

Regulation of Platelet α_{2A} -Adrenoceptors, Gi Proteins and Receptor Kinases in Major Depression: Effects of Mirtazapine Treatment

Jesús A García-Sevilla^{*1,4}, Pedro Ventayol¹, Víctor Pérez², Grégoire Rubovszky³, Dolors Puigdemont², Marcel Ferrer-Alcón⁴, Antonio Andreoli³, José Guimón⁴ and Enric Alvarez²

¹Laboratory of Neuropharmacology, Associate Unit of the Institute of Neurobiology 'Ramón y Cajal' (CSIC), Department of Biology, University of the Balearic Islands, Palma de Mallorca, Spain; ²Psychiatric Service, Sant Pau Hospital, Autonomous University of Barcelona, Barcelona, Spain; ³Emergency Psychiatric Unit, Department of Psychiatry, University of Geneva, Geneva, Switzerland; ⁴Clinical Research Unit, Department of Psychiatry, University of Geneva, HUG-Belle-Idée, Chêne-Bourg/GE, Switzerland

Major depression is associated with the upregulation of α_{2A} -adrenoceptors in brain tissue and blood platelets. The homologous regulation of these receptors by G-protein-coupled receptor kinases (GRKs) might play a relevant role in the pathogenesis and treatment of depression. This study was designed to assess the status of the complex α_{2A} -adrenoceptor/G α_i /GRK 2 in the platelets of depressed patients ($n = 22$) before and after treatment with the antidepressant mirtazapine, an antagonist at α_{2A} -adrenoceptors (30–45 mg/day for up to 6 months). A second series of depressed suicide attempters ($n = 32$) were also investigated to further assess the status of platelet GRK 2 and GRK 6. Platelet α_{2A} -adrenoceptors and G α_i protein immunoreactivities were increased in depressed patients (49 and 35%) compared with matched controls. In contrast, GRK 2 content was decreased in the two series of depressed patients (27 and 28%). GRK 6 (a GRK with different properties) was found unchanged. In drug-free depressed patients, the severity of depression (behavioral ratings with two different instruments) correlated inversely with the content of platelet GRK 2 ($r = -0.46$, $n = 22$, $p = 0.032$, and $r = -0.55$, $n = 22$, $p = 0.009$). After 4–24 weeks of treatment, mirtazapine induced downregulation of platelet α_{2A} -adrenoceptors (up to 34%) and G α_i proteins (up to 28%), and the upregulation of GRK 2 (up to 30%). The results indicate that major depression is associated with reduced platelet GRK 2, suggesting that a defect of this kinase may contribute to the observed upregulation of α_{2A} -adrenoceptors. Moreover, treatment with mirtazapine reversed this abnormality and induced downregulation of α_{2A} -adrenoceptor/G α_i complex. The results support a role of supersensitive α_{2A} -adrenoceptors in the pathogenesis and treatment of major depression.

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INTRODUCTION

The noradrenergic hypothesis of depression postulates a reduced function of the neurotransmitter norepinephrine at various adrenoceptors in specific brain regions (Ressler and Nemeroff, 1999). Recent functional and biochemical approaches further indicate that major depression is associated with noradrenergic dysfunction and upregulation of inhibitory $\alpha_{2A/C}$ -adrenoceptors in the brain (Callado *et al*, 1998; García-Sevilla *et al*, 1999; Fu *et al*, 2001; González-Maeso *et al*, 2002; Ordway *et al*, 2003). Chronic

psychosocial stress in monkeys (a model of depression) has also been shown to induce persistent increases of α_{2A} -adrenoceptors in the brain (Flügge *et al*, 2003). Moreover, depressed patients also display upregulation and supersensitive α_{2A} -adrenoceptors in blood platelets (García-Sevilla *et al*, 1981, 1986, 1987, 1990; Piletz *et al*, 1991; Gurguis *et al*, 1999). These observations suggest that patients with mood disorders might have an underlying disturbance of noradrenergic system, perhaps due to hyperactive α_2 -adrenoceptor function (Fu *et al*, 2001).

The regulation of α_{2A} -adrenoceptors is also an important issue in major depression because most antidepressant drugs have indirect regulatory effects on these receptors (Charney, 1998; Esteban *et al*, 1999). The homologous regulation of receptors is induced by a family of G-protein-coupled receptor kinases (named GRK 1–7), some of which translocate from the cytosol to the plasma membrane (eg GRK 2 but not GRK 6) to phosphorylate the agonist (endogenous ligand)-activated receptors (Pitcher *et al*,

*Correspondence: JA García-Sevilla, Unité de Recherche Clinique, Département de Psychiatrie, HUG Belle-Idée (Les Voirons), 2 Chemin du Petit-Bel-Air, Chêne-Bourg/GE CH-1225, Switzerland, Fax: +41-22-305-57-99, E-mail: jesus.a.garcia-sevilla@hcuge.ch

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1998). This process eventually results in receptor desensitization and downregulation and/or a change in receptor signaling (Lefkowitz, 1998). Since the α_{2A} -adrenoceptor is regulated by GRK 2/3 (Eason *et al*, 1995), a heuristic hypothesis in major depression is that a defect in GRK 2/3 function could lead to receptor supersensitivity, which in turn could predispose to illness. Alternatively, increased GRK 2/3 activity might help to compensate for excessive stimulation of α_{2A} -adrenoceptors. Few studies have dealt with the involvement of brain GRKs in major depression and in the mechanism of action of antidepressant drugs (García-Sevilla *et al*, 1999; Miralles *et al*, 2002; Grange-Midroit *et al*, 2003). In the prefrontal cortex of drug-free depressed suicides, the content of GRK 2/3, but not of GRK 6, was found increased in parallel with those of α_{2A} -adrenoceptors and G α i coupling proteins, which was interpreted as a compensation of the abnormal higher functioning of the α_{2A} -adrenoceptor/G α i complex in depression (García-Sevilla *et al*, 1999; Grange-Midroit *et al*, 2003).

