# Effects of a putative antidepressant with a rapid onset of action in defeated mice with different coping strategies

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## ABSTRACT

There is evidence suggesting that stressful social events may result in depressive-like disorders, but the development of these disorders depend on the way in which people cope with stress. Although antidepressants are useful their drawback is a delay in the therapeutic effects, moreover not all the patients show an adequate response to this treatment. The aim of this study was to analyse the effect of RS 67333, which is a 5-HT4 receptor partial agonist and a putative antidepressant which exhibits a rapid onset of action and to determine whether this drug reverses the behavioural and physiological effects that are generated by chronic defeat in subjects who manifest a more vulnerable profile in their response to stress. Male mice were exposed to defeat for 21 consecutive days using a sensorial contact model. After 18 days of defeat, 2 groups of subjects were established, active and passive, in accordance with the behaviour that was manifested during social confrontation, and drug treatment was initiated for 5 days. Finally, the animals were subjected to a forced swimming test (FST). The results revealed higher corticosterone levels in passive mice after the last defeat. Additionally, 3 days after the last defeat, they showed lower corticosterone levels and higher splenic IL-6 and TNF-α levels and hypothalamic GR mRNA levels when compared to their active and manipulated control counterparts. Passive mice had higher 5-HT1A receptor mRNA levels than the manipulated controls and a lower MR/GR ratio than active mice. Similar to stress, the drug increased hypothalamic GR mRNA levels, but it did not affect other measured physiological variables or social behaviour, which suggested that the mechanism of this drug is not the most adequate for reversing stress-induced effects in this model. Nevertheless, the treatment increased swimming and decreased immobility in the FST, suggesting an antidepressant potential for this drug.

Keywords: Coping strategies in social defeat, FST, Glucocorticoid receptors, RS 67333, Serotonergic receptors

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## 1. Introduction

Chronic social stress is considered a trigger factor for many affective disorders, including major depression. Research has demonstrated that stressful life events generate a series of behavioural and psychological effects that are similar in nature to the symptoms that are manifested by patients who are diagnosed with depression. As has been observed in depression, chronic social stress produces an increase in the release of proinflammatory cytokines such as interleukin-6 (IL-6) and tumour necrosis factor alpha (TNF-α) at a peripheral (Avitsur et al., 2003; Bailey et al., 2009; Stark et al., 2001) and central level (Wohleb et al., 2011), alterations in the hypothalamic pituitary adrenal (HPA) axis at a number of different levels (Blanchard et al., 1995; Buwalda et al., 1999, 2001) and changes in monoaminergic transmission (Van Praag, 2004). These alterations that are caused by stressful events can be considered biomarkers of depression (Dowlati et al., 2010; Hirschfeld, 2000; Howren et al., 2009; Lopez-Duran et al., 2009), and can be reversed by the administration of antidepressant treatments (Beitia et al., 2005; Reul et al., 1993; Wu et al., 2011).

Selective serotonin reuptake inhibitors (SSRIs), which are the most commonly prescribed antidepressants, may reestablish the activity of the HPA axis, probably by increasing the functionality of the glucocorticoid receptors (GRs) (Carvalho and Pariante, 2008; Pariante et al., 2004), and this neuroendocrine improvement is deemed necessary for clinical remission (Binder et al., 2009; Heuser et al., 1996; Ising et al., 2007; Ribeiro et al., 1993). SSRIs may also reestablish secretion patterns for proinflammatory cytokines such as IL-6 and TNF-α (Hannestad et al., 2011; Kubera et al., 2011).

However, a drawback of SSRIs is that their therapeutic effects are observed after a few weeks and, on occasions, after several months of treatment. This delay may correspond to the time that is required for these drugs to desensitise the 5-HT1A receptors at a presynaptic and postsynaptic level, thus increasing the release of serotonin, which produces the antidepressant effect (Berton and Nestler, 2006; Blier and De Montigny, 1994; Duman et al., 1997). For this reason, the search for fast-acting antidepressants is currently one of the top priorities within the field of biomedicine. Recently, 5-HT4 receptors have been the object of much attention with respect to their involvement in psychopathologies, and behavioural and neurochemical studies indicate that these receptors are involved in affective disorders and their treatments (Bijak et al., 1997, 2001; Duman, 2007; Vidal et al., 2009). Thus, it has been proposed that 5-HT4 receptor agonism may constitute a new fast acting antidepressant mechanism because it has been observed that these receptors exert excitatory control over the activity and firing of serotonergic neurons that are located in the dorsal raphe nucleus (Lucas and Debonnel, 2002; Lucas et al., 2005). In support of this proposal, various studies using animal models of depression have found that the administration of 5-HT4 agonists induces the same functional, morphological, molecular and behavioural changes as conventional antidepressants but within a shorter period of time (Licht et al., 2010; Lucas et al., 2007; Pascual-Brazo et al., 2011). Nevertheless, the effect of these agonists on the endocrine and immune alterations that are involved in depression has yet to be studied.

Despite the evident relationship among chronic social stress, the development of affective disorders and the validity of available treatments, it is important to highlight that individual differences exist in the way which people cope with stress and how they respond to these treatments. Many animal studies have found that individuals differ considerably in the manner in which they respond to stress and show a large degree of behavioural and physiological variability (Bartolomucci et al., 2005; Koolhaas et al., 1999; Veenema et al., 2003). For example, previous studies conducted in our laboratory have shown that mice that adopt a passive behavioural profile in response to chronic defeat-induced stress have higher levels of IL-6 and TNF-α in the spleen

than those who adopt a more active profile. Additionally, these individuals respond to stress with higher glucocorticoid levels after defeat and lower levels of this hormone when the chronic social stress ceases (Gómez-Lázaro et al., 2011). Nevertheless, the physiological mechanism involved in the alteration of the HPA axis, which was only observed in passive subjects, has yet to be determined. These different biological patterns in response to stress may be related to the fact that not all individuals respond similarly to drug treatments.

The aim of this study was to analyse the effects of administering a 5-HT4 receptor partial agonist, RS 67333, which has been described as a putative class of antidepressant with a rapid onset of action (Licht et al., 2010; Lucas et al., 2007; Pascual-Brazo et al., 2011), using a social stress model in mice. We hypothesised that a 3 and 5-day treatment with RS 67333 could reverse chronic defeat-induced HPA axis alterations in mice with a passive coping strategy and that this reversion may be mediated by hypothalamic GRs. Additionally, we expected the treatment to reduce the levels of the proinflammatory cytokines, IL-6 and TNF-α in the spleen and desensitise the 5-HT1A and 5-HT4 receptors in the hippocampus. Finally, we aimed to study the effectiveness of the treatment on the behavioural changes that are generated by chronic stress and on the behaviour that is manifested in the FST, which is the most widely used screening test for the antidepressant potential of novel compounds (Cryan and Holmes, 2005; Hunter et al., 2000; Nestler et al., 2002).

