*Revised Manuscript

Chronic effects of corticosterone on GIRK1-3 subunits and 5-HT_{1A} receptor expression in rat brain and their reversal by concurrent fluoxetine treatment

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Abstract

Dysregulation of the serotonergic system and abnormalities of the hypothalamic-pituitary-adrenal axis have been demonstrated in major depression. Animal studies indicate that 5-HT_{1A} receptor expression may be reduced by long-term administration of corticosterone. However, similar studies on the regulation of GIRK channels, one of the most important effectors of the neuronal 5-HT_{1A} receptor, are limited. In order to address these issues, slow-release corticosterone pellets were implanted subcutaneously to adrenal intact male rats (200 mg pellets, 35 days release). Starting on day 15, animals were treated for 21 days with fluoxetine (5mg/kg/day, i.p), or vehicle. Using in situ hybridization histochemistry and receptor autoradiography, we found that chronic corticosterone treatment was accompanied by a significant decrease on the mRNAs coding for mineralocorticoid receptors in hippocampal areas. Under these conditions, 5-HT_{1A} receptor mRNA expression decreased in dorsal raphe nucleus and dentate gyrus. However, 5-HT_{1A} receptor levels, as measured by [^{3}H]-8-OH-DPAT binding, diminished significantly only in dentate gyrus. It is noteworthy that chronic treatment with fluoxetine reversed the alterations on 5-HT_{1A} receptor mRNA levels only in dorsal raphe. Finally, chronic corticosterone treatment produced an increase on the mRNA coding for the GIRK2 subunit in several hypothalamic and thalamic areas, which was reversed by fluoxetine. Measurements of cell density and volume of the granular layer of the dentate gyrus did not reveal significant changes after corticosterone or corticosterone plus fluoxetine treatments. These data are relevant for a better understanding of the differential regulation of pre- and postsynaptic 5-HT_{1A} receptors by corticosterone flattened rhythm.

Key words (6): mineralocorticoid receptor; glucocorticoid receptor; corticosterone flattened rhythm; depression; [^{3}H]-8-OH-DPAT binding; hippocampus

1. Introduction

Major depression is, perhaps, one of the most compelling examples of a stress-related disorder with evidence of both reduced glucocorticoid responsiveness and dysregulation of the serotonergic system. Glucocorticoid hypersecretion, in conjunction with reduced glucocorticoid responsiveness, is a common and reliable finding in patients with major depression (Holsboer, 2000). Successful antidepressant treatment is associated with normalization of altered glucocorticoid-mediated inhibitory feed-back in these patients, as assessed by either the dexamethasone suppression test or dexamethasone-corticotropin releasing hormone test (Holsboer, 2000).

Animal models can provide important insights in order to address mechanisms of depression and anxiety. Overall, results from different gene knockout models implicate 5-HT$_{1A}$ receptors in anxiety behaviours (Parks et al., 1998; Santarelli et al., 2003). Interestingly, the expression of 5-HT$_{1A}$ receptors is decreased by corticosteroid hormones in a variety of animal studies, whereas removal of circulating corticosteroids by adrenalectomy up-regulates 5-HT$_{1A}$ receptor expression (Chalmers et al., 1993; Meijer et al., 1997b; Meijer and de Kloet, 1994; Mendelson and McEwen, 1992). In the literature there are also diverging findings as regarding to extent of 5-HT$_{1A}$ receptor expression regulation (see Holmes et al. (1995)). Over the last years, a rat model of flattened glucocorticoid-rhythm induced by subcutaneous implantation of corticosterone pellets has been carefully characterized and validated (Gartside et al., 2003a; Gartside et al., 2003b; Leitch et al., 2003). Such pellets provide a steady, continuous corticosterone exposure leading to the loss of circadian fluctuation (Akana et al., 1992; Leitch et al., 2003; Young et al., 1995). The levels of blood corticosterone achieved are within the physiological range (midway between nadir and zenith) but the overall glucocorticoid daily exposure is unchanged (Leitch et al., 2003). Elevated nadir and flattened rhythm are consistent with the pattern observed in depressed patients, although in humans the peak of the diurnal rhythm is preserved, or even elevated, resulting in an increase in total cortisol exposure during the day (Holsboer, 2000). Using the corticosterone pellet rat model, Leitch et al. (2003) demonstrated desensitization of somatodendritic 5-HT$_{1A}$ autoreceptors, but the mechanisms underlying these changes, i.e. expression of receptor and/or

effectors, have not been addressed yet. The effects of corticosterone upon 5-HT_{1A} receptors could result from actions of mineralocorticoid (MR) and/or glucocorticoid receptors (GR) directly on the promoter region of the 5-HT_{1A} receptor gene or through interactions with other regulatory proteins (Ou et al., 2001; Wissink et al., 2000). Nothing is known either about the effects of such treatments on G-protein inwardly rectifying potassium (GIRK) channels, which are activated by 5-HT_{1A} receptors (Zgombick et al., 1989) and are expressed in most 5-HT_{1A} receptor mRNA-containing cells in brain areas such as hippocampus, cerebral cortex, septum and dorsal raphe nucleus (Saenz del Burgo et al., 2008). In this respect, it is of note that the study by Karten et al. (1999) shows that the loss of CA1 hippocampal 5-HT_{1A} responsiveness induced by long-term exposure to corticosterone was not accompanied by alterations on 5-HT_{1A} receptor mRNA expression.

Considering the above evidence, the purposes of this study were *i*) to study the effects that these alterations in circulating corticosterone might have on the expression of MR and GR mRNAs; *ii*) to monitor possible impairments in the expression of brain mRNAs coding for GIRK1-3 subunits and 5-HT_{1A} receptors following long-term exposure to flattened corticosterone rhythm and *iii*) to analyze the effects of chronic fluoxetine treatment in this rat model, which shows elevated plasma corticosterone levels and presumably dysregulated MR and GR signalling. In this context, studies in "normal" animals (i.e. unstressed) have demonstrated that long-term fluoxetine administration increases the expression of corticosteroid receptors (Bjartmar et al., 2000; Brady et al., 1992) but does not alter 5-HT_{1A} receptor mRNA not ^{3}H-8-OH-DPAT binding levels (Hervás et al., 2001). However, no attempts have been made to assess the regulatory effects of fluoxetine upon corticosteroid receptors under the persistent presence of a relatively high circulating level of corticosterone. Since many depressed patients with a pre-existing flattening of their glucocorticoid rhythm will be prescribed fluoxetine as a treatment for their depression, this potential interaction may be of therapeutic importance. In this sense, previous studies show that flattened glucocorticoid rhythm compromises the ability of fluoxetine to elevate forebrain 5-HT levels (Gartside et al., 2003b), an effect that could be necessary for the up-regulation of corticosteroid receptors induced by fluoxetine, and that might explain the limited efficacy of fluoxetine in patients with abnormal hypothalamic-pituitary adrenal (HPA) axis function (Young et al., 2004).

2. Experimental procedures

2.1. Animals

Male albino Sprague-Dawley rats (200–250 g b.w.) were obtained from Harlan Iberica (Barcelona, Spain). Animals were kept in a controlled environment (12-hour light-dark cycle, lights on at 08:00 hours, and 22 ± 2°C room temperature) with ad libitum access to food and water. Rats were handled following the European Union regulations (O.J. of E.C. L358/1 18/12/1986).

2.2. Chronic treatments

Three groups of individually housed rats underwent implantation of corticosterone pellets or sham surgery under chloral hydrate anaesthesia. Commercial corticosterone pellets (Innovative Research of America, USA) designed to release 200 mg of corticosterone over 35 days (5.7 mg/day) were used. Rats were weighed every day. From day 15th after surgery on, at around 10:00 am, one group of animals treated with corticosterone received daily ip. injections of fluoxetine (5 mg/kg i.p. in saline) for 21 days. Another group of rats treated with corticosterone and the sham group received ip. injections of saline. Animals were killed at 12.00 pm on day 36 after surgery. The selected dose of fluoxetine is within the range used by others to produce plasma concentrations of fluoxetine similar to those obtained in patients treated with clinical doses of this antidepressant (Unceta et al., 2007).

