOD values of autoradiography bands were determined by image analysis using the

program SigmaGel (SPSS Science, Erkrath, Germany). To quantify the mRNA levels, the IOD values from the test samples were compared to those from the standards as described previously (Escribá *et al*, 1994). To avoid artifacts, mRNA expression for each receptor was corrected by the expression of β -actin, which was used as internal control, being determined from the same cDNA sample used to measure receptor expressions, as indicated above. Therefore, the mRNA expression for each receptor was calculated as $RE = [(R_R/R_T)/(A_R/A_T)] \times 100$, where R_R is the calculated mRNA content for a given receptor, obtained by interpolation of the autoradiography band IOD value into the standard control curve; R_T is the total amount of mRNA present in the PCR, calculated by spectrophotometry; and A_R and A_T are equivalent to R_R and R_T , but for β -actin.

Statistics

The results were finally expressed as the means \pm SEM. One-sample *t*-test (identical in its implementation to the paired *t*-test) was used for statistical evaluations. Pearson correlation coefficients were calculated to test for possible associations among variables. The level of significance was $p \leq 0.05$. All tests were two-tailed.

RESULTS

RT-PCR has been used to determine gene expression in many types of tissue, including brain tissue from both humans and animals (Cao *et al*, 2001; Li and Wang, 2000; Newman *et al*, 1988). Here, we have used this method to quantify the mRNA levels of various GPCRs in post-mortem brain tissue samples from suicide victims and control subjects. In contrast to other similar experimental approaches, such as 'real-time RT-PCR', the technique employed here separates undesired nonspecific reaction products from those amplified specifically, thereby eliminating a potential artifact from the analysis. However, under our experimental conditions, no nonspecific products were detected at all (Figure 1). In addition, the specificity of these primers was further confirmed by sequencing the reaction products generated on both strands.

GPCR mRNA Expression in the Brains of Suicide Victims

Major depression has been associated with alterations in the density of α_2A -adrenoceptors, 5-HT_{1A}, 5-HT_{2A}, and μ -opioid receptor proteins. Consistent with these findings, we found that the level of α_2A -adrenoceptor mRNA augmented in the prefrontal cortex of suicide victims ($42.5 \pm 10.5\%$ over control, $p = 0.03$; Figure 2). Similarly, quantitative analyses demonstrated marked and significant increases in the expression of mRNA for the 5-HT_{1A}, 5-HT_{2A} receptors ($131 \pm 33\%$ over control, $p = 0.02$ and $65 \pm 26\%$ over control, $p = 0.04$, respectively; Figure 2). Furthermore, expression of the μ -opioid receptor was also augmented in suicide subjects ($89 \pm 36\%$ over control, $p = 0.03$; Figure 2). A comparison between the increase in the levels of receptor expression in the frontal cortex of suicide victims with a known ($n = 9$) or unknown ($n = 3$) history of major