## 2. Methods

### 2.1. Animals

OF1 outbred mice, which are characterised by their aggressive behaviour, were used. One hundred and eighty, 6-week-old male mice (Charles River, Oncins, France) were individually housed for 7 days in transparent plastic cages that measured 24.5×24.5×15 cm. Food and water were available ad libitum, and the holding room was maintained at a constant temperature of 20 °C with a reversed 12-h light/ dark cycle (white lights on from 20:00 to 08:00 h) to enable the nocturnal animals to be tested during their active phase, which was 1 h after the dark cycle began. All experimental procedures were conducted under dim red light conditions in a room that was adjacent to the holding facility. All procedures involving mice were carried out according to the European Directive (2010/63/EU) on the protection of animals used for scientific purposes (22 September 2010). The procedures were approved by the Ethical Committee for animal welfare of the Basque Country University (CEBA).

### 2.2. Experimental procedures

After a period of adaptation (7 days), a control group (i.e., manipulated control) and a group of socially stressed mice were established. Next, a social stress period was initiated, which lasted for 21 consecutive days. After the end of the social stress period, the FST (Porsolt et al., 1977) was performed over 2 days for all mice. The socially stressed group was divided into two subgroups (active and passive) according to the behavioural profiles that weremanifested during defeat on day 18 (see below). The drug or vehicle treatment was initiated the next day and continued until the end of the experiment, which was 5 days later (days 19, 20 and 21 of chronic social stress and days 1 and 2 of the FST). The manipulated control group was also divided into drug and vehicle control groups. Therefore, a total of six groups were obtained. Three days before the experiment began and on day 21 of social stress, blood samples (50–100 μl) were collected from stressed mice (45 min after social defeat) andmanipulated controls by submandibular puncture. This new method, which was developed by Golde et al. (2005), allows researchers to obtain a sufficient volume of blood from the submandibular vein in a short time while holding the mouse and without the use of anaesthesia. Mice were euthanised by cervical dislocation 24 h after the second swimming session, which corresponded to 72 h after the last defeat experience.

Bloodwas immediately collected fromeachmouse by cardiac puncture, and only a fewseconds elapsed between cervical dislocation and blood collection. The brain was then quickly removed and the hypothalamus and hippocampus were dissected. The spleen was also removed under sterile conditions and immediately processed to determine the cytokine secretion in response to mitogenic stimulation in vitro (Fig. 1).

2.2.1. Socially stressed mice

Mice were socially stressed using the sensorial contact model (Kudryavtseva et al., 1991) to obtain mice that experienced repeated social defeat. Eighty pairs of mice, which were matched by weight, were exposed to a 10-min confrontation, where half of the animals were placed in their opponent's cage (i.e., in the cage of a resident mouse), for 3 successive days to establish a dominance–submission relationship. After the third day, only those pairs that had clearly established a dominance–submission relationship during their agonistic confrontations continued the experiment, and this relationship was observed to remain unchanged throughout the subsequent stress period. From the fourth day until the last day of chronic stress (21 days), subordinate mice were exposed daily to 5 min of agonistic interaction with a different resident dominant mouse. As a result, the subordinate mice were repeatedly defeated by a different aggressive resident dominant mouse every day. After each daily confrontation, the mice were separated by transparent partitions with holes, which permitted the mice to see, hear and smell each other but prevented physical contact. Although the defeated mice received some bites during the direct interaction period, most mice did not have evident wounds. When visible wounds were found, those mice were removed from the experimental procedure. The final number of defeated subordinate mice was 68. A cluster analysis (see Gómez-Lázaro et al., 2011) using the mean percentage of time that was allocated to each assessed behavioural element was carried out on all defeated mice in terms of the behavioural characteristics that they demonstrated during the social confrontation on day 18. Thus, two groups of defeated subjects were identified in accordance with the coping strategy that was adopted (active or passive). Half of the subjects from each group were assigned to the drug treatment group, while the other half were assigned to the vehicle treatment group. The administration of the drug and the vehicle began on day 19 of social stress and lasted for 5 days. The confrontation which took place on day 21 was also recorded for subsequent behavioural assessment (3 days of drug treatment).

2.2.2. Manipulated controls

The manipulated control group (n=20) was treated identically to the stressed group but was not exposed to agonistic interaction or sensorial contact with other mice. These mice were housed individually in cages that contained a transparent barrier to subject them to the same space restrictions as the experimental mice. Manipulated controls were moved daily to an experimentation room, where the barrier was removed for the same period of time that the confrontations lasted in the defeated mice. Additionally, as with the defeated subjects, starting on day 19 of the experiment, half (n=10) of the subjects were treated with the drug for 5 days, while the other half (n=10) received the vehicle for the same length of time.

2.3. Behavioural assessment

The behaviours that were manifested by defeated mice on days 18 and 21 were recorded using video cameras (JVC, GZ-MG77E), and behavioural assessment was carried out using an ethogram for the mouse. This method was developed by Brain et al. (1989) and describes 51 behaviours that are divided into the following behavioural categories: avoidance–flee (the anterior portion of the body moves with the head away from the opponent, running away when the opponent approaches), defence–submission (exhibits upright and sideways defensive postures, the head is pushed backwards, and the forelimbs are held rigid and widely splayed), digging/self-

grooming (moves sawdust with front or back legs and self-cleans), exploration at a distance (pays attention to the opponent from a distance), immobility (complete lack of movement of any portion of the body, and no attention is directed toward the opponent), non-social exploration (walking, running and scanning around the cage, and no attention is directed toward the opponent) and social exploration (crawling over and under, following, grooming, nose sniffing and walking around the opponent). The behavioural evaluation was carried out using The Observer 4.0 (Noldus, ITC, Wageningen, the Netherlands).

2.4. Forced swimming test

The mice were singly placed in glass cylinders (with a height of 18.5 cm and a diameter of 12.5 cm) that contained 13.5 cm of water at 25±1 °C (Duarte et al., 2006). The test was performed over 2 days; the pre-test session took place on 1 day (24 h after the last defeat, for 15 min) and the test session took place on the subsequent day (48 h after the last defeat, for 5 min) and was recorded for behavioural assessment. The behaviours that were assessed were immobility, swimming and climbing, and the time that was spent engaged in each behaviour and the latency period were considered. Mice were judged to be immobile when they ceased struggling and remained floating motionless in the water, making only those movements necessary to keep their head above water.