2.3. Tissue preparation

Rats were decapitated and trunk blood was collected for subsequent analysis of serum corticosterone. Brains were removed, frozen on dry ice and stored at -20ºC to be used for in situ hybridization and receptor autoradiography. Tissue sections, 14 μm thick, were cut using a microtome-cryostat (HM500 OM; Microm, Walldorf, Germany), thaw-mounted onto 3-aminopropyltriethoxysilane (APTS; Sigma) coated slides, and kept at -20°C.

2.4. Serum corticosterone assay

Trunk blood samples were left to coagulate on ice, centrifuged at 1,300xg for 15 minutes, and the resultant serum removed. Samples were stored at -80ºC until analysis for corticosterone using a commercial radioimmunoassay kit (Coat-a-count

Rat Corticosterone DPC, Los Angeles, CA, USA).

2.5. In situ hybridization

The oligodeoxyribonucleotide probes used to detect GIRK1, GIRK2, GIRK3 and $5\text{-}HT_{1A}$ receptor mRNA have been described elsewhere (Saenz del Burgo et al., 2008). For GR mRNA, two oligonucleotides were used simultaneously that were complementary to bases 2221-2265 and 2345-2389 (GenBank acc. no. M14053.1). MR mRNA was detected using one oligonucleotide probe complementary to bases 3356-3400 (GenBank acc. no. M36074).

The oligonucleotides were synthesized and HPLC-purified by Isogen Bioscience (Maarsden, The Netherlands). Evaluation of the oligonucleotide sequences with the basic local alignment search tool of EMBL and Gen-Bank databases indicated that the probes do not show any significant similarity with mRNAs other than their corresponding targets in the rat. Oligonucleotide probes were labeled at the 3´-end using [α-^{33}P]dATP (3,000 Ci/mmol; New England Nuclear, Boston, MA) and terminal deoxynucleotidyltransferase (Oncogene Research Products, San Diego, CA), and then purified by using QIAquick Nucleotide Removal Kit (Qiagen, Hilden, Germany).

The protocol for in situ hybridization histochemistry was based on previously described procedures and has been already published (Saenz del Burgo et al., 2008 and references therein). The incubated sections were exposed to Biomax-MR (Kodak) films for 1-2 weeks at -80°C with intensifying screens to obtain autoradiograms. The average densities of each mRNA in different brain regions were evaluated semiquantitatively on film autoradiograms with the aid of an image analysis system (AIS, Imaging Research Inc., St. Catherines, Canada).

The specificity of the hybridization signal obtained was confirmed in a series of routine controls including 1) different oligonucleotide probes directed against different regions of the same mRNA gives identical hybridization patterns when used independently; 2) hybridization signal of a given ^{33}P-labeled probe is abolished in the presence of an excess of the same unlabeled probe in the hybridization solution; 3) thermal stability of the hybrids examined by washing at increasing temperatures shows a sharp decrease in hybridization signal at a temperature consistent with their Tm. Autoradiographic images of control experiments are shown in supplementary Figs. S1 and S2..

2.6. Receptor autoradiography

Sections were preincubated for 10 minutes at room temperature in 170 mM Tris-HCl (pH 7,6), 4 mM $CaCl_2$, and 0.01% ascorbic acid and then incubated for 1 hour at room temperature in [^{3}H]-8-OH-DPAT (230 Ci/mmol; Amersham, U.K.) in the same buffer (Pazos and Palacios, 1985). Non-specific binding was defined in the presence of 10^{-5} mM 5-HT. Sections were washed twice (5 min each) in the same buffer at 4°C, dipped in distilled water at 4°C, and rapidly dried under cold air. Tissues were exposed to Biomax MR (Kodak) films together with plastic standards (^{3}H-Microscales; Amersham) for 2 months. Quantitative analysis of the autoradiograms was performed with a computerized image analysis system (AIS, Imaging Research Inc., St. Catherines, Canada).

2.7. Figure preparation

The AIS image analysis system was used to capture pseudocolor images from in situ hybridization or receptor autoradiography films. The images were prepared for publication using Adobe Photoshop software (Adobe Systems, San Jose, CA) without modifications.

2.8. Analysis of the volume of the granular layer of the dentate gyrus

A set of animals from the three treatment groups (n=5 each) was processed for analysis of volume and cell density in the granular layer of the dentate gyrus (DGgl). The rats were deeply anesthetized with an overdose of chloral hydrate (1 g/kg, i.p.) and perfused transcardially with 0.1 M phosphate-buffered saline (PBS), pH 7.4, followed by 4% paraformaldehyde in 0.1 M PB, pH 7.4. Subsequently, the brains were removed and immersed in the same fixative for 4 h at 4ºC, and then transferred to PBS containing 30% sucrose, maintained in the fridge until they sunk and then frozen in isopentane at -80°C. Whole series of 40 µm thick frozen coronal sections were obtained using a microtome (Leitz-Wetzlar 1310, Wetzlar, Germany) provided with a specific temperature sensor (5MP BFS-Physitemp Controller, Clifton, New Jersey, USA). A section-sampling fraction of 1/10 was used for volume estimation. Tissue sections from all groups were mounted on gelatin-coated slides, allowed to dry, and processed simultaneously for Nissl staining with identical incubation solutions. Sections were then coverslipped with DPX and examined with

an Olympus BX50F optic microscope (Olympus, Tokyo, Japan) equipped with a high-resolution digital camera (Olympus and Soft Imaging Systems, Tokyo, Japan). 8-bit gray scale images of the hippocampus were taken under a 4X objective and digitised using CellA software for image acquisition with identical illumination and exposure settings (Olympus and Soft Imaging Systems, Tokyo, Japan). Thereafter, all images were converted to black and white particles by setting an identical "threshold" using ImageJ image analysis software (ImageJ, NIH, Bethesda, MD, USA). The resulting binary images were edited further in Photoshop to remove particles outside the DGgl. Finally, percent area fraction measurements using ImageJ allowed us to calculate the area corresponding to DGgl in each of the acquisitions. The total volume for the DGgl was estimated using Cavalieri's principle (Gundersen and Jensen, 1987), by multiplying the total area measured in each section by the distance between consecutive sections of the 1/10 sampling fraction (400 µm). See supplementary figures for further details concerning measuring methods.

2.9. Analysis of cell density in the granular layer of the dentate gyrus

Four 40 µm thick frozen coronal sections per animal, collected between bregma -4.0 and 5.5 mm (with 400 µm distance between sections), were mounted on gelatin-coated slides, allowed to dry, and simultaneously processed with Hoescht 33342 (Sigma-Aldrich; 1 µg/ml in PBS containing 0.1% Triton X-100 for 15 min). Sections were then coverslipped with Mowiol and images of lateral and medial blades of the DGgl were captured with a fluorescence microscope (Carl Zeiss Axio Observer) equipped with a structured illumination module (ApoTome) and a XYZ motorized stage. The objective used was a 20X Zeiss Plan-Apochromat 0.80 NA. Acquired images were rotated and cropped to fit the DGgl in a counting box of 300 µm x 100 µm. For the analysis, two sampling areas per section and side (left and right) were selected. Thus, a total of 8 sampling areas per animal and side (right and left) in a total of 15 specimens (n=5 per group) were used for quantification. An observer blind to the treatment counted directly on microscope images the number of neuronal nuclei, which could be distinguished from glial nuclei based on their larger size, slightly granulated staining and looser chromatin. Data were expressed as number of cells per mm^2. See supplementary figures for further details concerning measuring methods.