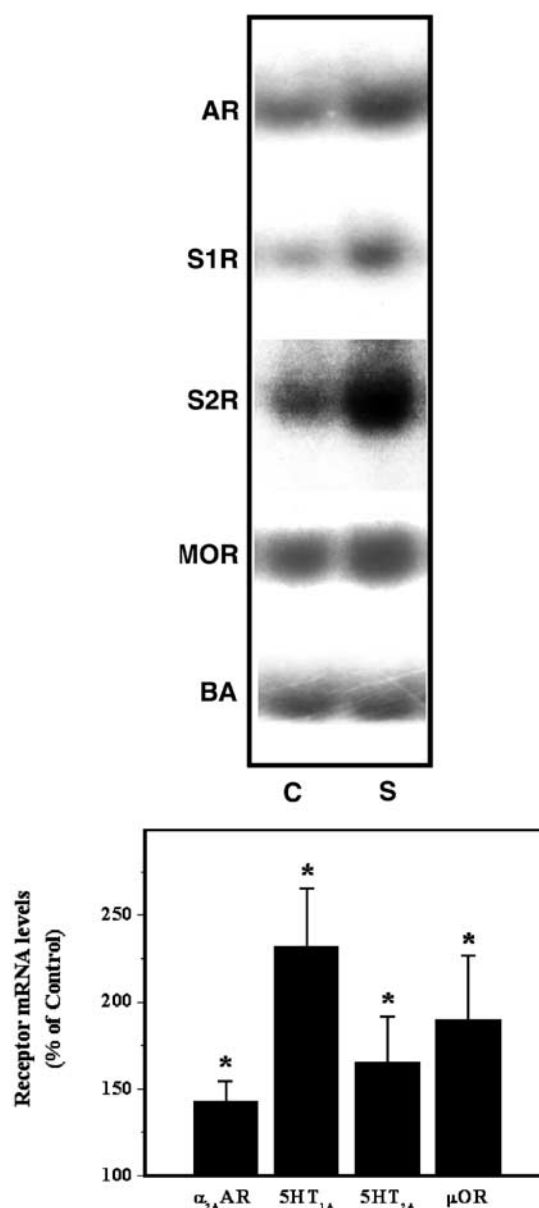


Figure 2 GPCRs expression in the human brain. Upper panel: Comparative expression of α_2A -adrenoceptors (AR), 5-HT_{1A} (S1R), 5-HT_{2A} (S2R), and μ -opioid receptors (MOR) in the brain of a depressed suicide victim (S) and an age-, sex-, and post-mortem-delay-matched control (C). In both cases, 100 ng of total RNA was used for RT-PCR. The expression of β -actin (BA) is also shown. Lower panel: Relative expressions of GPCRs (α_2A -AR, α_2A -adrenoceptors; 5HT_{1A}, 5-HT_{1A} receptors, 5HT_{2A}, 5-HT_{2A} receptors; μ OR, μ -opioid receptors) in the brains of suicide victims. Data are the percentage values (mean \pm SEM) with respect to the matched control group (100%). * $p < 0.05$.

depression did not show any significant differences ($2-12\%$, $p > 0.05$). Nor were significant changes observed in the relative levels of β -actin in the suicide group ($92 \pm 16\%$) with respect to the control group ($99 \pm 14\%$). Thus, in this study, the levels of receptor expression in each brain sample were corrected for the relative levels of β -actin in the same brain sample to avoid possible artifacts originated by RNA degradation, differences in RNA concentration measurements, etc.

Correlation between the Levels Of α_{2A} -adrenoceptor mRNA and Protein in the Human Brain

In previous studies, we have used other biochemical and immunological approaches to investigate α_2 -adrenoceptor protein in suicide and depressed subjects (Callado *et al*, 1998; García-Sevilla *et al*, 1999). However, due to differences in the regulation of the translational and post-translational machinery, changes in the cellular levels of an individual mRNA species and its corresponding protein are not always equivalent. For this reason, we analyzed the relationship between α_{2A} -adrenoceptor mRNA and protein levels. A significant correlation ($r=0.79$; $p<0.05$) was found between the expression of α_{2A} -adrenoceptor mRNA when compared with the corresponding levels of protein (measured by immunoblotting). The data corresponding to adrenoceptor protein were measured previously in the same brain area of those subjects in which both molecular species were measured (Brodman's area 9, $n=9$; Figure 3) (García-Sevilla *et al*, 1999).

Relationships Between the mRNA Levels of GPCRs

The GPCRs here studied share signaling entities and expression regulatory DNA sequences (see below), so that we investigated possible relationships in the expressions of these receptors. In the human prefrontal cortex, expression of α_{2A} -adrenoceptors was closely correlated with that of 5-HT_{1A} receptors ($r=0.6$, $p=0.014$, $n=19$; Figure 4). The expression of the latter also showed a significant correlation with the expression of 5-HT_{2A} receptors ($r=0.65$, $p=0.007$, $n=18$; Figure 4). In addition, 5-HT_{2A} receptor expression showed a trend for correlation with the expression of both the α_{2A} -adrenoceptors ($r=0.38$, $p=0.12$, $n=18$) and