Changes in receptor function in blood platelets have often been used as an indirect marker of neurotransmitter receptor alterations in psychiatric disorders (Stahl, 1985; Camacho and Dimsdale, 2000). Moreover, recent findings indicate that the platelets can synthesize proteins in response to external stimuli and that these cells contain messenger RNA for a wide diversity of proteins (Lindemann *et al*, 2001), which allows the investigation of post-transcriptional receptor regulatory mechanisms. Therefore, this study was designed to assess the biological status of the complex α_{2A} -adrenoceptor/G α i/GRK in the platelets of patients with major depression. The aim was also to determine whether treatment with the antidepressant mirtazapine (Holm and Markham, 1999; Millan *et al*, 2000), an antagonist at α_{2A} -adrenoceptors that enhances the release of norepinephrine and serotonin, results in the modulation (eg reversal of baseline abnormalities) of platelet α_{2A} -adrenoceptors and associated regulatory proteins in depressed patients.

SUBJECTS AND METHODS

Patient Selection and Treatment

A first series of depressed patients ($n = 22$) were examined and treated (mirtazapine) in the Affective Disorders Unit of the Sant Pau Hospital, Autonomous University of Barcelona (ADU patients), and a second series of depressed suicide attempters ($n = 32$) were examined in the Emergency Psychiatric Unit, Department of Psychiatry, University of Geneva (EPU patients). The study protocols were approved by the respective institutional review boards.

In the ADU series, 22 consecutive eligible outpatients 18 years or older were recruited, examined, and treated by three research psychiatrists (VP, DP, and EA), with high inter-rated reliability and good internal consistency (Alvarez *et al*, 1997). Each patient was rated by the same clinician along the study. The inclusion criteria were: (1) a diagnosis of major depressive disorder according to DSM-IV criteria (APA, 1994), single episode (diagnostic code 296.2, 14 patients), or recurrent episodes (diagnostic code 296.3, eight patients); (2) absence of any active antidepressant treatment during a minimum of 2 months before admission;

and (3) provision of informed consent after complete description of the study. All selected depressed patients (20 women, two men, mean age \pm SD: 42 ± 3 years) were free of other psychiatric disorders (except personality disorder, $n = 2$, which was assessed by medical history and clinician's judgment) or severe organic pathology, and were permitted their regular habits but were warned not to use psychotropic drugs, except for moderate doses of prescribed benzodiazepines. The baseline clinical ratings (the 17-item Hamilton Rating Scale for Depression, HRSD, and the Montgomery-Åsberg Rating Scale for Depression, MARSD) and blood sampling were performed on the same day and before the onset of mirtazapine treatment. The mean global score on the HRSD was 19 ± 2 (range, 18–24, $n = 22$) and on the MARSD was 26 ± 4 (range, 20–33, $n = 22$). The mean duration of the depressive episode was 3.1 ± 0.8 months. Whenever possible, depressed patients were studied again after treatment with mirtazapine (30–45 mg/day for an initial period of 6 months). Clinical ratings and biochemical assays were repeated 1 ($n = 22$), 4 ($n = 21$), 12 ($n = 16$), 24 ($n = 12$), and 28 ($n = 12$) weeks after the onset of treatment. Plasma concentrations of mirtazapine were monitored by a HPLC/ultraviolet detection method at 1, 4, 12, and 24 weeks, and were found in the therapeutic range (Dodd *et al*, 2000). At week 24, mirtazapine was substituted by placebo during 4 weeks (study end point: 28 weeks) to assess the clinical and biochemical effects of drug withdrawal. Safety was evaluated by the assessment of treatment-emergent adverse events, routine clinical laboratory tests, and vital signs. Therapeutic response was defined as a 50% reduction in the baseline HRSD, and clinical remission as a reduction of the score to 9 or below. In all, 12 depressed patients completed the 6-month treatment phase, and showed a good therapeutic response and remission of the depressive episode. Mirtazapine withdrawal (4 weeks) did not induce reappearance of depressive symptoms. Four patients did not respond to mirtazapine treatment and were excluded from the study (from week 4 to 12), two patients were withdrawn for excessive drug side effects (anxiety, weight-gain), and four patients abandoned the study for personal reasons. In all, 15 normal volunteers who were matched with one or two of the selected patients for sex and as closely as possible for age (± 4 years) were also studied. The healthy controls (13 women, two men, mean age: 43 ± 14 years) were recruited among hospital employees and written informed consent was also obtained. Control subjects were interviewed by a research psychiatrist to exclude those with a history of psychiatric disorders, medical illness, or drug or alcohol abuse. Control subjects were drug free for a minimum of 2 weeks before the assays, and only one blood sample was obtained.