2.5. Drug

The drug used was the 5-HT4 receptor agonist drug RS 67333 hydrochloride (1-(4-amino-5-chloro-2-methoxyphenyl)-3-[1-butyl-4- piperidinyl]-1-propane hydrochloride) (Tocris Cookson Ltd., Bristol, United Kingdom), which was dissolved in the same sterile physiological saline (Grifols, Barcelona, Spain) that was used for vehicle injections. All injections were administered intraperitoneally in a volume of 5 ml/kg body weight. The utilised dose was 1.5 mg/kg/day and was administered as a single injection 1 h after the start of the dark cycle.

2.6. Physiological determinations

2.6.1. Determination of plasma corticosterone concentrations

The blood collected by submandibular vein puncture (3 days before the experiment and on day 21 of social stress) and by cardiac puncture after death was collected in heparinised containers and centrifuged at 1800 g for 15 min at 4 °C. The resulting plasma was collected and stored at −70 °C until it was assayed for corticosterone. Plasma corticosterone concentrations (ng/ml) were determined using a commercially available enzyme immunoassay kit (Assay Designs, Ann Arbor, MI, USA) and by employing an ELx 800 plate reader (Bio- Tek Instruments, Inc.). The sensitivity of the assay was 5 pg/ml and the intra- and inter-assay variation coefficients were 7% and 8%, respectively.

2.6.2. Determination of IL-6 and TNF-α content in the spleen

The spleen was immediately placed in sterile RPMI-1640 medium (Sigma-Aldrich, Madrid, Spain) and then passed through a sterile wire mesh to produce single cell suspensions. The resulting cells were washed three times in sterile medium and viable cells were counted using the Trypan blue dye (0.5% v/v) exclusion technique. Viability was always greater than 80%. Mononuclear cells were isolated from the splenic cell suspension using Ficoll-Paquee™ PLUS (GE Healthcare Bio Sciences, Uppsala, Sweden), and then the cells were centrifugated (400 g for 30 min) at room temperature. The density gradient that was established using the Ficoll–Paque enabled the sedimentation of erythrocytes, granulocytes and the dead cells, with mononuclear cells (lymphocytes and monocytes) being distinguished as an interface between Ficoll–Paque and the RPMI-1640 medium. The cells that were collected at the interfaces were washed three times, their viability was redetermined and their concentrations were adjusted to 2.5×106 cell/ml with

RPMI- 1640 medium supplemented with 10% foetal calf serum (Gibco, Life Technologies, Carlsbad, California, USA), 25 mM HEPES, 2 mM L-glutamine, 5×10−5 M 2-mercaptoethanol (Sigma-Aldrich) and 2 g/l sodium bicarbonate (Sigma). Once the mononuclear cells from the spleen had been obtained, cell cultures were prepared to determine the levels of IL-6 and TNF-α proinflammatory cytokines in vitro. On a flat 96 well Falcon plate (Becton–Dickinson), six wells were sown per mouse with 100 μl of the medium in which the cells obtained in the extraction process were suspended. Next, three of these wells were sown with 100 μl of the RPMI-1640 medium that was supplemented with 20% foetal calf serum and 5 μg/ml of Con-A (Sigma-Aldrich), to measure the secretion of IL-6. Finally, the remaining three wells were sown with 100 μl of the RPMI-1640 medium supplemented with 20% foetal calf serum and 1 mg/ml LPS (Sigma-Aldrich) to measure the secretion of TNF-α. Once all of the samples had been sown, they were cultured for 48 h in a 95% air/5% CO2, humidified atmosphere in an incubator (Jouan) at 37 °C. After this incubation period, the supernatant was removed and frozen at −70 °C until the cytokine determination could be conducted. The IL-6 and TNF-α content from the spleen was determined using commercially available enzyme-labelled immunosorbent assay kits obtained from BD OptEIA™ (Pharmigen, San Diego, CA, USA). According to the manufacturer's instructions, the plates were read at 450 nm for both cytokines using an ELx 800 plate reader (BioTek Instruments, Inc.). The detection limits were 3.8 pg/ml (with intra- and inter-assay variation coefficients of 6.4–6.9 and 4.0–9.6%) and 5 pg/ml (with intra- and inter-assay variation coefficients of 6.4–9.5 and 6.0–13.2%) for the IL-6 and TNF-α assays, respectively.

2.6.3. Real time RT-PCR measurements of the mRNA expression of the GRs and MRs in the hypothalamus and the 5-HT1A and 5-HT4 receptors in the hippocampus

Brain tissue from the hypothalamus and the hippocampus was homogenised using Trizol reagent (Invitrogen, Madrid, Spain) and total RNA was isolated by utilising the standard phenol:chloroform extraction method (Chomczynski and Sacchi, 1987). UV spectrophotometric analysis of nucleic acid was performed at 260 nm to determine the RNA concentrations, and the 260:280 absorbance ratio was utilised to assess nucleic acid purity. The samples were Dnasetreated (DNase I, Invitrogen, Madrid, Spain) to remove contaminating DNA prior to cDNA synthesis, and the total RNA was reversetranscribed using Superscript II reverse transcriptase (Invitrogen, Barcelona, Spain). The resulting cDNA levels were quantified by SybrGreen-based (QuantiTect SYBER Green PCR, Qiagen Iberia S.L., Madrid, Spain) real time PCR, and the formation of PCR products was monitored in real time using the Applied Biosystems 7500 Real Time PCR System. The sequences of the cDNA were obtained from Genbank at the National Center for Biotechnology Information (NCBI; www.ncbi.nlm.nih.gov), and glyceraldehyde-6-phosphate dehydrogenase (GAPDH) served as a housekeeping gene. Primer sequences (Table 1) were designed using the Primer Express Software v3.0 (Applied BioSystems). Primers were obtained from Applied Biosystems (Madrid, Spain) and specificity was verified by melt curve analysis. The relative gene expression was determined using the $2^{-\Delta\Delta t}$ method (Livak and Schmittgen, 2001).

3. Statistical analysis

All statistical analyses were carried out using SPSS 15.0 for Windows (SPSS Inc., Chicago, IL, USA), and the level of significance was set at b0.05. The social behavioural variables were analysed using hierarchical cluster and multivariate discriminant analyses. The behavioural and physiological variables were analysed using a two-way or the three-way ANOVA for repeated measures. When appropriate, specific comparisons were made using Tukey's post hoc tests.