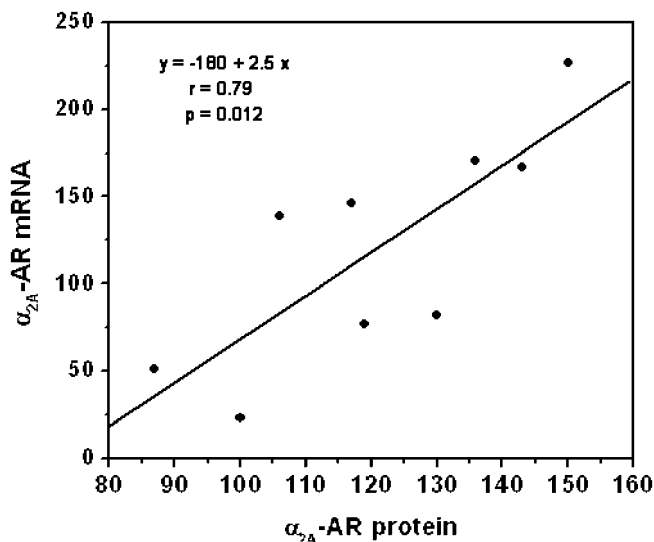


Figure 3 Correlation between α_{2A} -adrenoceptor density (protein levels) and expression (mRNA levels) measured in the same brain area (Brodman's area 9) of the same subjects. The density of α_{2A} -adrenoceptors was determined by immunoblotting, as previously described (García-Sevilla *et al*, 1999). Data are expressed as the percentage of immunoreactivity or expression with respect to control samples. Each point represents a different subject and the solid line represents the regression of the correlation. The data were best described by the expression $y = -180 + 2.5 \times$ ($r=0.79$, $n=9$, $p=0.012$).

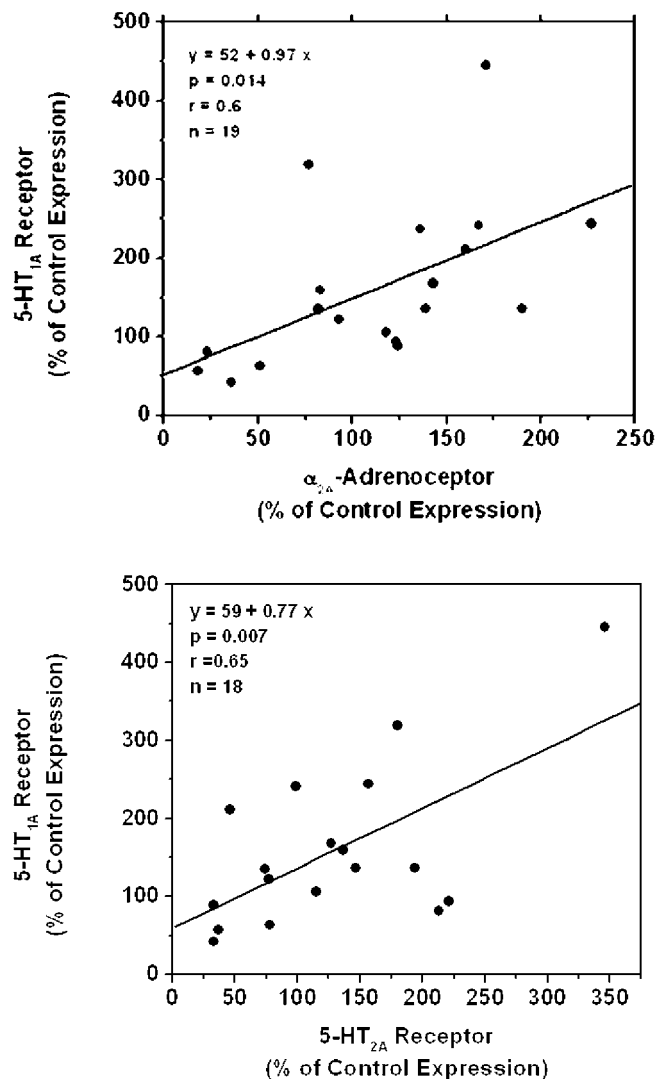


Figure 4 Correlations between the expression of α_{2A} -adrenoceptors and 5-HT_{1A} receptors (upper panel), and 5-HT_{1A} and 5-HT_{2A} receptors (lower panel) in the brains of the same subjects (controls and suicides). Each point represents a different subject and the solid lines represent the regression of the correlations. Both axes are expressed as percentages of receptor mRNA levels with respect to a control subject (100%). Significant correlations were found between the two variables in both cases. The data were best described by the expressions: α_{2A} -adrenoceptors vs 5-HT_{1A} receptors, $y = 52 + 0.97 \times$ ($r=0.6$, $n=19$, $p=0.014$) and 5-HT_{1A} vs 5-HT_{2A} receptors, $y = 59 + 0.77 \times$ ($r=0.65$, $n=18$, $p=0.007$). Correlations between other variables were not significant (see the Results section).