In the EPU series, 32 consecutive eligible hospitalized suicide attempters 18 years or older were recruited and examined by two research psychiatrists (GR and AA). The inclusion criteria were: (1) an immediate suicide attempt, defined as a self-destructive act carried out with at least some intent to end one's life, which required hospitalization for medical treatment of the sequelae of the attempt; (2) a DSM-IV diagnosis of major depressive disorder (single episode, 31 patients; recurrent episodes, one patient); (3) absence of antidepressant treatment during a minimum of 2 weeks before admission; and (4) provision of informed

consent after description of the study. All selected depressed suicide attempters (27 women, five men, mean age \pm SD: 34 ± 10 years) were free of other psychiatric disorders (except personality disorder, $n = 19$, which was assessed by medical history and clinician's judgment) or severe organic pathology. The methods of suicide attempts were drug overdose ($n = 28$ with positive urine toxicology, including antidepressant drugs, $n = 7$, and benzodiazepines plus ethanol, $n = 18$) and venesection ($n = 4$). Suicidal ideation and the actual risk of suicidal behavior (RSB) at the time of hospital admission was assessed with an adaptation of the Suicidal Thinking and Behavior Questionnaire (Chiles *et al*, 1989), where the scale scores from 0 (low risk, 0–2) to 5 (high risk, 3–5). The mean global score for RSB was 2.8 ± 1.6 (range, 0–5, $n = 32$). The mean global score on the HRSD was 25 ± 5 (range, 13–34, $n = 32$). A total of 17 normal volunteers who were matched with one or two of the selected patients for sex and as closely as possible for age (± 5 years) were also studied. The healthy controls (14 women, three men, mean age: 34 ± 10 years) were recruited among hospital employees and screened as above.

Platelet Samples And Membrane Preparation

Venous blood (35 ml) was obtained by venipuncture (fasting subjects, 0830–1000) and the platelets were immediately isolated by centrifugation on a specific cell separation medium as described previously (García-Sevilla *et al*, 1997). The pellets of the platelets were stored at -80°C until assays. Crude platelet membranes (and protein content) were prepared for the immunodetection of the target proteins as reported in detail previously (García-Sevilla *et al*, 1997, 1999).

Immunoblot Assays And Quantitation Of Target Protein Contents

The relative densities of platelet α_{2A} -adrenoceptors and associated regulatory proteins were assessed by quantitative immunoblotting by using specific polyclonal antibodies and in-gel standard curves as described previously (García-Sevilla *et al*, 1997). In brief, solubilized target membrane proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (6×8 -cm minigels, 15 wells, Mini 2-D cell, Bio-Rad Laboratories, Hercules, CA, USA), transferred to nitrocellulose membranes (Western blotting),

labeled with the specific primary antibody (overnight at 4°C), and subsequently incubated for 2 h at room temperature in a blocking solution containing the secondary antibody, horseradish peroxidase-linked donkey anti-rabbit IgG antiserum (1:5000 dilution). The following primary antibody were used: anti- α_{2A} -adrenoceptor, 1:500 dilution (produced and characterized in this laboratory (Ozaita *et al*, 1999; García-Sevilla *et al*, 1999); anti-G α i1/2, 1:7000 dilution (AS/7, Du Pont-NEN Research Products, Boston, MA, USA), anti-GRK 2, 1:5000 dilution (C-15, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-GRK 6, 1:1000 dilution (C-20, Santa Cruz Biotechnology), and anti-PP-2A catalytic subunit, 1:20 000 dilution (clone 46, Transduction Laboratories, Lexington, KY, USA). Bound antibody (immunoreactivity) was detected using the enhanced chemiluminescence Western blot detection system (Amersham International, Little Chalfont, Buckinghamshire, UK) and visualized by the exposure to autoradiographic film (Amersham ECL Hyperfilm) for 1–15 min (autoradiograms). The autoradiograms were quantitated by densitometric scanning (GS-700 Imaging Densitometer, resolution: $42 \mu\text{m}$, Bio-Rad Laboratories) by measuring the integrated optical density (IOD) of the immunoreactive bands. In previous studies, the antibodies used had been tested and characterized in human platelets and/or human and rat brain tissue, and labeled bands with molecular masses of 70 kDa (mature α_{2A} -adrenoceptor), 40 kDa (G α i2 subunit), 80 kDa (GRK 2), 66 kDa (GRK 6), and 36 kDa (PP-2A) (García-Sevilla *et al*, 1997, 1999; Ozaita *et al*, 1999; Grange-Midroit *et al*, 2002, 2003).

The procedure for the quantitation of target proteins in human platelet and brain membranes by immunoblotting has been described in detail previously (García-Sevilla *et al*, 1997; Grange-Midroit *et al*, 2003). Briefly, two platelet samples of different protein content, in duplicate or triplicate, from one or two depressed patient (eg drug free and after mirtazapine treatment) were evaluated using standard curves (ie total protein loaded *vs* IODs), which consisted of five points of different protein content (resulting in a linear relationship) of a control subject matched for sex and age, all loaded on the same gel (see Figure 1 for quantitation of GRK 2). Given a known amount of protein from a problem sample loaded in the gel well (PR, the real amount), the percentage change with respect to control samples was calculated as the ratio between the amount of protein corresponding to the IOD value of the