2.10. Statistical analysis

GraphPad Prism 4.01 (GraphPad Software Inc., San Diego, CA) was used to organize and analyze data. Statistical significance of treatment effects on weight gain, plasma corticosterone and adrenal gland weight was determined using one-way ANOVA followed by Tukey–Kramer's test, with $p<0.05$ considered significant in all cases. Overall effects of corticosterone treatment with or without fluoxetine administration on the in situ hybridization signal for each mRNA species and ^{3}H-8-OH-DPAT binding in each brain region analyzed were tested by two-way ANOVA. The data were analyzed considering the effect of treatment (sham, corticosterone and corticosterone plus fluoxetine), the anatomical region and treatment×region interaction. Since the anatomical region factor was statistically significant for all variables examined (mRNAs encoding MR, GR, 5-HT_{1A} receptor and GIRK1-3, as well as ^{3}H-8-OH-DPAT binding) ($P<0.0001$), we report only P values corresponding to treatment×region interaction when $P<0.05$, or treatment factor (if $P<0.05$) when treatment×region interaction was $P<0.05$. Further analysis was done with post hoc Bonferroni test to determine the source of the detected significances in the ANOVAs. Statistical data are reported in the figure legends.

3. Results

3.1. Effects of treatments on animal-growth

Animals were monitored daily at 9 a.m. to control body weight. The time course of all animal groups is shown in Supplementary Fig. S3. Over the postoperative period all experimental groups suffered a marked weight loss, which was consistently more pronounced in corticosterone-treated animals. As shown in Fig. 1A, weight gain was significantly smaller in rats that received corticosterone (groups CORT and CORT+FLX) than in the corresponding sham-operated animals between days 5 and 15 of treatment ($P<0.01$ in all cases). While sham animals started to recover weight around day 5 after surgery, the two groups of animals that received chronic corticosterone treatment showed no weight gain or even weight loss during days 5 to 15, and a reduced weight gain between days 5 to 36 (in all instances $P<0.01$ in comparison to sham group 1) (Fig. 1A).

3.2. Determination of plasma corticosterone levels and effects of treatments on adrenal weight

In order to validate our animal model, hormone plasma levels were evaluated by radioimmunoassay in blood samples collected at the moment of sacrifice. As shown in Fig. 1B, plasma corticosterone levels were significantly higher in corticosterone-treated animals than in sham-operated animals. Plasma corticosterone levels were elevated in both chronically treated groups, independently of the concomitant fluoxetine treatment. As shown in Fig. 1C, there was a significant reduction in the adrenal glands' average weight after chronic corticosterone treatment, independently of the concomitant fluoxetine treatment.

3.3. Effects of treatments on mineralocorticoid and glucocorticoid receptor mRNA expression

Mineralocorticoid (MR) and glucocorticoid (GR) receptor mRNA expression was examined in several areas across the brain, as reported in Fig. 2. Chronic corticosterone treatment induced significant and selective decreases in MR mRNA expression in the hippocampal CA1-4 region (CA) and dentate gyrus (DG). In contrast, chronic corticosterone treatment failed to alter GR mRNA levels in the hippocampus and dentate gyrus but selectively reduced expression in the granular layer of the cerebellum (CbGr). Concurrent treatment with fluoxetine did not modify corticosterone effects on MR and GR mRNA expression since no significant differences were found between groups CORT and CORT+FLX.

3.4. Effects of treatments on serotonin 5-HT$_{1A}$ receptor

Figure 3 reports 5-HT$_{1A}$ receptor mRNA levels and [^{3}H]-8-OH-DPAT binding densities as measured in the different experimental groups. Colour-coded images from autoradiograms illustrate areas where significant changes were detected. After chronic corticosterone treatment there was a decrease in 5-HT$_{1A}$ receptor mRNA levels in the DG (which reached statistical significance only in animals that received fluoxetine) (Fig. 3B) and in [^{3}H]-8-OH-DPAT binding in the two groups treated with corticosterone (Fig. 3D). In contrast, in the dorsal raphe nucleus (DR) corticosterone elicited a fluoxetine-reversible decrease in 5-HT$_{1A}$ receptor mRNA expression (Fig. 3C), while it did not affect [^{3}H]8-OH-DPAT binding".

3.5. Effects of treatments on GIRK channel mRNA expression

The expression of GIRK1, GIRK2 and GIRK3 subunit mRNA in several brain areas is reported in Fig. 4. Chronic hypercorticosteronemia significantly increased GIRK2 subunit mRNA expression in the ventromedial (VMH) and dorsomedial (DMH) hypothalamic areas. However, concomitant fluoxetine treatment counteracted the effect of chronic corticosterone and GIRK2 mRNA levels remained indistinguishable from sham-controls.

3.6. Effects of chronic treatments on granular layer of the dentate gyrus

In order to quantitatively determine the potential effects that chronic hypercorticosteronemia might have on neuronal loss in our animal models, we measured the volume of the granular layer of the DG (DGgl) (see supplementary Fig. S4) in control, chronic corticosterone and chronic corticosterone with fluoxetine treated animals (n=5 each). Our volume calculations were in the range of 1.74±0.11 to 1.87±0.10 mm^3, and showed that neither corticosterone nor corticosterone with fluoxetine treatment caused significant differences among groups (supplementary Fig. S5). To further investigate the impact on structural integrity of DGgl, neuronal density was measured in the DGgl of the above-mentioned groups (supplementary Figs. S6 and S7). Once again we found no statistically significant effects of corticosterone or corticosterone with fluoxetine treatments on neuronal density in the DGgl.

4. Discussion

The present results support and extend previous observations showing that manipulation of corticosterone levels by slow-release corticosterone pellets produces a functional desensitization of somatodendritic 5-HT_{1A} autoreceptors in dorsal raphe nucleus (DR) (Gartside et al., 2003a; Gartside et al., 2003b; Leitch et al., 2003). Our work provides novel and useful evidence regarding the regulation of cellular expression of mRNAs coding for the 5-HT_{1A} receptor and GIRK1-3 subunits in this animal model and in particular during the concurrent administration of corticosterone and fluoxetine. As previously discussed (Gartside et al., 2003b; Holsboer, 2000; Young et al., 2004), interactions between corticosterone and fluoxetine may be critical in the origin of reduced antidepressant efficacy of selective serotonin reuptake

inhibitors (SSRIs) in patients with pre-existing abnormalities of the HPA axis. Moreover, we have monitored the expression patterns of the mRNAs coding for mineralocorticoid (MR) and glucocorticoid (GR) receptors at which the effects of corticosterone alone and plus fluoxetine were observed.

The present experimental paradigm differs from that of Leitch et al. (2003), who used corticosterone pellets to provide continuous slow release of corticosterone in adrenally intact animals, both in terms of the daily dose being higher (25 mg/kg compared to 18 mg/kg) and in terms of treatment duration (35 days compared to 14 days). However, the average daily dose of corticosterone, although did vary over the 5 weeks because of increase in the body weight of the rats (25-31 mg/kg/day), was within the range used by Akana et al. (1992), Young et al. (1995) and Young (1996), and was slightly lower than that used by Meijer et al. (1997b). In accord with all the aforementioned studies, we found that the adrenal glands in rats treated for 35 days with corticosterone showed a degree of atrophy compared to controls, probably via a decrease in ACTH, the major determinant of adrenal weight via its trophic effects (Fig. 1). It is of note that the corticosterone treatment regime did not appear to adversely affect the general health of the animals, although corticosterone-treated animals were lighter at the end of the treatment period. This was accounted for by low weight gain 5 to 15 days after pellet implantation, and their weight gain was normal for the latter part of the treatment period (Fig. S3). Although, the regime of corticosterone treatment used here produced corticosterone levels (Fig. 1) that did not exceed the physiological levels, we cannot reject that probably some degree of hypercorticism could have taken place during the first week of treatment. In this context, thymus weight changes could have confirmed the potential hypercorticism.

Effects of treatments on corticosteroid receptor mRNAs.

Both MR and GR mRNA were abundant in the granule cell layer of the DG and the pyramidal cell layer of the Ammon's horn (CA). However MR mRNA was present in all fields CA1-4 whereas GR mRNA was restricted to fields CA1-2. GR mRNA showed also a very strong labelling over the granule cell layer of the cerebellar cortex (CbGr). These patterns of hybridization agree with the previously described cellular distribution of the two types of corticosteroid receptors (Herman et al., 1989).