μ -opioid receptors ($r=0.43$, $p=0.09$, $n=17$). Similarly, the expression of 5-HT_{1A} receptors showed a trend for correlation with that of the μ -opioid receptors ($r=0.42$, $p=0.1$, $n=17$). α_{2A} -Adrenoceptor expression did not appear to be related with that of μ -opioid receptors ($r=0.18$, $p=0.47$, $n=17$).

DISCUSSION

To our knowledge, this is the first time that alterations in the expression of α_{2A} -adrenoceptor, serotonin (1A and 2A subtypes) receptors, and the μ -opioid receptor have been

reported in the frontal cortex of the same suicide victims. In addition, there is no information about adrenoceptor and opioid receptor mRNA levels and very few reports about the expression of 5-HT receptors and associated signaling elements in suicides (Arango *et al*, 2001; Pandey *et al*, 2002). This study was designed to evaluate the molecular alterations in the post-mortem brain of subjects with mood disorders who had committed suicide. Depression is the most important risk factor for suicide and, therefore, a common approach to study neurochemical alterations in mood disorders is the analysis of brain samples from suicide victims (García-Sevilla *et al*, 1996a). Indeed, no significant differences were found between GPCR expression in the brains of suicide victims with known ($n=9$) and unknown ($n=3$) history of depression. Therefore, the conclusions drawn from the present results might be relevant to both suicide and depression. As the molecular bases of suicide and depression are not fully understood, we studied the expression of various GPCRs associated with these human pathologies. In these studies, a number of issues were raised and answered.

Alterations in α_{2A} -AR Expression in the Brains of Suicides

Presynaptic inhibitory α_{2A} -adrenoceptors control neuronal norepinephrine and 5-HT release. Previous studies, using specific antibodies, have demonstrated that the density of α_{2A} -adrenoceptor is increased in suicide subjects with major depression (García-Sevilla *et al*, 1999). In addition, other studies using radioligand agonists revealed an elevation in the density of α_{2A} -adrenoceptor (high-affinity state) in the post-mortem brains of suicide victims (Callado *et al*, 1998; De Paermentier *et al*, 1997; González *et al*, 1994; Meana *et al*, 1992; Meana and García-Sevilla, 1987). In addition, higher levels of G_i proteins (García-Sevilla *et al*, 1996b, 1997, 1999) and lower levels of adenylyl cyclase activity (Reiach *et al*, 1999) were reported in the platelets of depressed subjects and post-mortem brains of suicide victims who previously suffered from depression. Finally, the activity of α_{2A} -adrenoceptors has recently been found to be enhanced in the brains of depressed suicide victims, as determined by [35 S]GTP γ S binding (González-Maeso *et al*, 2002). This is in agreement with studies demonstrating increased receptor and G_i protein densities. These results further support the hypothesis that adrenoceptor supersensitivity explains in part the molecular alterations related to suicide and depression (García-Sevilla *et al*, 1986). Thus, increases of α_2 -adrenoceptor and G_i protein density/activity, and decreases of adenylyl cyclase density/activity would be involved with reductions in cytosolic levels of cAMP. Moreover, antidepressant drug treatment has been shown to decrease the levels of α_{2A} -adrenoceptors and G_i proteins, further implicating these receptors in the pathophysiology, and thus, in the therapy of suicide, and depression (De Paermentier *et al*, 1997; García-Sevilla *et al*, 1986; Piletz *et al*, 1996).

All these data indicate that the α_{2A} -adrenoceptor signal pathway is enhanced at each molecular step, from receptor to second messenger in the brains of suicide victims. In this context, significant increases in α_{2A} -adrenoceptor mRNA levels were found in the post-mortem brains of suicides.

This demonstrates that increases in the receptor protein detected by immunoblotting and radioligand binding studies originate from an increase in adrenoceptor transcription and not by other causes, such as altered receptor turnover. In addition to protein studies, it is advisable to investigate gene expression, since discrepancies between protein and mRNA levels in signaling proteins have been previously reported and associated with post-translational regulatory processes (Young *et al*, 1987; Busquets *et al*, 1997; Ventayol *et al*, 1997).