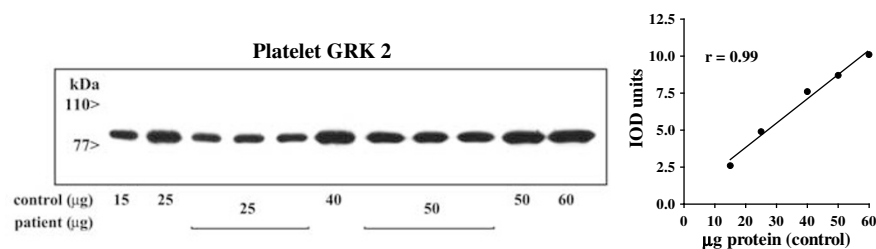


Figure 1 Autoradiograph of a Western blot depicting labeling of immunodetectable GRK 2 in platelet membranes of a patient with major depressive disorder (46-year-old woman) and a matched-control subject (45-year-old woman). The standard curve (right), constructed with samples from the control subject (15–60 μg protein), was used to quantitate the content of GRK 2 (25 and 50 μg protein in triplicates) in the depressed patient (mean value for GRK 2: 46% of matched control). The molecular mass of GRK 2 (80 kDa) was determined by calibrating the blot with prestained molecular weight markers as shown on the left-hand side.

problem sample interpolated in the control standard curve (PT, the theoretical amount) and PR. Thus, the percentage change is $(PT/PR) \times 100$, where this value is 100 for a control sample used as the standard. Initially, the target proteins in depressed patients (ADU series) were quantitated in the same gel at baseline (drug free) and 1 and 4 weeks after mirtazapine treatment. Then the proteins were quantitated 12, 24, and/or 28 weeks after treatment in different gels, which always included baseline samples from the corresponding depressed patient. For representative immunoblots (Figure 3), new experiments were performed that included all samples from the chosen patient. In a selected group of depressed patients (drug free or treated with mirtazapine), the density of α -tubulin, used a negative control, was not changed compared with the content in matched controls (data not shown) (García-Sevilla et al, 1997).

Data Analysis

All series of data were analyzed with the program GraphPad Prism™, version 3.0, and all of them followed Gaussian distributions (Kolmogorov–Smirnov normality test). Results are expressed as mean values \pm SD (text) or SEM (figures). The one-sample *t*-test (identical in its implementation to the paired *t*-test), direct paired *t*-test, or analysis of variance (ANOVA) with repeated measures was used for the statistical evaluations. In the 12 patients who completed the treatment, ANOVA with repeated measures (before, during, and after mirtazapine) allowed an assessment of drug effects (basal vs protein change in immunoreactivity) and time (changes with the duration of treatment). Significant interactions revealed by ANOVA were further examined with Bonferroni's multiple comparison test to determine when significant effects occurred and the magnitude of the changes. Changes in behavioral rating (score on the HRSD) were assessed by the χ^2 test. Pearson's correlation coefficients were calculated to test for possible association among variables (eg relation between platelet GRK 2 and behavioral rating). The level of significance was chosen at $p \leq 0.05$. All tests were two-tailed.

Drugs and Materials

Mirtazapine (Rexer^R) and placebo were prepared by the manufacturer (Akzo Nobel, Organon Española, S.A., Barcelona, Spain). Acrylamide (Protogel) was from BDH Brunswick (Dorset, UK). Nitrocellulose membranes were from Schleicher & Schuell GmbH (Dassel, Germany). Secondary antibody, chemiluminescence reagents, and film for autoradiography were from Amersham International. Other chemicals were from Sigma Chemie (Buchs, Switzerland).

RESULTS

α_{2A} -Adrenoceptors and Associated Regulatory Proteins in Patients with Major Depressive Disorder

In platelet membranes of drug-free depressed patients (ADU series), the immunodensities of α_{2A} -adrenoceptors and G α i2 coupling proteins were increased (mean \pm SD:

$49 \pm 37\%$, $n = 22$, $t = 5.86$, $p < 0.001$, and $35 \pm 28\%$, $n = 22$, $t = 5.68$, $p < 0.001$, respectively) compared with those of age- and sex-matched controls. In contrast, the content of regulatory receptor kinase GRK 2 was decreased (mean \pm SD: $27 \pm 28\%$, $n = 22$, $t = 4.30$, $p < 0.001$) compared with the controls (Figures 2 and 3; for GRK 2 see also individual values in Figure 4). Platelet GRK 2 was not altered in the two depressed men studied (decreases of