Previous studies have shown that high doses of corticosterone trigger a down-regulation of GR mRNA and GR protein in the CA1 subfield of the hippocampus

(Sapolsky and McEwen, 1985; Sarabdjitsingh et al., 2010), and removal of circulating glucocorticoids by adrenalectomy induces an up-regulation of GR mRNA and MR mRNA in CA1-2 subfields, which may be reversed by dexamethasone replacement (Herman et al., 1989). In our rat model, after 35 days of continuous corticosterone release, the MR and GR mRNA levels were significantly down-regulated in the DG and CA hippocampal areas and the CbGr, respectively. The lack of GR mRNA down-regulation at the hippocampus is at odds with the aforementioned studies (Sapolsky and McEwen, 1985; Sarabdjitsingh et al., 2010), but agrees with previous studies that used comparable corticosterone pellets in adrenally intact rats (Akana et al., 1992; Herman and Spencer, 1998; Young et al., 1995). Thus, unlike the corticosterone pellet treatment that produces a flat circadian rhythm of corticosterone, the chronic corticosterone injection paradigm produces a GR down-regulation in the hippocampus (Sapolsky and McEwen, 1985), suggesting that physiological increases in corticosterone levels may not be sufficient to down-regulate the GR. The differential pattern of MR and GR occupancy profiles by corticosterone (de Kloet and Reul, 1987; Reul and de Kloet, 1985) has led to the well-reasoned hypothesis that regulation of MR-dependent actions of corticosterone will take place primarily at the level of regulation of MR protein expression (de Kloet and Reul, 1987). Our data suggest that MR gene transcription in the hippocampal formation may be more sensitive to regulation by corticosterone than GR gene. This was also inferred from studies in which animals were subjected to unpredictable stress or corticosterone treatment for a considerable period (Herman and Spencer, 1998; Lopez et al., 1998). Therefore, chronic corticosterone treatment-induced down-regulation of MR is likely to be a result of autologous regulation of the MR gene expression, whereas in the case of GR mRNA, expression appears to be inhibited by corticosterone acting through both MR and GR (Herman and Spencer, 1998; Kalman and Spencer, 2002). In agreement with this view, overexpression of MR in the mice forebrain decreases the basal mRNA expression patterns of GR in the CA1 region (Rozeboom et al., 2007).

Finally, it has been demonstrated that long-term fluoxetine administration increases the expression of MR (Bjartmar et al., 2000; Brady et al., 1992). However, it is important to note that most animal studies investigating fluoxetine effects are performed in animals under baseline conditions. Since many depressed patients with a pre-existing flattening of their glucocorticoid rhythm will be prescribed fluoxetine as

a treatment for their depression, this potential interaction may be of therapeutic importance. Our results show that long-term treatment with fluoxetine was unable to reverse the hippocampal down-regulation of MR mRNA expression induced by corticosterone. In this sense, previous studies show that flattened glucocorticoid rhythm compromises the ability of fluoxetine to elevate forebrain 5-HT levels (Gartside et al., 2003b), an effect that could be necessary for the up-regulation of corticosteroid receptors induced by fluoxetine, and that might explain the limited efficacy of fluoxetine in patients with abnormal HPA axis function (Young et al., 2004).

Effects of treatments on 5-HT$_{1A}$ receptor

Our results show that chronic corticosterone administration without fluoxetine produces decreases in 5-HT$_{1A}$ receptor mRNA (in DR) and binding sites (only in DG), and it is noteworthy that concomitant fluoxetine treatment reverses the alterations on 5-HT$_{1A}$ receptor mRNA levels in dorsal raphe and induces a significant decrease of this mRNA in the DG. It is well established that the 5-HT$_{1A}$ receptor is subject to strong glucocorticoid repression (Chalmers et al., 1993; Meijer et al., 1997a; Meijer et al., 1997b; Meijer and de Kloet, 1994; Mendelson and McEwen, 1992). Regulation of the 5-HT$_{1A}$ receptor gene at the transcriptional level has been studied by analysis of its promoter region, where hormone response elements have been described (Ou et al., 2001; Wissink et al., 2000). At present, two possible mechanisms have been demonstrated: via binding of corticosteroid receptors to a negative glucocorticoid responsive element (nGRE) (Ou et al., 2001), and via protein-protein interactions with transcription factors such as NF-κB and AP-1, which up-regulate 5-HT$_{1A}$ receptor gene transcription (Wissink et al., 2000). Thus, MR and GR are able to interact by forming heterodimers, whereas additional GR can repress 5-HT$_{1A}$ receptor gene transcription in the absence of DNA binding through direct protein-protein interactions with AP-1 and NF-kB. Both pharmacologic and gene knockout studies have provided evidence that MR is involved in corticosterone-induced repression of 5-HT$_{1A}$ receptor gene transcription (Meijer et al., 1997a; Rozeboom et al., 2007). In particular, knockout of the murine GR gene did not impair corticosterone-mediated repression of hippocampal 5-HT$_{1A}$ receptor mRNA suggesting that MR alone is sufficient for trans-repression of the 5-HT$_{1A}$ receptor gene by glucocorticoids (Meijer et al., 1997a).

In fact, on this molecular basis, and assuming that in our rat model the

decreases in MR mRNA result in decreased levels of the corresponding protein, we could expect an up-regulation of the 5-HT_{1A} receptor mRNA expression. However, in the last decade, a family of transcriptional repressors of the basal expression of this serotonin receptor, Freud-1 and Freud-2, and its inhibition by increasing intracellular calcium levels has been characterized (Ou et al., 2003). Importantly, the effects of calcium were reversed by calmodulin or calcium/calmodulin-dependent kinase (CAMK) inhibitors (Ou et al., 2003). Previous data have shown reduced expression of CAMKII mRNA in the DG of rats following implantation of corticosterone pellets (Gartside et al., 2003a). Since chronic treatment with corticosterone could be modifying at least three molecular mechanisms operative at the promoter region of the 5-HT_{1A} receptor gene (nGRE, NF-kB and Freud-1), future studies on the expression of the CAMKII and Freud-1 in serotonergic projection areas, such as hippocampus and hypothalamus, and their localization in 5-HT_{1A}-positive cells might help to characterize the role of these transcriptional repressors of the 5-HT_{1A} receptor gene in this rat model of flattened corticosterone rhythm.

Another important issue involves the effects of concurrent administration of fluoxetine both on the expression of 5-HT_{1A} receptor mRNA and binding levels. As previously discussed, interactions between corticosterone and fluoxetine may be critical in the origin of reduced antidepressant efficacy of SSRIs in those patients with pre-existing abnormalities of the HPA axis (Gartside et al., 2003b). Our results support the contention since the concurrent treatment with fluoxetine could only reverse the changes on 5-HT_{1A} receptor mRNA levels in the DR. The mechanism for these preferential effects on somatodendritic 5-HT_{1A} autoreceptors remains unclear.

Effects of treatments on GIRK1-3 subunit expression.

GIRK channels mediate the synaptic actions of numerous neurotransmitters in the mammalian brain and play an important role in the regulation of neuronal excitability in most brain regions through activation of various G protein-coupled receptors including the serotonin 5-HT_{1A} receptor (Zgombick et al., 1989*).* In fact, GIRKs are the only effectors for 5 HT_{1A} autoreceptors in the DR, although in other brain regions 5-HT_{1A} receptors are frequently coupled to adenylate cyclise (De Vivo and Maayani, 1986; Luscher et al. 1997). Recently, we have demonstrated that the mRNAs encoding GIRK1-3 subunits are present in most 5-HT_{1A} receptor mRNA-expressing cells in hippocampus, cerebral cortex, septum and DR (Saenz del Burgo

et al., 2008). Since GIRK channels can act as effectors of 5-HT_{1A} receptors, we intended to determine whether GIRK1-3 subunit expression is altered by corticosterone-treatment. We did not find significant effects, with the exception of increases in GIRK2 mRNA in hypothalamic areas, which are reversed by fluoxetine treatment. To date, there is only scarce evidence that GIRK1 and GIRK2 subunits may be under negative regulation at the transcriptional level by corticosterone (Fairchild et al., 2003; Muma and Beck, 1999). The GIRK1 gene contains a potential binding site for the glucocorticoid receptor (GRE) (Schoots et al., 1997), but the presence of hormone response elements in the promoter region of GIRK2 and GIRK3 subunit genes remains to be established. Taking into account that fluoxetine, at low micromolar concentrations, inhibits the GIRK channel activity of xenopus oocytes injected with GIRK1-2 subunit mRNAs (Kobayashi et al., 2003), it is plausible that during the chronic and concurrent administration of corticosterone and fluoxetine, the acute blocking actions of fluoxetine on GIRK channels also play a role in the regulation of the mRNAs coding for GIRK1-3 subunits. A number of G-protein-coupled receptors have been shown to activate GIRK channels. In fact it has been shown that postsynaptic K+ conductance increase induced by specific agonists of GABA, adenosine A1, and serotonin 5-HT_{1A} receptors, is markedly reduced or absent in mice lacking the GIRK2 channel (Luscher et al., 1997). Therefore, by downregulating GIRK rather than one specific receptor, fluoxetine could reduce the inhibitory inputs from a number of metabotropic receptors and could increase the excitability of DRN neurons in a greater extent than of hippocampal neurons.