4. Results

4.1. Analysis of the coping strategy adopted in response to chronic defeat and the effect of RS 67333 administration

A cluster analysis, using the mean percentage of time allocated to each assessed behavioural element, was carried out on all defeated mice on day 18 to separate the mice into groups based on the behavioural characteristics they demonstrated during social confrontation. This analysis resulted in two final clusters. The multivariate discriminant analysis confirmed the statistical validity of the established groups and accounted for 98.6% of the cases obtained by the cluster solution, thus confirming their behavioural descriptions. The nonsocial exploration was the variable that best discriminated between the two clusters, followed by digging/self-grooming. Cluster 1 (n=28), which was designated the "active group", was characterised by high exploratory behaviours, especially those of non-social exploration. Cluster 2 (n=40) was characterised by mice that spent most of their time immobile. The group of mice that belonged to this second cluster was termed the "passive group". When a three-way ANOVA (i.e., time, group and treatment) using repeated measures was performed, significant differences were observed for the group factor ($F[2,57]=9.818$; $p<0.0001$) and for the time×group interaction ($F[1,57]=4.565$; $p<0.0001$). However, no significant effect of the drug treatment was observed. In relation to the group factor, the analysis of variance revealed that on day 18 of social stress, the passive subjects had higher levels of immobility ($F[1,67]=56.477$; $p<0.0001$) and avoidance-flee ($F[1,67]=18.321$; $p<0.0001$) behaviours and lower levels of digging/self-grooming ($F[1,67]=6.728$; $p<0.02$), non-social exploration ($F[1,67]=107.107$; $p<0.0001$) and social exploration ($F[1,67]=7.252$; $p<0.01$) behaviours than their active counterparts. Additionally, on day 21 of social stress, the passive subjects were found to have higher levels of immobility behaviour ($F[1,67]=4.566$; $p<0.0001$) and lower levels of non-social ($F[1,67]=10.274$; $p<0.0001$) and social exploration behaviours ($F[1,67]= 4.441$; $p<0.0001$) than their more active counterparts. The analysis of the time×group interaction revealed that active subjects increased their level of non-social exploration behaviour ($F[1,27]=4.195$; $p\leq0.05$) from day 18 to 21 of social stress. Immobility behaviour ($F[1,38]=27.547$; $p<0.0001$) increased in passive subjects from day 18 to 21 of social stress, while non-social exploration behaviour decreased ($F[1,38]=17.990$; $p<0.0001$) (Table 2) in these animals.

### 4.2. The effect of the coping strategy adopted in response to chronic defeat and treatment with RS 67333 on the neuroendocrine system

#### 4.2.1. Plasma corticosterone levels after the last defeat (day 21)

When the corticosterone data obtained after the defeat challenge (day 21) were analysed, a two-way ANOVA (group and treatment) showed a significant effect on only the group factor ($F[2,78]=6.944$; $p<0.01$). Specifically, the post hoc analysis revealed that passive subjects had higher levels of corticosterone in their plasma than the active subjects ($p<0.02$) and manipulated controls ($p<0.01$). No significant differences between the corticosterone levels of the active subjects and manipulated controls were found, and no effect on the interaction between the factors was observed (Fig. 2).

#### 4.2.2. Repeated measures of corticosterone levels prior to and three days after the end of the stress period

The three-way ANOVA (time, group and treatment) using repeated measures revealed an effect of the time ($F[1,80]=12.901$; $p\leq0.001$) and the group factors ($F[2,80]=5.366$; $p<0.01$) but not of the treatment factor. Analysis of the time factor revealed an increase in corticosterone levels in all of the analysed groups, and the post hoc analysis revealed that this increase was similar in all groups. Analysis of the group factor revealed that three days after the last defeat, passive subjects exhibited lower levels of corticosterone than the active subjects ($p<0.05$) and manipulated controls ($p<0.04$). However, no differences were observed between the two latter groups. The analysis failed to reveal any differences between the corticosterone levels of the groups observed three days before the application of chronic stress, and no effect on the interaction between these factors was observed (Fig. 3).

### 4.3. mRNA expression of the GR and MR and the MR/GR ratio in the Hypothalamus

The two-way ANOVA (group and treatment) revealed a significant effect of the group ($F[2,54]=5.035$; $p\leq0.01$) and the drug treatment ($F[2,54]=6.420$; pb0.02) on the mRNA expression of the GR. Post hoc analysis revealed that passive subjects had a higher GR mRNA expression than the active subjects (pb0.03) and manipulated controls (pb0.03) (Fig. 4a), and the administration of RS 67333 increased the mRNA expression of the GR (pb0.01) (Fig. 4b). Furthermore, no effect on the interaction between the factors was observed. Additionally, a significant effect of drug treatment onMRmRNA expression was found ($F[2,54]=80.882$; pb0.001), and the results indicated that the administration of RS 67333 reduced themRNA expression of the MR when compared to the administration of the vehicle (pb0.05) (Fig. 5a). An interaction was also observed between the two factors analysed (group×treatment) ($F[2,54]=4.251$; pb0.02) (Fig. 5b). Specifically, the analysis revealed that active subjects that received treatment had lower MR mRNA levels than active subjects that did not receive treatment (pb0.05), passive subjects that did not receive treatment (pb0.001) and manipulated controls that did not receive treatment ($p\leq0.04$). Additionally, passive subjects that received treatment were found to have lower MR mRNA levels than active subjects (pb0.04), passive subjects (pb0.001) and manipulated controls that did not receive treatment (pb0.04). Finally, passive subjects that did not receive treatmentwere observed to have higherMRmRNA levels than the manipulated controls that received treatment (pb0.001). Analysis of the MR/GR ratio revealed an effect of the group factor ($F[2,54]=3.251$; pb0.05); passive subjects had a lower ratio than the manipulated controls ($p\leq0.05$) (Fig. 6a), but this differences was not found between active subjects and the manipulated controls. Additionally, an effect of the treatment on the MR/GR ratio was observed ($F[2,54]=72.275$; pb0.001); specifically, the administration of RS6733 reduced the MR/GR ratio (pb0.05) (Fig. 6b).

### 4.4. Effect of the coping strategy adopted in response to chronic defeat and treatment with RS 67333 on IL-6 and TNF-α levels in the spleen

Data analysis revealed a significant effect of the group factor on the two cytokines: IL-6 ($F[2,80]=17.052$; pb0.0001) and TNF-α ($F[2,80]=18.013$; pb0.0001). Post hoc analysis was conducted to determine the differences between IL-6 levels of the three groups, and passive subjects were observed to have higher levels than active subjects (pb0.02) and manipulated controls (pb0.0001). The active subjects had higher levels of this cytokine than manipulated controls (pb0.01). Post hoc analysis of TNF-α levels revealed that passive subjects had higher levels than the active subjects (pb0.04) and the manipulated controls (pb0.0001), and active subjects had higher TNF-α levels than the manipulated controls (pb0.01). An effect of treatment with RS 67333 or an interaction between the two factors analysed was not observed (Fig. 7).