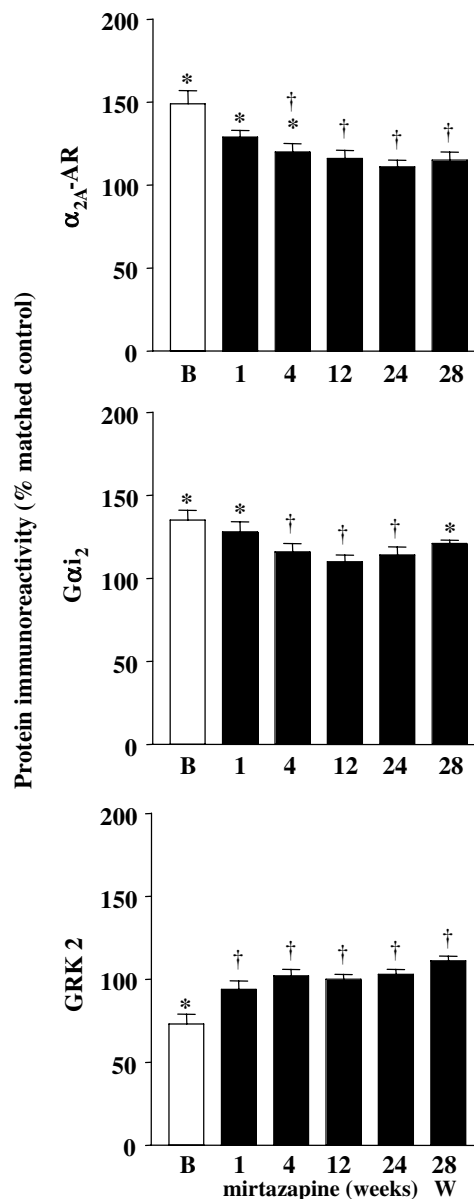


Figure 2 Immunodensities of α_{2A} -adrenoceptors (α_{2A} -AR), guanine nucleotide-binding proteins (G α i2) and GRK 2 in platelet membranes of patients with major depressive disorder before (B, basal drug-free values, $n = 22$), during mirtazapine treatment (1–24 weeks, $n = 22-12$), and after drug withdrawal (W, $n = 12$). Data are expressed as means \pm SEMs (bars) percentages of the contents in matched controls. * At least $p < 0.05$ when compared with the corresponding protein density in matched controls (one-sample *t*-test). In the 12 patients who completed the antidepressant treatment (24 weeks) and the period of drug withdrawal (4 weeks), ANOVA with repeated measures detected significant drug effects on α_{2A} -AR ($F = 4.35$, $p = 0.002$), G α i2 ($F = 5.85$, $p = 0.0002$), and GRK 2 ($F = 4.98$, $p = 0.0006$). † At least $p < 0.05$ when compared with basal (B) values (ANOVA followed by Bonferroni's multiple comparison test).

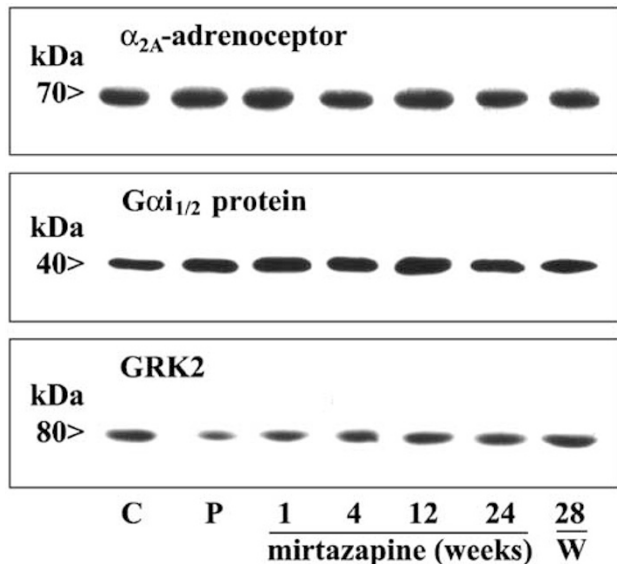


Figure 3 Representative immunoblots of α_{2A} -adrenoceptors, guanine nucleotide-binding proteins ($G\alpha_{i2}$) and GRK 2 in platelet membranes of a patient (32-year-old woman) with major depressive disorder before (P, basal drug-free values), during mirtazapine treatment (1–24 weeks) and after drug withdrawal (W). Matched-control subject (C, 31-year-old woman). The molecular masses of α_{2A} -adrenoceptors (70 kDa), $G\alpha_{i2}$ (40 kDa), and GRK 2 (80 kDa) were determined by calibrating the blots with pre-stained molecular weight markers as shown on the left-hand side. Note that in the depressed patient the basal immunodensities (P vs C) of α_{2A} -adrenoceptors and $G\alpha_{i2}$ proteins were increased (31 and 22%, respectively) and that of GRK 2 was markedly decreased (54%). Mirtazapine treatment progressively reversed these changes toward basal values (weeks 12–24). Substitution of mirtazapine by placebo (W, 4 weeks) did not alter the immunodensities of the target proteins.

9 and 3%). The changes in immunoreactive proteins were significant both in patients with a single depressive episode ($n=14$) and in those with the recurrent type of depression ($n=8$) (data not shown). Moreover, protein changes were similar in depressed patients with low suicidal ideation ($n=16$) and in those with moderate suicidal behavior ($n=6$) (item 3 of the HRSD) (data not shown).

For the 22 patients, the severity of depression on the HRSD (total score) correlated inversely with the immunodensity of platelet GRK 2 ($r=-0.46$, $n=22$, $F=5.28$, $p=0.032$) (Figure 4). A similar negative correlation between clinical behavioral ratings and platelet GRK 2 was also obtained when the severity of depression (total score) was measured with the Montgomery-Åsberg rating instrument ($r=-0.55$, $n=22$, $F=5.70$, $p=0.009$) (not shown). In contrast, no statistically significant correlation was observed between the density of α_{2A} -adrenoceptors ($r=0.08$, $n=22$, $p>0.05$) or $G\alpha_{i2}$ proteins ($r=-0.11$, $n=22$, $p>0.05$) and the severity of depression (HRSD). Platelet α_{2A} -adrenoceptors correlated weakly with $G\alpha_{i2}$ proteins ($r=0.38$, $n=22$, $p=0.08$) or GRK 2 ($r=0.22$, $n=22$, $p=0.33$) in the same samples of depressed patients. A multiple regression analysis between GRK 2 enzyme (dependent variable) and α_{2A} -adrenoceptors and $G\alpha_{i2}$ proteins (independent variables) also resulted in a weak positive relationship ($r=0.41$, $n=22$, $p=0.18$).