Relevance of 5-HT_{1A} receptor changes in the dentate gyrus

Regarding 5-HT_{1A} receptor, the general consensus is that chronically elevated corticosterone down-regulates 5-HT_{1A} receptors in the DG (Lopez et al., 1998; Meijer et al., 1997b; Meijer and de Kloet, 1994). Neuroanatomical studies have shown that prolonged (lasting for months) elevations of glucocorticoids, induced by chronic stress or high doses of corticosterone, are toxic to hippocampal neurons (Sapolsky, 2004). This so-called “dendritic pruning” has been observed mainly for CA3 pyramidal cells (Woolley et al., 1990), while granule cells of DG appear to be largely unaffected. More recently, cell loss has been shown among CA3 pyramidal neurons and DG granule cells (Sousa et al., 1999). Since the studies cited above used very large doses of glucocorticoids, it remains unclear whether more moderate doses (our

rat model) would cause similar effects. Interestingly, previous results in this animal model showed that MAP2b mRNA was reduced in CA3 but not in CA1 or DG (Gartside et al., 2003a). This finding would be consistent with a selective decrease in dendritic extent and/or synaptic strength in the CA3. The changes observed here are somehow selective -in terms of mRNA species (decreases in 5-HT_{1A} receptor mRNA but unchanged GIRK mRNAs) and hippocampal region affected (DG versus CA3)- suggesting that moderate doses of corticosterone cause quite specific effects rather than generalized effects, such as loss of neurons or decrease in hippocampal volume.

In the DG and CA3 field of the rat hippocampus the vast majority of 5-HT_{1A} receptor-expressing cells showed GIRK1-3 subunit mRNAs (Saenz del Burgo et al., 2008). Therefore, it is difficult to explain our data simply as a consequence of a partial loss of neurons in these areas. Our volume calculations are in line with previous studies in the adult rat (Bayer et al., 1982; Brummelte and Galea, 2010), and, in fact, there were no obvious volumetric changes or changes in overall cell number of the DGgl after treatment with corticosterone pellets (see supplementary Figs. S4-S7). In this sense, our findings are consistent with several studies that have shown neither volumetric changes nor changes in overall cell number of the hippocampus or dentate gyrus after treatment with corticosterone pellets or injections (Bodnoff et al., 1995; Brummelte and Galea, 2010; Sousa et al., 1998). It is also established that glucocorticoid excess decreases neurogenesis in the adult DG (Cameron and Gould, 1994). Much of that is due to the sensitivity of progenitor cells to glucocorticoids (Cameron and Gould, 1994). Fluoxetine stimulates proliferation of progenitor cells in the DG through a 5-HT_{1A} receptor-dependent mechanism (Huang and Herbert, 2005; Santarelli et al., 2003). Interestingly, fluoxetine action is also dependent on the presence of a diurnal rhythm of corticosterone. Thus, flattening glucocorticoid levels by implanting a subcutaneous pellet, prevents the stimulating actions of fluoxetine (Huang and Herbert, 2006). Therefore, our data support that the postsynaptic effects of 5-HT on 5-HT_{1A} receptors in the DG to modulate cell proliferation will be compromised after corticosterone treatment, being the concurrent fluoxetine treatment unable to restore the functionality of the 5-HT_{1A} receptor signalling. As proposed (Huang and Herbert, 2006), these findings may point to a new approach to the problem of treatment resistance, or to improved therapeutic sensitivity to fluoxetine in depression.

Abbreviations used in text and figures

5-HT	serotonin
CbGr	granular layer of the cerebellum
CA	pyramidal cell layer of the hippocampus (CA1, CA2, CA3)
DG	dentate gyrus of the hippocampus
DGgl	granular layer of the dentate gyrus
DMH	dorsomedial hypothalamic area
DR	dorsal raphe nucleus
GIRK	G-protein inwardly rectifying potassium channel (Kir3)
LC	locus coeruleus
PFCx	prefrontal cortex
PVN	paraventricular nucleus
SO	supraoptic nucleus
Sp	septum
SSRIs	selective serotonin reuptake inhibitors
Th	thalamus
VMH	ventromedial hypothalamic nucleus

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Role of the funding source

Funding for this study was provided by Instituto de Salud Carlos III, Spanish Ministry of Science and Basque and Catalan Governments. These public entities had no further role in the study design, collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the paper for publication.

Contributors

RC and JS conceived the study and coordinated the project. RC, GM, GGC and JS designed the experimental protocols. LSB, MM, RC, and GGC carried out the experimental work, data collection, and statistical analyses. RC and JS wrote the final draft of the manuscript. All the authors discussed the results and approved the final manuscript.

Conflict of interest

All authors declare that they have no conflicts of interest

Acknowledgements

This work was supported by grants SAF2004-02784 and SAF-2006-10243 from the Spanish Ministry of Science, grants S-PE08UN29 and IT-332-10 from the Basque Government, grant 2009SGR220 from the Catalan Government and the Instituto de Salud Carlos III, Centro de Investigación Biomédica en Red de Salud Mental (CIBERSAM) and Centro de Investigación Biomédica en Enfermedades Neurodegenerativas (CIBERNED). Laura Saenz del Burgo and Mario Montaña were recipients of fellowship from the Basque Government and from the UPV/EHU respectively. We gratefully acknowledge Juan Manuel Rodriguez for technical assistance.

Figure 1

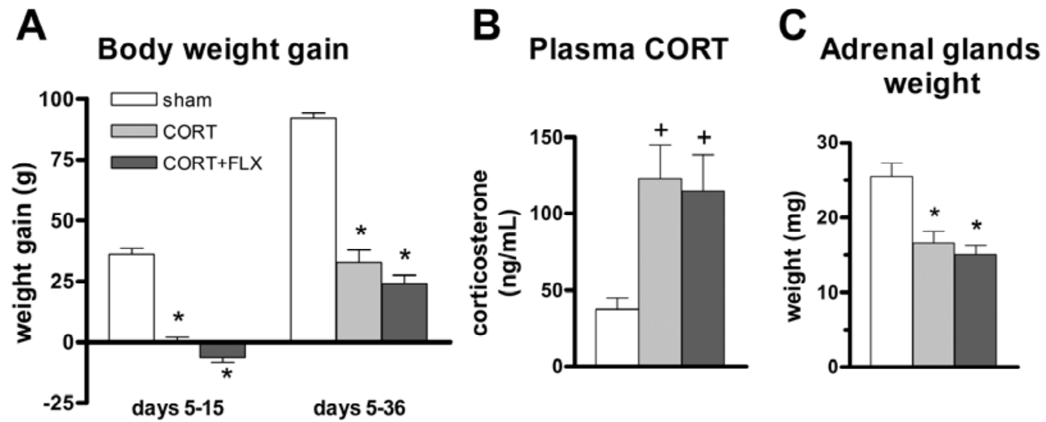


Fig. 1.- Effects of corticosterone treatment on body weight gain, plasma corticosterone levels and adrenal glands weight. (A) Reduction in weight gain observed in both corticosterone (CORT) and corticosterone plus fluoxetine (CORT+FLX) treated rats in comparison to sham control animals (n=9-10) during days 5-15 (One-way ANOVA: $F_{2,26}$=116.9, P<0.0001) and days 5-36 (One-way ANOVA: $F_{2,26}$=102.5, P<0.0001). (B) Elevation in plasma corticosterone levels in both treated animal groups (n=8-10) (One-way ANOVA: $F_{2,24}$=5.909, P=0.0082). (C) Reduction in adrenal gland weight after corticosterone treatment (with or without fluoxetine) (n=9-10) (One-way ANOVA: $F_{2,26}$=13.58, p<0.0001). Data are mean±SEM. Tukey's test: $^{+}$P<0.05, *P<0.001 versus control.