### 4.5. Effect of the coping strategy adopted in response to chronic defeat and treatment with RS 67333 on the expression of the 5-HT1A and 5-HT4 receptors in the hippocampus

The two-way ANOVA (group and treatment) revealed a significant effect of the group factor ($F[2,79]=3.378$; pb0.04) on the expression of 5-HT1A receptors. In the post hoc analysis, passive subjects were observed to have higher 5-HT1A receptor mRNA levels than the manipulated controls (pb0.05) (Fig. 8). However, this analysis failed to reveal any significant differences between passive subjects and manipulated controls and their active counterparts. No effect of treatment with RS 67333 or an interaction between the two factors analysed was observed. Furthermore, the analysis revealed that no effect on the mRNA levels of the 5-HT4 receptor occurred and no significant interaction was found between the two factors analysed.

### 4.6. Effect of the coping strategy adopted in response to chronic defeat and treatment with RS 67333 in the forced swimming test

The analysis revealed a significant effect of the group factor on climbing behaviour ($F[2,81]=13.432$; $p<0.0001$), and the post hoc analysis indicated that active and passive subjects spent significantly less time engaged in this behaviour than the manipulated controls ($p<0.0001$; in both cases). No differences were observed between the three groups in relation to the other behaviours analysed (i.e., immobility and swimming). Fig. Although no effect of the treatment was observed, the analysis did reveal a significant interaction between the two factors (group×treatment) on immobility ($F[2,81]=3.131$; $p<0.05$) (Fig. 9a) and swimming behaviours ($F[2,81]=4.895$; $p<0.02$) (Fig. 9b). In the case of immobility the post hoc analysis revealed that active and passive subjects that did not receive treatment remained immobile for a longer period of time than the manipulated controls that did not receive treatment ($p<0.04$, in both cases). The treatment reduced the amount of time in which passive subjects remained immobile, compared with their passive counterparts that did not receive treatment. Finally, no differences were observed between active subjects that received treatment and the controls. In the case of swimming behaviour, the post hoc analysis revealed that passive subjects that were treated spent more time swimming than their passive counterparts that were not treated ($p<0.02$) and the manipulated controls that were treated ($p<0.01$). Finally, the treated manipulated controls were observed to spend less time swimming than the manipulated controls that did not received treatment ($p<0.04$).

## 5. Discussion

In accordance with previous results (see Gómez-Lázaro et al., 2011), the results obtained in this study corroborate the existence of two different behavioural profiles in response to chronic defeat that are associated with different physiological characteristics. After 18 days of defeat, subjects with a passive behavioural profile differ from their active counterparts mainly by responding to social stress through engaging in high levels of immobility and low levels of non-social and social exploration. Three days later, on day 21, the results showed that these behavioural differences actually became more pronounced. The administration of RS 67333 for 5 days increased the expression of GR mRNA but decreased MR (mineralocorticoid receptor) mRNA levels and the MR/GR ratio in the hypothalamus in all groups, which indicates that these effects are characteristic of the drug. Other studies have found that antidepressants increase the expression and protein levels of hypothalamic GRs and reestablish the deficit of these receptors and the MR/GR balance disturbance that is associated with melancholic depression (Okugawa et al., 1999; Peiffer et al., 1991; Pepin et al., 1989). Data exist for the MR that indicate a normal function of or even an increase in these receptors in depressed patients (Mason and Pariante, 2006; Young et al., 2003) and that antidepressants can have opposing actions on their expression depending on, among other factors, the duration of treatment (Reul et al., 1993; Yau et al., 2001). Nevertheless, it is important to bear in mind that, in this study, stress as well as RS 67333 increased the expression of hypothalamic GRS (see Fig. 4a) and did not change the expression of hypothalamic MRs in passive subjects. This may be the reason that could explain why the drug failed to reverse the alterations triggered by stress in this group. It is known that depression is a biologically heterogeneous disorder and that hypocortisolemia is a characteristic of atypical depression that distinguishes this disorder from other subtypes of depression (Antonijevic, 2006; Gold and Chrousos, 2002). In certain cases in humans, hypocortisolemia has been observed to occur after a period of stress and as a consequence of hyperactivity of the HPA axis and an excessive release of glucocorticoids (Fries et al., 2005; Hellhammer and Wade, 1993). In our study, an increase in the expression of the hypothalamic GR was observed in passive subjects that had been exposed to high levels of glucocorticoids during repeated experiences of defeat. This could give rise to an increase in the negative feedback of the HPA axis, which is responsible for low corticosterone levels (Gupta et al., 2007), without a reduction in the sensitivity of the HPA axis to stress, which is mediated by MRs (De Kloet et al., 1998). Thus, in this case, the blocking of GRs may constitute a more appropriate mechanism for inducing an antidepressant effect