A second series of depressed patients who had attempted suicide (EPU series) was studied to assess the relevance of

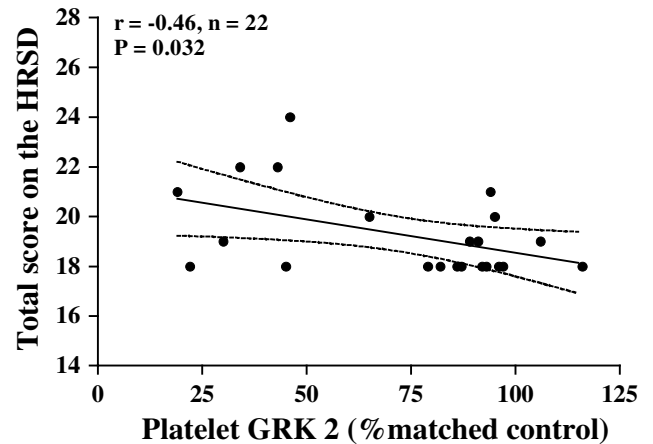


Figure 4 Scatterplot depicting a significant inverse correlation between the immunodensity of GRK 2 (as percentages of matched controls) in platelet membranes of patients with major depressive disorder and the severity of depression (HRSD, total score on the Hamilton rating scale for depression). Each circle represents a different depressed patient and the solid line ($y=21.2-0.027x$) is the best fit of the correlation ($r=-0.46$, $F=5.28$, $n=22$, $p=0.032$). The dotted curves indicate the 95% confidence interval for the regression line.

the unexpected observed decrease in the content of platelet GRK 2 in major depression. In this group of depressed suicide attempters, the immunodensity of platelet GRK 2 was also found decreased (mean \pm SD: $28 \pm 37\%$, $n=32$, $t=4.37$, $p=0.0001$) when compared with that of age- and sex-matched controls (Figure 5). Two patients exhibited a very high (176%) or a very low (13%) density of platelet GRK 2 corresponding to 2.8 SD above and 1.6 SD below the mean of the depressed group (Figure 5). After removal of these two patients, the decrease of platelet GRK 2 was similar: $30 \pm 31\%$ ($n=30$, $t=5.33$, $p<0.0001$). However, no experimental factors were identified that would justify excluding these observations (the patients were tested twice with similar results using different matched controls). The decrease of platelet GRK 2 in depressed suicide attempters was more pronounced in women ($30 \pm 36\%$, $n=27$, $t=4.39$, $p=0.0002$) than in men ($18 \pm 43\%$, $n=5$, $t=0.91$, $p=0.41$), although no significant difference was found between sexes ($t=0.70$, $df=30$, $p=0.49$). Moreover, the decreases of platelet GRK 2 were similar in high-risk ($32 \pm 37\%$, $n=18$, $t=3.72$, $p=0.002$) and in low-risk ($23 \pm 37\%$, $n=14$, $t=2.34$, $p=0.036$) suicide attempters. The downregulation of platelet GRK 2 was also similar in depressed suicide attempters with ($35 \pm 31\%$, $n=19$, $t=4.94$, $p<0.0001$) or without ($25 \pm 40\%$, $n=13$, $t=2.24$, $p=0.04$) a concomitant diagnosis of personality disorder ($t=0.77$, $df=30$, $p=0.45$). For the 32 patients, the severity of depression on the HRSD (total score) also correlated inversely, but weakly, with the immunodensity of platelet GRK 2 ($r=-0.27$, $n=32$, $p=0.13$) (not shown). Suicidal ideation (score on the RSB) did not correlate with platelet GRK 2 ($r=0.08$, $n=32$, $p=0.65$).

In depressed suicide attempters, the immunodensity of platelet GRK 6 (a receptor kinase that belongs to a different subgroup of the GRK family) was not significantly different from that in age- and sex-matched controls ($7 \pm 24\%$ decrease, $n=32$, $t=1.68$, $p=0.10$) (Figure 5). Similarly, the

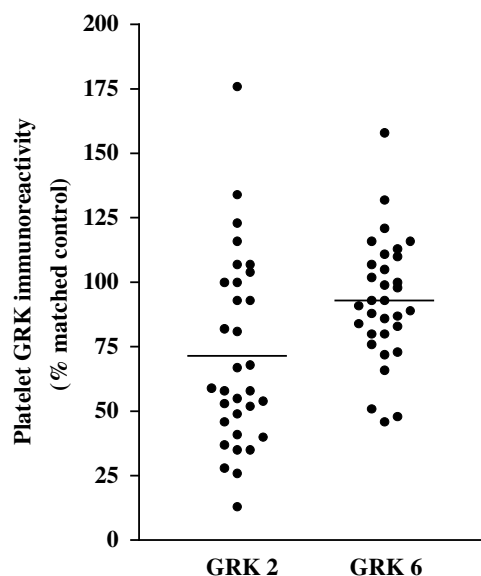


Figure 5 Immunodensities of GRK 2 and GRK 6 in the same platelet membranes of depressed suicide attempters. Each circle represents a different depressed patient. The lines represent the mean values of the groups (GRK 2: $72 \pm 37\%$, $n=32$, $t=4.37$, $p=0.0001$; GRK 6: $93 \pm 24\%$, $n=32$, $t=1.68$, $p=0.10$ when compared with matched controls by one-sample t -test).

abundance of phosphatase type PP-2A in the platelets did not differ from that in the controls ($7 \pm 28\%$ decrease, $n=29$, $t=1.37$, $p=0.18$). In these patients, however, there were positive and significant correlations between the contents of GRKs and PP-2A in the same platelet samples (for GRK 2: $r=0.45$, $n=29$, $F=6.72$, $p=0.015$; for GRK 6: $r=0.38$, $n=29$, $F=4.57$, $p=0.042$). In depressed suicide attempters, clinical behavioral ratings (HRSD and RSB) did not correlate significantly with platelet GRK 6.