Figure 2

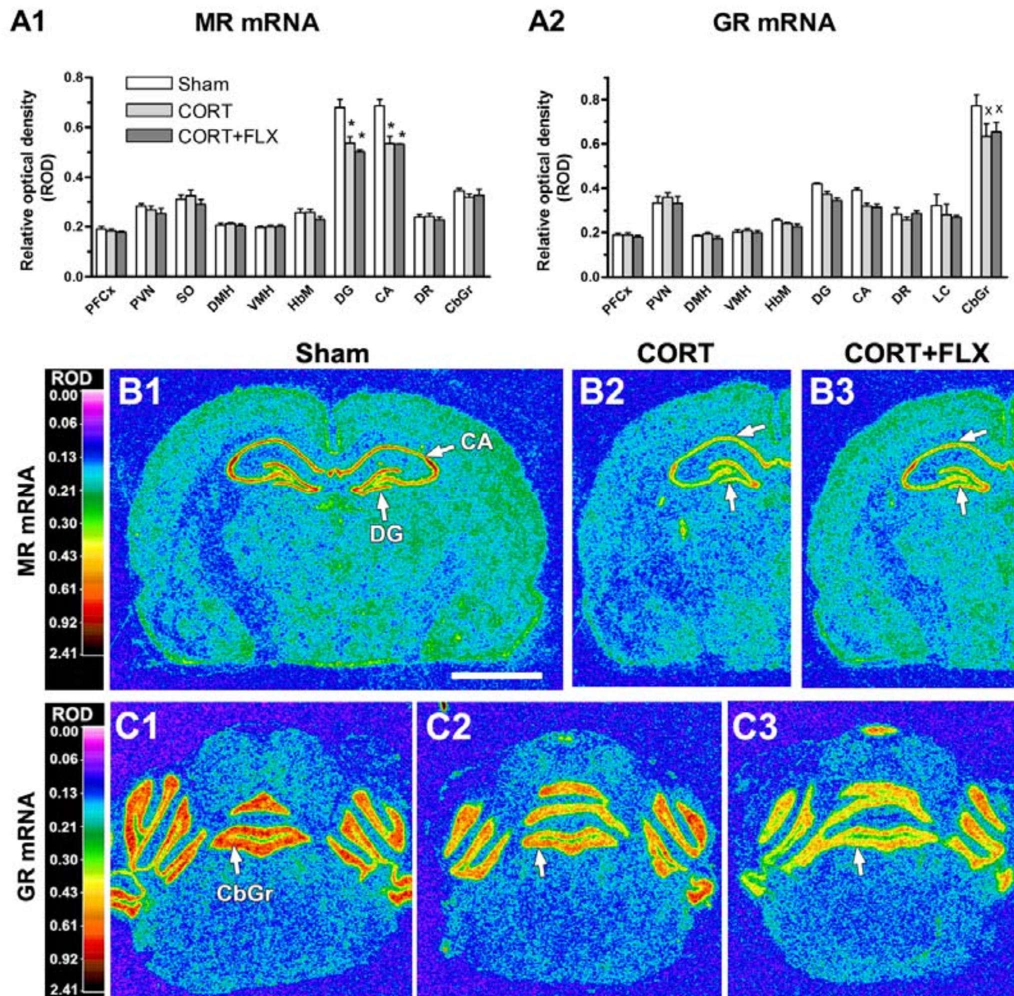


Fig. 2.- Effects of corticosterone treatment on mineralocorticoid (MR) and glucocorticoid (GR) receptor mRNA expression. Microdensitometric measurements (mean±SEM, n=4 per group) of MR mRNA (A1) and GR mRNA (A2) levels in several brain areas after treatment with corticosterone alone (CORT) or in combination with fluoxetine (CORT+FLX). Two-way ANOVA analysis revealed significant effects on MR mRNA (treatment×region interaction $F_{18,82}$=5.48, P<0.0001) and GR mRNA expression (effect of treatment $F_{2,89}$=7.484, P=0.001). Results of Bonferroni posttest: ^{X}P<0.01; *P<0.001. Significant changes in hippocampal MR mRNA (DG and CA) and in GR mRNA in the cerebellum (CbGr) are shown in digital colour-coded images obtained from autoradiograms of coronal brain sections: (B1-B3) MR mRNA and (C1-C3) GR mRNA. Arrows point to structures where average mRNA levels were significantly reduced. Bar=3 mm.