(DeBattista et al., 2006; Flores et al., 2006; Young et al., 2004). Treatment with RS 67333 for 5 days resulted in no changes in the expression of the 5-HT4 and 5HT1A receptors in the hippocampus. One might argue that because stress failed to modify the 5HT4 receptors in the hippocampus, no changes should be expected after treatment with the drug. However, we did expect the treatment to down-regulate the 5HT1A receptors in the hippocampus because an increase in this type of receptor was observed after 21 days of defeat in subjects that adopted a passive coping strategy (see Fig. 8). This increase may reflect the serotonin deficit that has commonly been associated with depression (Coppen, 1967; Lanni et al., 2009; Maes and Meltzer, 1995; Schildkraut, 1965). In contrast to the findings of other studies (Licht et al., 2010; Lucas et al., 2007), our results suggest that short-term treatment with RS 67333 is insufficient to increase serotonergic activity, as reflected by changes in receptor expression. This inability to increase serotonergic activity could also explain why no effects of the treatment were observed on proinflammatory cytokine levels in the spleen. It is known that antidepressants that work by increasing serotonin levels suppress the production of these cytokines, while triggering an increase in antiinflammatory cytokines such as interleukin-10 (IL-10) and interleukin-4 (IL-4) (Kubera et al., 2001, 2006; Shen et al., 1999). Bearing in mind recent data that indicate that inflammatory processes may contribute to the reduction in the availability of serotonin (O'Connor et al., 2009; Raison et al., 2010; Zhu et al., 2006), may be the high IL-6 and TNF- α levels observed after episodes of defeat facilitate this deficit in serotonin. Moreover, existing data indicate that high levels of these two interleukins are a characteristic trait of depressed patients who are resistant to conventional pharmacological treatments (Eller et al., 2008; Lanquillon et al., 2000; O'Brien et al., 2007). Although the drug failed to produce observable serotonergic changes in this study, it did result in an increase in the expression of GRs in the hypothalamus, which may be explained by recent in vitro and in vivo findings, which show that antidepressants have a direct effect, i.e., independent of an intervention by serotonin, on the expression and function of GR receptors (Carvalho and Pariante, 2008; Mukherjee et al., 2004). The treatment for 3 days with RS 67333 failed to trigger changes in the social behaviour observed during defeat on day 21. Other studies using animal models of depression, such as chronic mild stress and olfactory bulbectomy, have observed that the administration of this drug for 3 days improved certain behavioural variables that had been altered in these models (Lucas et al., 2007). However, it was noted that a more prolonged treatment was required to completely reverse these alterations (Lucas et al., 2007). Therefore, it is possible that, in our case, a longer treatment is also required to observe the antidepressant effect on individuals subjected to chronic defeat. The data obtained in the forced swimming test revealed that a 5 day pharmacological treatment with RS 67333 had a positive effect on immobility and swimming behaviours, but the drug had no effect on climbing behaviour. This finding, in accordance with those of other authors, predicts a certain therapeutic potential of this compound (Cryan et al., 2002). Specifically, the drug reduced immobility time in passive subjects, when compared to their passive counterparts that did not receive treatment. This indicates that an antidepressive effect occurred in passive group, which was also the group that was most affected by stress. Moreover, the drug was also observed to have an effect on the immobility of active subjects because, following treatment, the immobility behaviour observed in active subjects that received treatment was no different from the control group that received vehicle (see Fig. 9a). It has been proposed that in the forced swimming test active behaviours such as swimming, unlike climbing, which has been related to noradrenergic activity (Detke et al., 1995), are important variable when analysing the antidepressive effects of serotonergic receptor agonist drugs (Cryan and Mombereau, 2004). In this sense, we observed that treated, passive subjects spent significantly more time engaging in swimming behaviour than their passive counterparts in the non-treatment group. The increase in swimming induced by the drug may be due to the activation of serotonergic neurotransmission (Tamburella et al., 2009), which, as discussed earlier, may be diminished in these subjects but not to a degree to which the other studied variables were affected. Additionally, it is known that the effects of partial agonist drugs

vary in accordance with serotonin levels, and because these levels may differ in the three groups analysed, this finding may explain the different observed effects of the drug in the tested groups.

## 6. Conclusion

In conclusion, the data show that, similar to other antidepressants, RS 67333 increased the expression of hypothalamic GRs. However, considering the alterations triggered by this stress model, such as the increase in the expression of GR in the hypothalamus and of IL- 6 and TNF-α levels, the mechanism of this drug does not appear to be the most adequate for reversing stress-induced effects in this model. This may also be the reason for the absence of any effects on the other biological variables analysed in this study. Nevertheless, we cannot dismiss the possibility that a more prolonged treatment may prove more effective, since the data gained from the FST suggest that this drug has a certain degree of antidepressant potential, increasing swimming behaviour and decreasing immobility in subjects with a passive coping strategy.

## Conflict of interest

All authors declare that they have no conflicts of interest.

## Contributors

Larraitz Garmendia and Amaia Arregi proposed the study; Larraitz Garmendia, Amaia Arregi and Eneritz Gómez-Lázaro designed the study protocol; Eneritz Gómez-Lázaro, Joana Perez-Tejada, Arantza Azpiroz and Garikoitz Beitia conducted the experiment; Larraitz Garmendia, Amaia Arregi and Eneritz Gómez-Lázaro contributed for data analysis and interpretation, wrote the manuscript and approved the final article.

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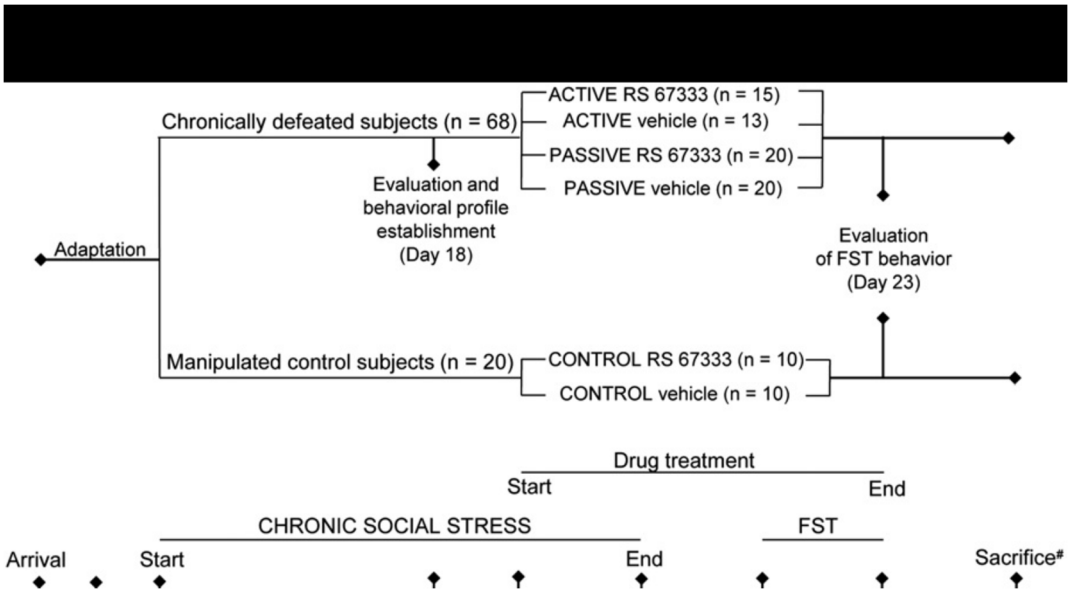


Fig. 1. Schematic representation of the experimental procedure. (*) The blood sample obtained by submandibular puncture. (#) The following samples were obtained: blood by cardiac puncture, and tissue from the spleen, hypothalamus and hippocampus. (FST=forced swimming test).