α_{2A} -Adrenoceptors and Associated Regulatory Proteins During and After Mirtazapine Treatment

Treatment of depressed patients with mirtazapine (30–45 mg/day for 1–24 weeks) induced a time-dependent regulation of the initially abnormal immunodensities of target proteins in the platelets toward basal values (Figures 2 and 3). Thus, mirtazapine treatment for 4 and 24 weeks was associated with the downregulation of α_{2A} -adrenoceptors (percentage change \pm SD: $-29 \pm 41\%$, $n=21$, $t=3.22$, $p<0.005$, and $-34 \pm 42\%$, $n=12$, $t=2.74$, $p<0.02$ by paired t -test) and Gzi2 proteins ($-20 \pm 23\%$, $n=21$, $t=4.25$, $p<0.001$, and $-28 \pm 24\%$, $n=12$, $t=3.92$, $p<0.005$), and with the upregulation of GRK 2 ($27 \pm 27\%$, $n=21$, $t=4.17$, $p<0.001$, and $30 \pm 24\%$, $n=12$, $t=4.01$, $p<0.005$) (Figure 2). During the treatment (1–24 weeks), the plasma concentrations of mirtazapine were found in the therapeutic range (22.4 ± 7.2 – 35.7 ± 13.6 ng/ml). In most patients, mirtazapine treatment was also associated with good clinical response (HRSD \pm SD, basal: $19 \pm 2\%$, $n=22$; 4-week treatment: $11 \pm 4\%$, $n=21$, $p<0.001$; 24-week treatment: $6 \pm 6\%$, $n=12$, $p<0.05$). Substitution of mirtazapine by placebo (4 weeks) in remitted patients with major depression ($n=12$) was not associated with the reappearance of depressive

symptoms (HRSD at week 28: $4 \pm 1\%$, $n=12$), or any other serious withdrawal reaction or with alterations in the immunodensities of target proteins in the platelets (Figure 2).

DISCUSSION

The results of the first part of the study indicate the existence of an increased density of the α_{2A} -adrenoceptor/Gzi protein complex in blood platelets of drug-free depressed patients, which is in agreement with previous reports in postmortem brains (Callado *et al*, 1998; García-Sevilla *et al*, 1999; Ordway *et al*, 2003) and platelets (García-Sevilla *et al*, 1981, 1986, 1987, 1990; Piletz *et al*, 1991; Gurguis *et al*, 1999) from depressed subjects. Therefore, these results further support the existence of supersensitive α_{2A} -adrenoceptors in major depression (Fu *et al*, 2001; González-Maeso *et al*, 2002; see also Flügge *et al*, 2003). Unexpectedly and in marked contrast to previous observations in the brains of drug-free depressed suicides (García-Sevilla *et al*, 1999; Grange-Midroit *et al*, 2003), the content of platelet GRK 2 was found reduced in drug-free depressed patients. In fact, the combined results of the two series of patients investigated (ADU and EPU) clearly indicate that the abundance of platelet GRK 2 is decreased ($28 \pm 34\%$, $n=54$, $p<0.0001$) in major depression. The similar results obtained in the ADU (drug-free patients; at least 2 months) and EPU (suicide attempters mainly by drug overdose) series of depressed patients indicate that possible acute drug effects (EPU series, for example, cyclic antidepressant drugs) were not involved in the decrease of GRK 2 (see Miralles *et al*, 2002). Moreover, the reduced platelet GRK 2 was not related to suicidal behavior, but it showed a clear tendency to correlate inversely with the severity of depression (total score on the HRSD or MARSD). In this context, GRK 6 (a GRK with different properties) and protein phosphatase PP-2A (involved in receptor resensitization) (Pitcher *et al*, 1998) were found unchanged in the platelets of depressed patients (current results) and in the brains of depressed suicides (Grange-Midroit *et al*, 2003), suggesting some specificity for GRK 2 in major depression.

Recent functional genomic approaches (ie changes in gene expression after methamphetamine treatment in rats using oligonucleotide GeneChip microarrays) have identified several novel candidate genes that may be involved in mood disorders or schizophrenia (Niculescu *et al*, 2000). Interestingly, one of these induced genes coded for GRK 3 and the immunodensity of this kinase (a close homolog of GRK 2) was shown to be decreased in lymphocytes from a small group of subjects ($n=4$) with bipolar disorder (Niculescu *et al*, 2000). In this context, the observed reduction in the content of platelet GRK 2 in depressed patients could suggest that a defect in the transcriptional regulation of GRK 2 may contribute to the upregulation of α_{2} -adrenoceptors and/or to the pathogenesis of major depression (ie inability of GRK to desensitize the receptor and hence a greater inhibitory response of norepinephrine). As mentioned, however, an increased expression of GRK 2/3 was observed in the prefrontal cortex of drug-free depressed suicides (García-Sevilla *et al*, 1999; Grange-Midroit *et al*, 2003), and most probably this is an adaptive mechanism to