Besides these novel alterations in the expression of these GPCRs in the brains of suicide victims, we also studied the correlation between mRNA and protein levels in the brains of the same subject and brain area. A positive correlation was found between both molecular entities (α_{2A} -adrenoceptor protein and mRNA). This result indicates that post-translational mechanisms and proteolytic degradation do not play important roles in the regulation of this receptor *in vivo*, as previously suggested from studies of the adrenoceptor stability (Ozaita *et al*, 1999).

Altered 5-HT Receptor Expression in the Brains of Suicide Victims

Brain 5-HT receptors have also been implicated in the pathophysiology of suicide. A large number of studies have demonstrated alterations in the abundance and/or function of these receptor proteins in depressive/suicide subjects, but there is a lack of information regarding the mRNA levels of these receptors in the brains of suicide victims. The levels of the 5-HT $_{2A}$ receptor protein have been consistently found to be increased in depressed subjects, (Mendelson, 2000) and increased coupling and activity of 5-HT $_{2A}$ receptors has been demonstrated in depressed patients (Serres *et al*, 1999). As a consequence, this receptor has been proposed as a marker for depression/suicide (Mendelson, 2000; Pandey *et al*, 1995). In this work, we found a significant degree of 5-HT $_{2A}$ receptor overexpression in the frontal cortex of depressed suicides, in agreement with data recently published (Pandey *et al*, 2002). In addition, positron emission tomography studies suggest that depressive and suicidal states are associated with increases of 5-HT $_{2}$ receptors and low levels of the 5-HT metabolite 5-hydroxyindoleacetic acid, which suggest reduced neurotransmitter concentrations (Meyer *et al*, 1999, 2003). Thus, as for α_{2A} -adrenoceptors, these increases in 5-HT $_{2A}$ receptor density and function could be attributed to an increased rate of transcription. On the other hand, a significant correlation between the expression of 5-HT $_{2A}$ receptors and α_{2A} -adrenoceptors suggests that they might share common regulatory elements and further supports their involvement in the pathophysiology of suicide. However, it is not clear what the origin of the common regulation of these two presynaptic receptors might be, and whether heterologous regulation, cross-talk mechanisms, or common transcription regulatory processes could be involved in this phenomenon (see below).

In the present study, it has also been shown that the expression of 5-HT $_{1A}$ receptors increases in the brain of suicide victims. This result is in agreement with a recent report, showing that the promoter region of the 5-HT $_{1A}$ gene contains a polymorphism that accounts for increased

receptor expression in association with depression and suicide (Lemondé *et al*, 2003). Studies on the alterations of 5-HT_{1A} receptors in suicide/depressed subjects have generated certain controversy, since both increases and decreases in receptor protein abundance have been reported. It has been suggested that impaired receptor sensitivity (Cowen *et al*, 1994) or altered signaling (Lesch *et al*, 1990) could account for the apparent decreases in receptor abundance, although post-transcriptional regulation and protein degradation (Young *et al*, 1987; Busquets *et al*, 1997; Ventayol *et al*, 1997) could also be involved in the differences observed between receptor mRNA and protein levels. In fact, a functional impairment of 5-HT_{1A}-associated signaling is originated by alteration of downstream transduction proteins in the brains of suicides (Hsiung *et al*, 2003). Moreover, a lower serotonergic activity associated with suicidal behavior has been also shown through positron emission tomography studies in depressed subjects and radioligand binding studies in post-mortem brain of suicides (Oquendo *et al*, 2003; Stockmeier *et al*, 1998). On the other hand, many antidepressant drug treatments downregulate 5-HT_{1A} and 5-HT_{2A} receptors, supporting the idea that increases in the activity of both receptors are associated with the development of suicidal/depressive behaviors (Stahl, 1994). In the present study, a significant correlation between the expression of 5-HT_{1A} receptors and 5-HT_{2A} receptors was also found. Gene expression is modulated by proteins acting at DNA regulatory sequences. In this context, it has been shown that SP1- and cAMP responsive (CRE)-related DNA sequences regulate 5-HT_{1A}, 5-HT_{2A}, and α_{2A} -adrenoceptor expression (Handy and Gavras, 1996; Tamotsu *et al*, 1995; Meijer *et al*, 2000; Zhu *et al*, 1995). Thus, regulation by common transcription activators and repressors could be involved in the coordinated changes in α_2 -adrenoceptor and 5-HT receptor mRNA levels that we observed.