Table 1
PCR primer specifications.

| Gene | Function | Primer sequence (5′→3′) | Genbank accession |
|---|---|---|---|
| GR | Glucocorticoid receptor | F-CCCATGGAGGTAGCGATTGT<br>R-TGTAAAGGCTGCCCAATGTGT | X66367.1 |
| MR | Mineralocorticoid receptor | F-ACCTGCAGAGAGGACCAATGA<br>R-GGAGTAATTCGTGTTTTTCTTTGCT | AJ311855.1 |
| 5-HT1A | Serotonin receptor | F-CTGTGCTGCACTTCGTCCAT<br>R-GGTCGGTGATTGCCCAGTAC | NM_008308.4 |
| 5-HT4 | Serotonin receptor | F-CACACATCGCATGAGGACAGA<br>R-GCAGCCCATGATGACACATAA | NM_008313.4 |
| GAPDH | Glycolysis | F-TGGCCTCCAAGGAGTAAGAAAC | NM_008084.2 |

Table 2
Mean of percentage of time (±SEM) dedicated to each of the behaviours analysed in terms of group membership, active (n=28) and passive (n=40) on 18 and 21 days of chronic social stress.

| | Day 18<br>Day 18 vs. day 21 | | | Day 21 | | | | |
|---|---|---|---|---|---|---|---|---|
| Social behaviour (Day 21) | Active | Vs. | Passive | Active | Vs. | Passive | Active | Pass |
| Digging/self-grooming | 2.64±1.04 | >* | 0.30±0.15 | 0.80±0.29 | = | 0.80±0.29 | = | = |
| Defence–submission | 4.06±0.92 | = | 5.56±0.60 | 3.38±0.67 | = | 5.13±0.57 | = | = |
| Avoidance–flee | 2.65±0.28 | b*** | 7.37±0.41 | 4.26±0.58 | = | 6.38±0.42 | = | = |
| Exploration at a distance | 13.46±1.44 | = | 13.31±1.49 | 14.51±1.36 | = | 15.16±1.36 | = | = |
| Non social exploration | 38.30±2.82 | >*** | 18.77±1.51 | 44.76±1.49 | >## | 12.85±1.22 | >▪ | b† |
| Social exploration | 3.74±1.30 | >** | 1.06±0.55 | 4.75±1.75 | >## | 0.57±0.39 | = | = |
| Immobility | 35.23±2.32 | b*** | 53.95±2.51 | 28.02±3.49 | b## | 60.01±2.23 | = | b††† |

The following symbols represent the differences between the two groups on 18 days of social stress: * pb0.05; ** pb0.01; *** pb0.001. The following symbol represents the differences between the two groups on 21 days of social stress: # pb0.001. The following symbol represents the differences in groups between 18 and 21 days of social stress in the active: ▪ pb0.001. The following symbols represent the differences in groups between 18 and 21 days of social stress in the passive: p b0.05; ††† pb0.001

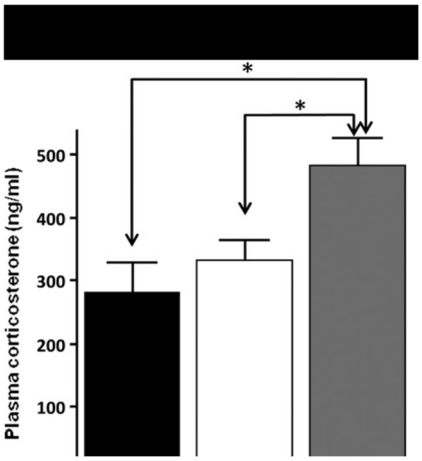


Fig. 2. Plasma corticosterone concentrations (ng/ml) obtained by submandibular puncture in the manipulated controls (n=20) and in the subjects subjected to repeated social defeat on day 21 and 45 min after defeat. Both active (n=28) and passive (n=40) groups are shown. Data are expressed as the mean±SEM. * pb0.05 (Tukey's tests).

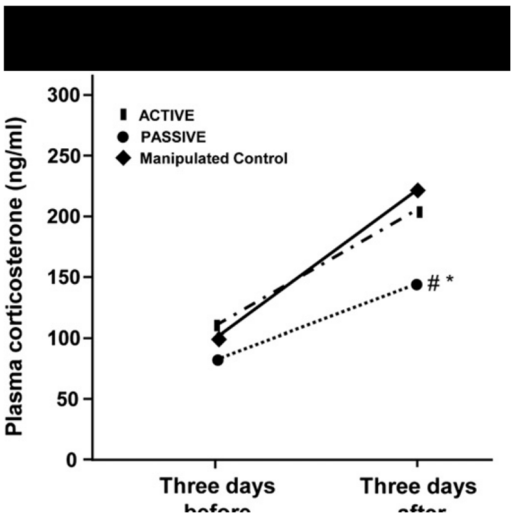


Fig. 3. Plasma corticosterone concentrations (ng/ml) obtained three days before and three days after applying chronic social stress in the manipulated controls (n=20) and in the subjects subjected to repeated social defeat: active (n=28) and passive (n=40). Data are expressed as the mean±SEM. (#) relative to the manipulated control, pb0.05; (*) relative to active subjects, pb0.05 (Tukey's tests).

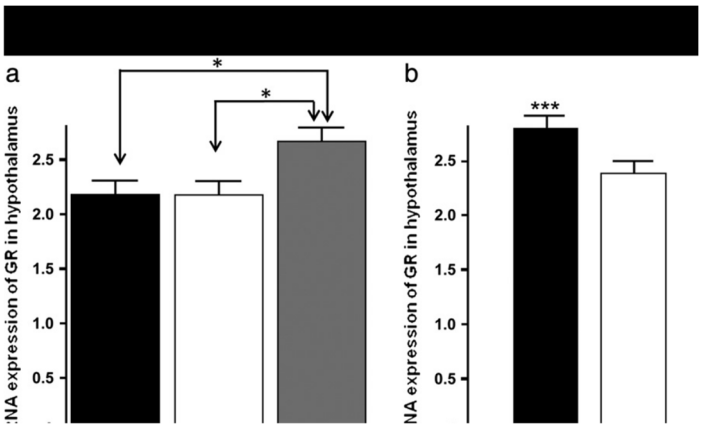


Fig. 4. (a) mRNA expression of the GR in the hypothalami of the different groups analysed three days after their last defeat: manipulated controls (n=20), active (n=28) and passive (n=40). (b) Effects of RS 67333 (1.5 mg/kg) on hypothalamic mRNA expression of the GR in stressed subjects (n=68) and manipulated controls (n=20) three days after their last defeat. Data are expressed as the mean±SEM. * pb0.05 and *** pb0.001 (Tukey's tests).