compensate the abnormal higher functioning of some receptors in the brain (eg homologous regulation of the α_2 -adrenoceptor/G α_i complex) (García-Sevilla *et al*, 1999). In the current study, the decrease of platelet GRK 2 in depressed patients was more pronounced in women than in men, suggesting that hormonal factors could also play a role. Thus, treatment of female rats with estradiol reduced protein content, enzyme activity, and messenger RNA of GRK 2 in the hypothalamus (Ansonoff and Etgen, 2001). On the other hand, the platelet aggregation (induced by epinephrine) and reactivity are increased in depressed patients (García-Sevilla *et al*, 1986, 1990; Musselman *et al*, 1996, 2000), and activated platelets produce reactive oxygen species, such as hydrogen peroxide (Maresca *et al*, 1992) that can modulate the expression of GRKs. Thus, the exposure of lymphocytes to hydrogen peroxide resulted in marked decreases in GRK 2 expression and in agonist-induced desensitization of β_2 -adrenoceptors (Lombardi *et al*, 2002). This latter mechanism could also explain the observed decrease of GRK 2 and upregulation of α_{2A} -adrenoceptors in the platelets of depressed patients. Clearly, further studies are still needed to clarify the molecular mechanisms of the reduced platelet GRK 2 in major depression.

The results of the second part of the study indicate that the treatment of depressed patients with mirtazapine (1–24 weeks) was associated with good clinical response and with a time-dependent downregulation of the complex α_{2A} -adrenoceptor/G α_i proteins and with the upregulation of GRK 2 in the platelets. The antidepressant mirtazapine (Holm and Markham, 1999; Delgado *et al*, 2002) is a potent α_{2A} -adrenoceptor antagonist that enhances the release of norepinephrine (acting at α_{2A} -autoreceptors) and serotonin (acting at α_2 -heteroreceptors) in nerve terminals (Millan *et al*, 2000; Haddjeri *et al*, 1997). Therefore, the observed clinical efficacy and downregulation of α_{2A} -adrenoceptors induced by mirtazapine also support a relevant role of supersensitive α_{2A} -adrenoceptors in the pathogenesis of major depression (Fu *et al*, 2001; González-Maeso *et al*, 2002). Chronic antidepressant treatments have been repeatedly shown to induce downregulation and desensitization of α_2 -adrenoceptors and/or G α_i proteins in the platelets of depressed patients (García-Sevilla *et al*, 1986, 1987, 1990, 1997; Piletz *et al*, 1991; Gurguis *et al*, 1999), and in the rat (Smith *et al*, 1981; Barturen and García-Sevilla, 1992; Kovachich *et al*, 1993; Mongeau *et al*, 1994; Esteban *et al*, 1999; Mateo *et al*, 2001) and human (De Paermentier *et al*, 1997; García-Sevilla *et al*, 1999) brains. Similar to other antidepressant drugs, mirtazapine could also induce the downregulation of platelet α_2 -adrenoceptors, indirectly, through the rise in plasma norepinephrine induced by the drug. In healthy subjects and in depressed patients, the α_2 -adrenoceptor antagonists yohimbine and idazoxan have been shown to increase the plasma concentrations of norepinephrine (Osman *et al*, 1989; Andrews *et al*, 1999) and of its main neuronal metabolite, dihydroxyphenylglycol (Andrews *et al*, 1999), indicating significant blockade at presynaptic $\alpha_{2A/C}$ -adrenoceptors in peripheral adrenergic nerves. Concomitantly, yohimbine reduced the orthostatic (upright posture) increase in the platelet aggregation response induced by the increased norepinephrine, also indicating blockade of α_{2A} -adrenoceptors in the platelets

(Andrews *et al*, 1999). Similar results should be expected after the systemic administration of mirtazapine, a more selective and potent α_{2A} -adrenoceptor antagonist (Millan *et al*, 2000).

Mirtazapine treatment in depressed patients (1–24 weeks) was also associated with a rapid and sustained upregulation of platelet GRK 2, which closely paralleled the downregulation of α_{2A} -adrenoceptors, suggesting that the expression of these proteins are regulated in a concert manner by the antidepressant. Thus, mirtazapine treatment would reverse, through direct or indirect mechanisms (see above), the basal deficit of platelet GRK 2 to finally allow the homologous regulation of the α_{2A} -adrenoceptor. However, during receptor desensitization and downregulation, GRKs are in turn subjected to complex regulatory mechanisms (eg increased activity of GRK 2 by its phosphorylation by other kinases) (Kohout and Lefkowitz, 2003), which also could be involved in the observed upregulation of GRK 2 induced by mirtazapine. Future studies should clarify whether other antidepressant drugs (eg cyclic antidepressants) are also able to reverse, and by which mechanism, the reduced expression of GRK 2 in the platelets of depressed patients.

In conclusion, the results of this study demonstrate that major depression is associated with increased α_{2A} -adrenoceptor/G α_i complex and decreased receptor kinase GRK 2 in blood platelets. This suggests that a defect in the regulation of platelet GRK 2 may contribute to the upregulation of α_{2A} -adrenoceptors. In drug-free depressed patients, the severity of depression correlated inversely with the expression of platelet GRK 2. Treatment with the α_2 -adrenoceptor antagonist mirtazapine was associated with good clinical response and downregulation of α_{2A} -adrenoceptor/G α_i complex and upregulation of GRK 2 in the platelets. Together, the results support a role of supersensitive α_{2A} -adrenoceptors in the pathogenesis and treatment of major depression.

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