Figure 3

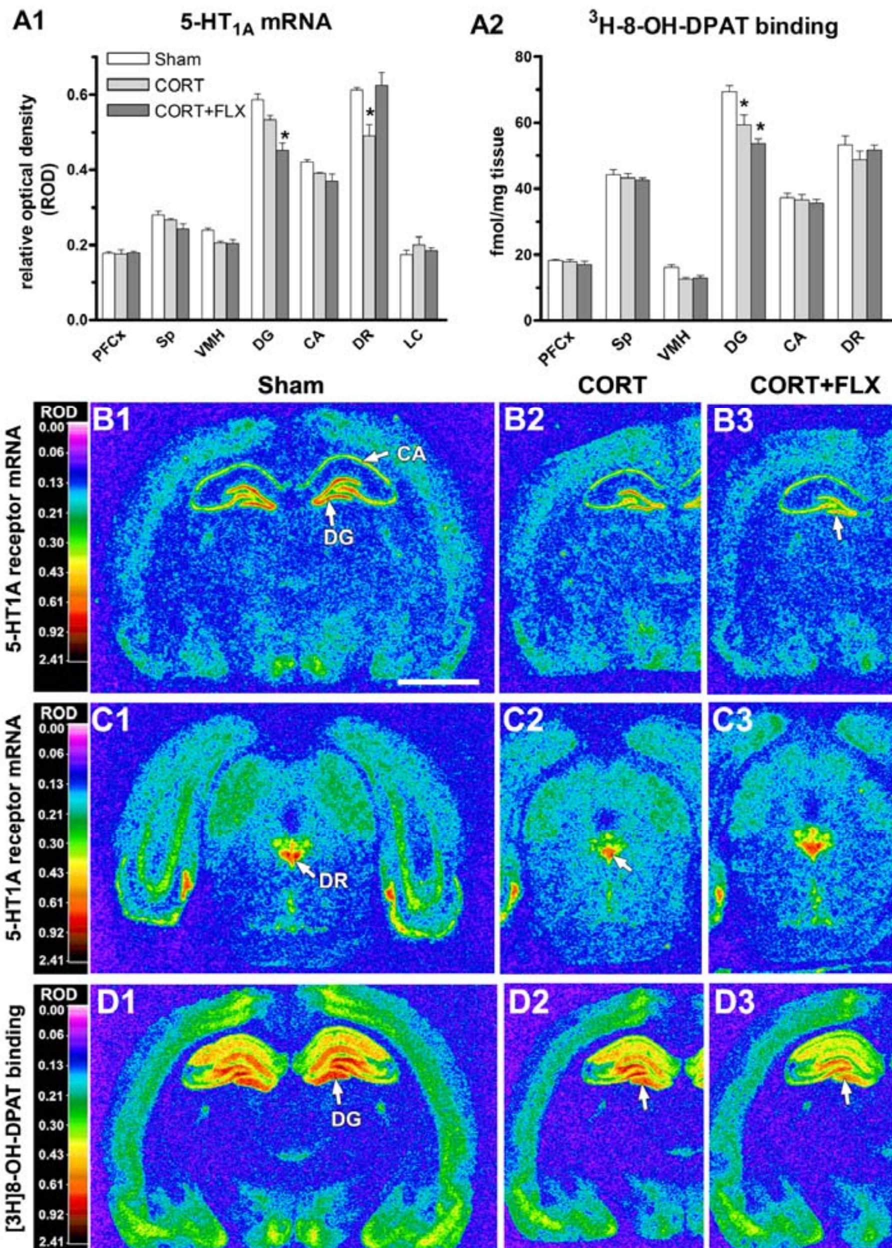


Fig. 3.- Effects of corticosterone treatment on serotonin 5-HT$_{1A}$ receptor mRNA expression and [^{3}H]8-OH-DPAT binding. Microdensitometric measurements (mean±SEM, n=4 per group) of 5-HT$_{1A}$ receptor mRNA levels (A1) and [^{3}H]8-OH-DPAT binding sites (A2) in several brain areas after treatment with corticosterone alone (CORT) or in combination with fluoxetine (CORT+FLX). Two-way ANOVA analysis revealed significant effects on 5-HT$_{1A}$ receptor mRNA expression (treatment×region interaction $F_{12,63}$=5.48, P<0.0001) and on [^{3}H]8-OH-DPAT binding densities (treatment×region interaction $F_{10,54}$=3.771, P=0.0007). Results of Bonferroni posttest: *P<0.001. Corticosterone treated animals (CORT) showed a decrease in 5-HT$_{1A}$ receptor mRNA expression in the dorsal raphe nucleus (DR) that was reversible by fluoxetine (CORT+FLX). This combined treatment also reduced 5-HT$_{1A}$ receptor mRNA levels in the hippocampus (DG). In the DG corticosterone treatment also decreased 5-HT$_{1A}$ receptor binding, but this effect was not reversed by fluoxetine. All changes are shown in digital colour-coded images obtained from autoradiograms of coronal brain sections: reductions in 5-HT$_{1A}$ receptor mRNA expression in DG (B1-B3) and DR (C1-C3), and also in receptor binding in DG (D1-D3). Arrows point to structures where average mRNA levels or receptor binding were significantly reduced. Bar=3 mm.

Figure 4

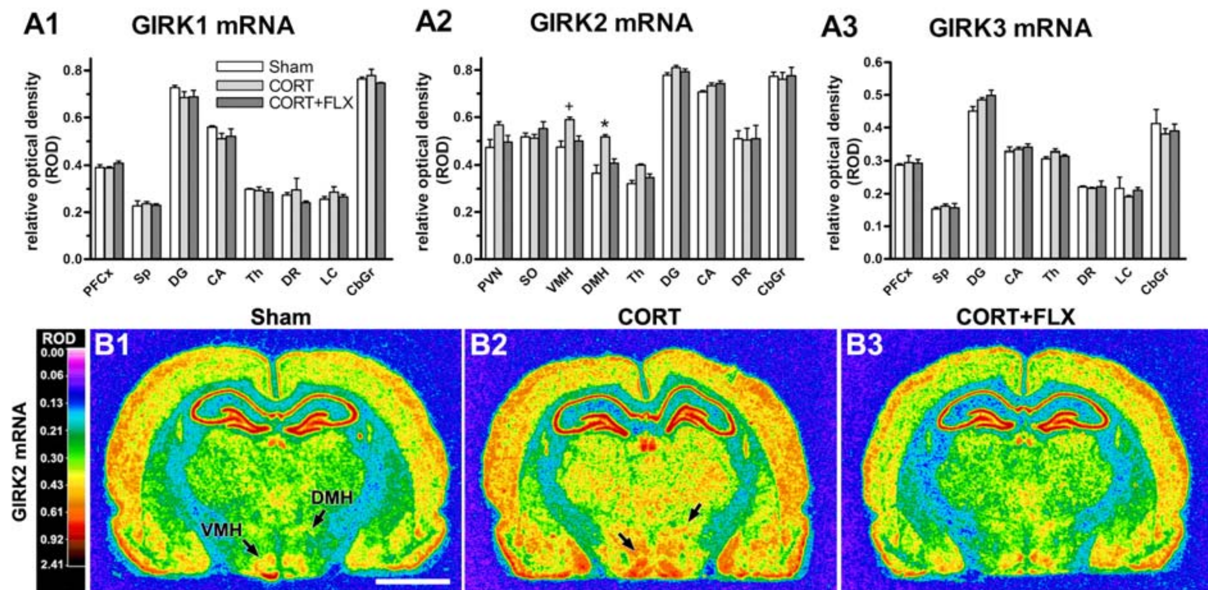
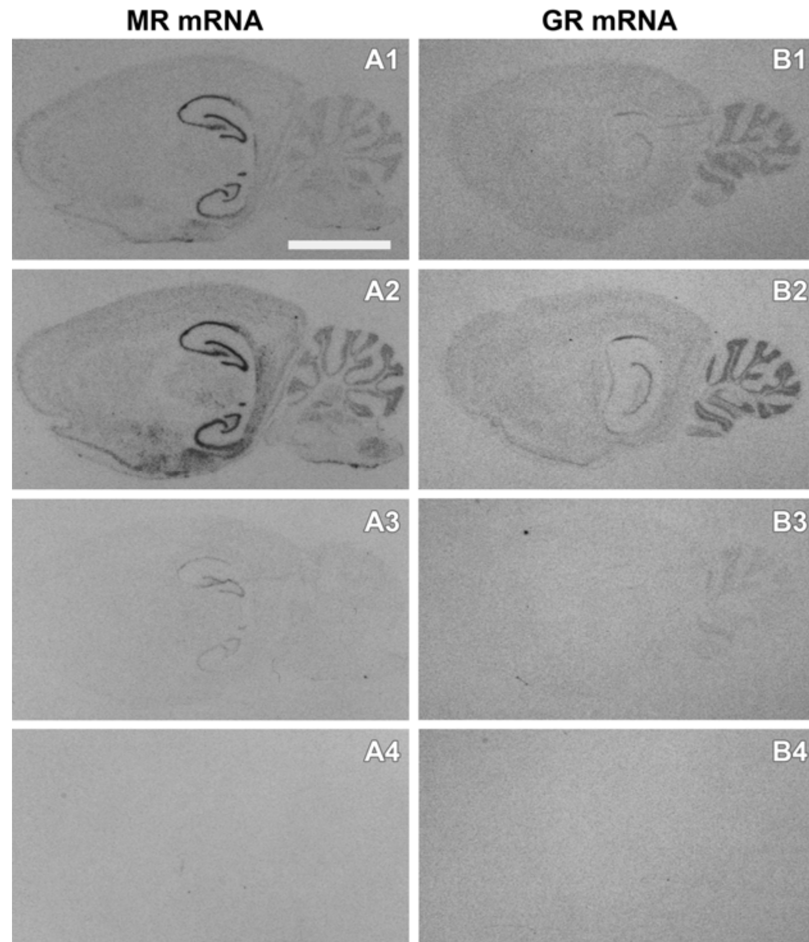


Fig. 4.- Effects of corticosterone treatment on GIRK1, GIRK2 and GIRK3 mRNA expression. Microdensitometric measurements (mean±SEM, n=4 per group) of GIRK1 (A1), GIRK2 (A2) and GIRK3 (A3) mRNA levels measured in different brain areas in sham controls, and in animals treated with corticosterone alone (CORT) or in combination with fluoxetine (CORT+FLX). Two-way ANOVA analysis revealed significant effects on GIRK2 mRNA expression (treatment effect $F_{2,81}$=10.31, P=0.0001). Bonferroni posttest indicated a significant increase in GIRK2 mRNA expression in the hypothalamic ventromedial (VMH, $^{+}P<0.05$) and dorsomedial (DMH, *P<0.001) nuclei elicited by corticosterone treatment, that was reversed by concomitant fluoxetine treatment. Changes in GIRK2 mRNA expression are shown in digital colour-coded images obtained from autoradiograms of coronal brain sections (B1-B3). Bar=3 mm.