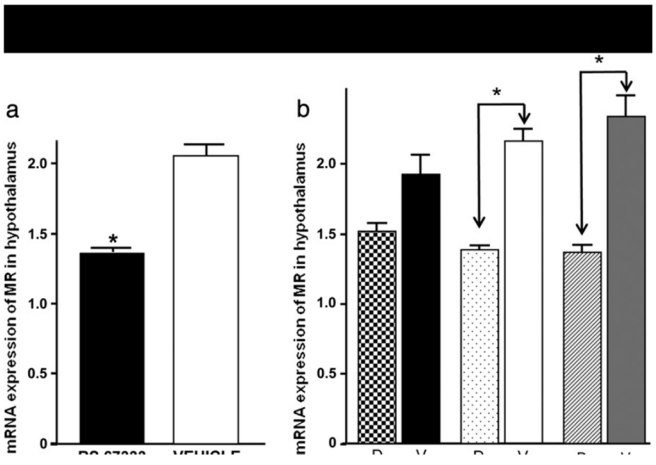


Fig. 5. (a) Effects of RS 67333 (1.5 mg/kg) on hypothalamic mRNA expression of the MR in stressed subjects (n=68) and manipulated controls (n=20) three days after their last defeat. (b) Effects of the interaction between chronic defeat and the pharmacological treatment (D=RS 67333; V=vehicle) in the three groups that were analysed: manipulated controls (n=20), active (n=28) and passive (n=40) three days after their last defeat. Symbols indicating significant differences are only shown for the most relevant data. For the other significant differences, see the detailed information given in the Results section. Data are expressed as the mean±SEM, and * pb0.05 (Tukey's tests).

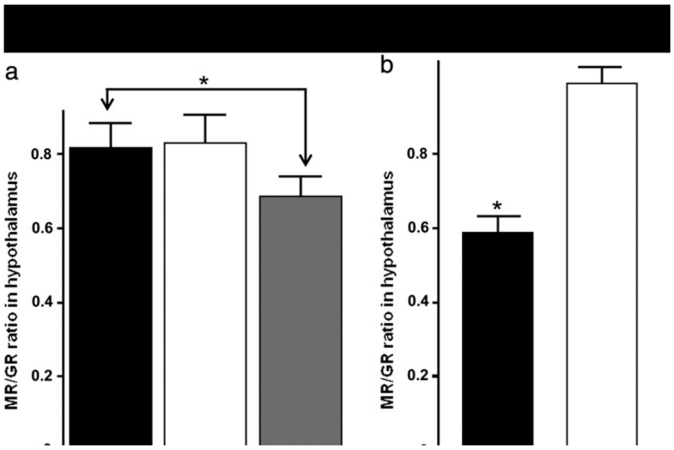


Fig. 6. (a) The hypothalamic MR/GR ratios of the three groups analysed three days after their last defeat: manipulated controls (n=20), active (n=28) and passive (n=40). (b) The effects of RS 67333 (1.5 mg/kg) on the hypothalamic MR/GR ratio in stressed subjects (n=68) and manipulated controls (n=20) three days after their last defeat. Data are expressed as the mean±SEM, and * p≤0.05 (Tukey's tests).

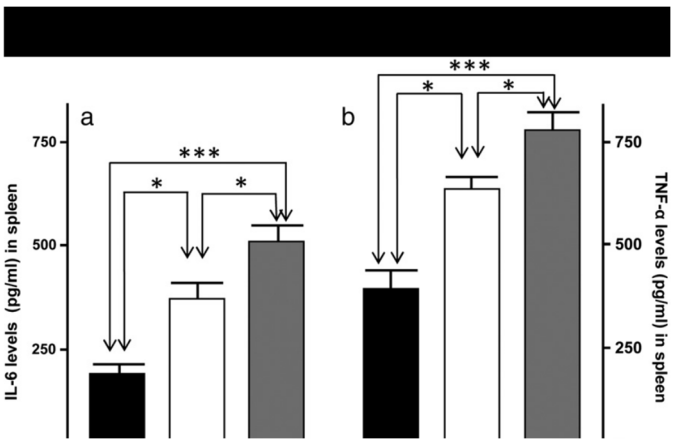


Fig. 7. (a) Splenic IL-6 levels and (b) splenic TNF-α levels in the three groups analysed three days after their last defeat: manipulated controls (n=20), active (n=28) and passive (n=40). Data are expressed as the mean±SEM, and * pb0.05, and *** pb0.001 (Tukey's tests).

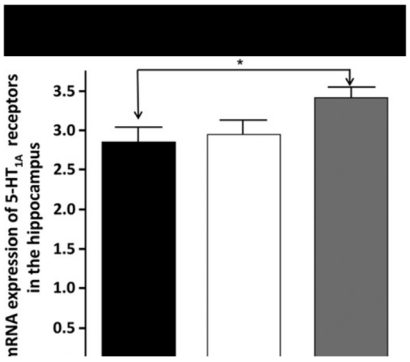


Fig. 8. mRNA expression of the 5-HT1A receptor in the hippocampus of the manipulated controls (n=20) and subjects exposed to repeated defeat: active (n=28) and passive (n=40) three days after their last defeat. Data are expressed as the mean±SEM, and * pb0.05 (Tukey's tests).

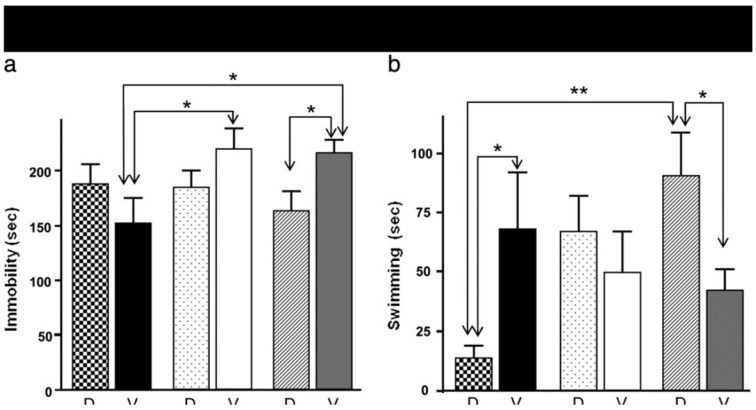


Fig. 9. (a) Effects of the interaction between chronic defeat and the pharmacological treatment (D=RS 67333; V=vehicle) on immobility time and (b) swimming time during the FST two days after their last defeat in the three groups that were analysed: manipulated controls (n=20), active (n=28) and passive (n=40). Data are expressed as the mean± SEM, and * pb0.05 and ** pb0.01 (Tukey's tests).