Altered Expression of μ -Opioid Receptors in the Brains of Suicide Victims

Most of the biochemical studies on neuroreceptor alterations in suicide brain tissue have been focused on adrenoceptors and serotonin receptors. However, clinical and biochemical evidence of alterations of the μ -opioid receptor in suicide also exists. In line with the present results, it was previously shown that the density of μ -opioid receptor sites is augmented in the post-mortem brain of depressed suicides (Gross-Isseroff *et al*, 1990; Gabilondo *et al*, 1995). Moreover, treatments with antidepressant drugs induce decreases in the receptor (and associated neuropeptide) levels (Hamon *et al*, 1987). Here we have shown that the expression of the μ -opioid receptor is significantly and markedly increased in the frontal cortex of suicide victims, supporting the involvement of this receptor in the pathophysiology of suicide. In this sense, it has been recently shown that stress, related to certain suicidal behaviors, induces increases in μ -opioid receptor mRNA expression in the rat brain (Yamamoto *et al*, 2003). In addition, positron emission tomography studies in humans demonstrate the *in vivo* involvement of these receptors in mood and behavior, further suggesting their potential implication in suicide behaviors (Zubieta *et al*, 2003).

Recent [³⁵S]GTP γ S binding studies, however, indicate that the activity of the μ -opioid receptor signaling is not altered in the brains of suicide victims (González-Maeso *et al*, 2002), despite the higher mRNA and protein levels found in this and other reports. The same reasons discussed above for 5-HT receptors might be involved in this phenomenon. On the other hand, no correlation was found between the expression of μ -opioid receptors and those of 5-HT receptors and α_{2A} -adrenoceptors, suggesting that some divergent regulatory mechanisms control the expressions of these receptors.

Is RT-PCR a Suitable Technique to Measure Receptor Expression in Human Post-mortem Brain Samples?

A number of techniques have been used to evaluate the rate of gene transcription in cells and tissues. However, more recently PCR-based methods have been used to detect smaller amounts of mRNA in human samples (Cao *et al*, 2001; Li and Wang, 2000; Newman *et al*, 1988). In this study, we have shown that RT-PCR is suitable for the quantification of certain mRNA species in post-mortem human brain tissue. However, when performing such analyses, several factors should be taken into account. Firstly, mRNA degradation after death can lead to the fragmentation of RNA species (Barton *et al*, 1993; Leonard *et al*, 1993). The longer the time spent by the corpses at high outdoor temperatures, the more severe mRNA degradation is. RNA fragmentation makes it difficult to detect gene expression by Northern blot or other hybridization techniques. However, fragmented mRNA may still be a good substrate for RT-PCR, since amplicons usually correspond to short regions of mRNA (100–300 bases), whose length might be smaller than that of the RNA fragments. Additionally, cuts within these regions can still yield amplification products. Furthermore, mRNA degradation (and other possible artifacts) can be corrected for by measuring the expression of a housekeeping gene, such as β -actin, and expressing the levels of expression of the mRNA of interest relative to the expression of such genes, as performed here.

The effect of other variables (age, sex, post-mortem delay, drugs, etc.) was also evaluated. For the receptors here studied and under the experimental conditions used, only elevated post-mortem delay periods appeared to induce decreases in the amount of mRNA in the human post-mortem brain. However, when receptor mRNA content was corrected by β -actin mRNA content, no difference in the relative content of mRNA was detected among subjects with different post-mortem delays. This result further supports the convenience to use an internal reference rather than an external cDNA to evaluate the overall experimental process. In addition, the very small difference between the post-mortem delay values of the study and control groups (only about 40 min) discounted possible influences of this variable on the final result obtained.

One of the advantages of using RT-PCR for this type of analysis is that it is very sensitive, and thus, small amounts of template mRNA can be detected and measured. Moreover, mRNA levels for a given receptor are not masked by other mRNA species, whereas when measuring GPCR protein levels through functional properties (eg agonist

radioligand binding), the behavior of these receptors can be modified by G protein coupling, receptor phosphorylation, internalization, etc. (which may result in alterations in the apparent protein levels). Finally, the close correlation between template (mRNA/cDNA) concentrations and the signal detected (IOD values), as well as the high signal-to-noise ratio obtained demonstrated that RT-PCR is a suitable technique for the study of receptor expression in post-mortem human brain samples. In contrast, other techniques are not so advantageous and often give qualitative results difficult to quantify.

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