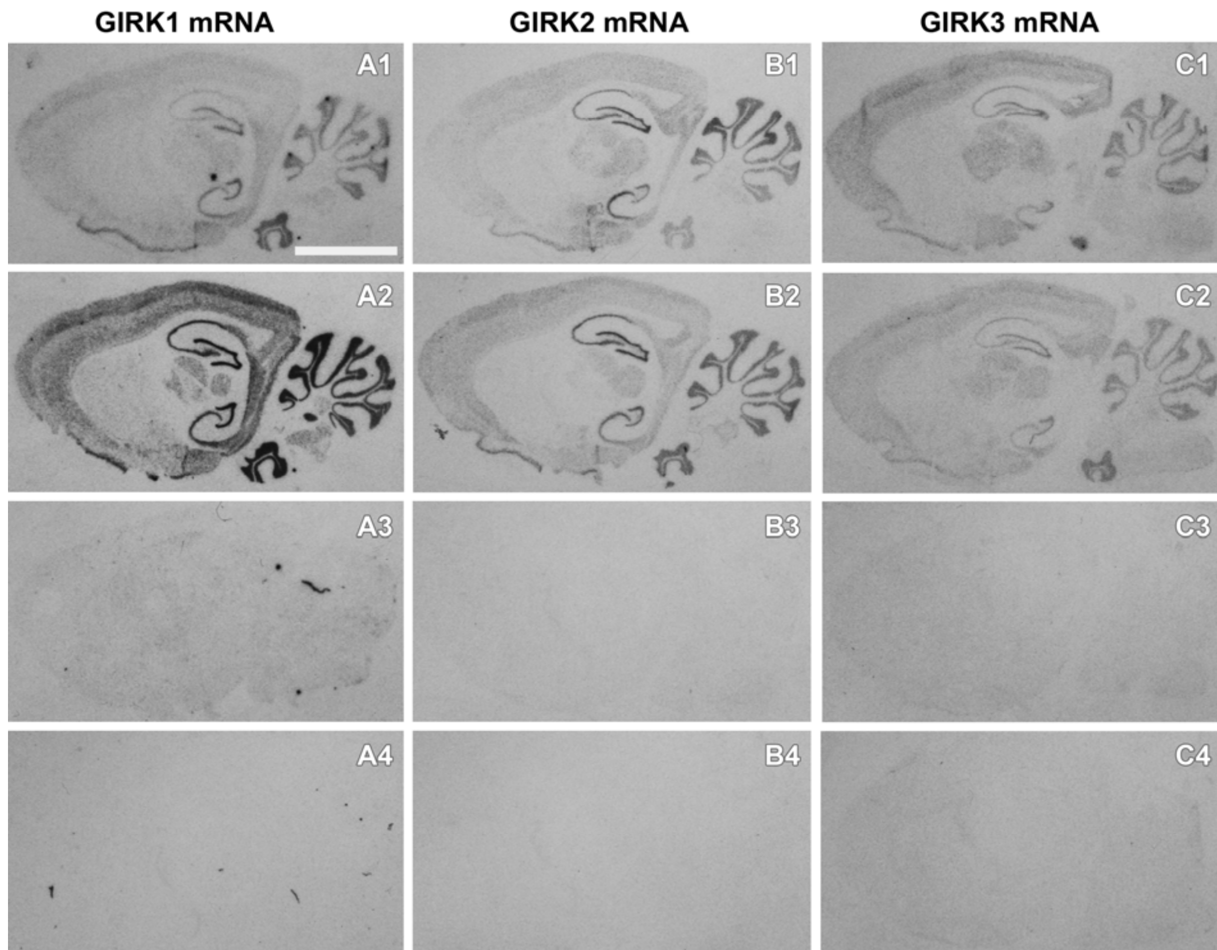
Supplementary Material:



Supplementary Figure S1. Specificity controls of MR and GR oligonucleotide probes.

The control tests illustrated here were performed using probes complementary to bases 3116-3160 (A1) and 3356-3400 (A2-A4) of MR mRNA (GenBank acc. no. M36074.1), and bases 70-120 (B1) and 2221-2265 (B2-B4) of GR mRNA (GenBank acc. no. M14053.1).

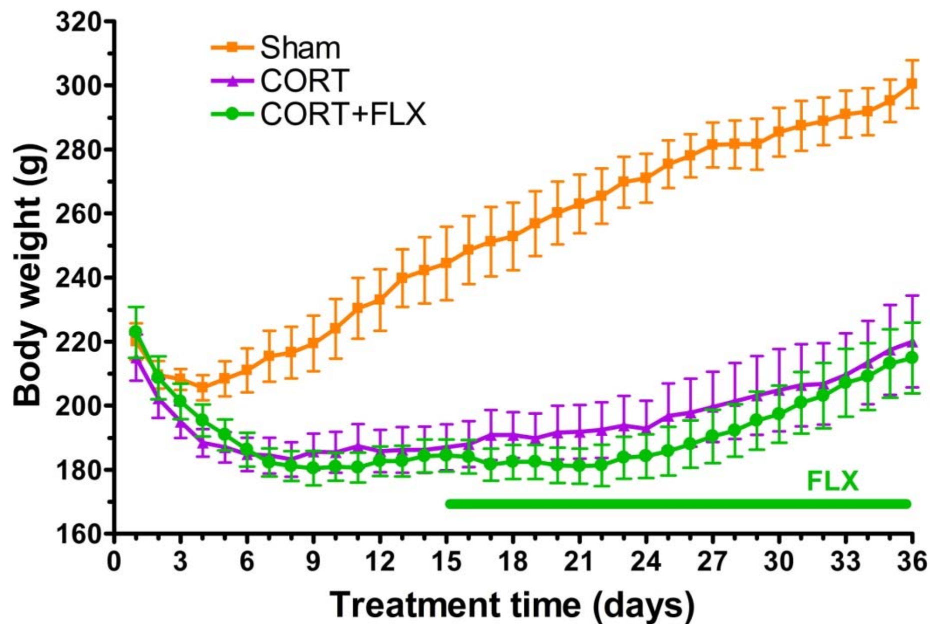
Identical patterns of hybridization signal distribution are obtained when using separately two different ^{33}P-labeled oligonucleotide probes specific for MR mRNA (**A1, A2**) or for GR mRNA (**B1, B2**). Hybridization signal shown is abolished when washing is performed at 80°C (**A3, B3**). Hybridization of ^{33}P-labeled probe is prevented in the presence of an excess (50-fold) of the same unlabeled oligonucleotide probe in the incubation buffer (**A4, B4**). Bar= 5 mm.



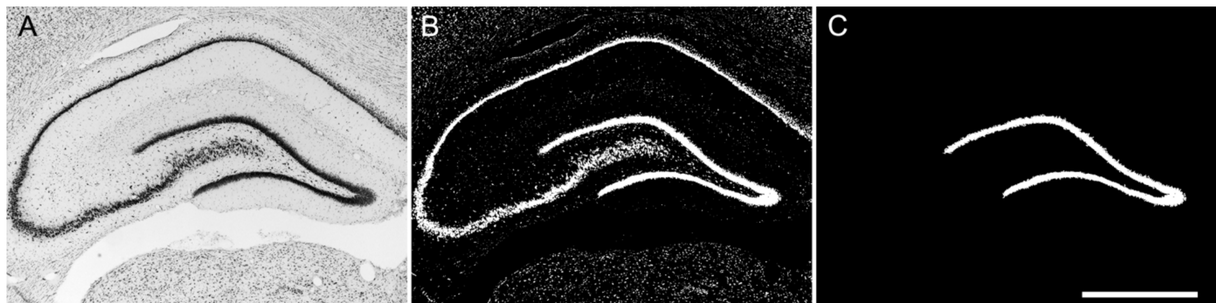
Supplementary Figure 2. Specificity controls of GIRK1-3 oligonucleotide probes.

The control tests illustrated here were performed using probes complementary to bases 8-31 (A1) and 137-181 (A2-A4) of GIRK1 mRNA (GenBank acc. no. U01071.1), bases 1401-1446 (B1) and 219-263 (B2-B4) of GIRK2 mRNA (GenBank acc. no. AB073755.1), and bases 1398-1442 (C1) and 276-320 (C2-C4) of GIRK3 mRNA (GenBank acc. no. L77929.1).

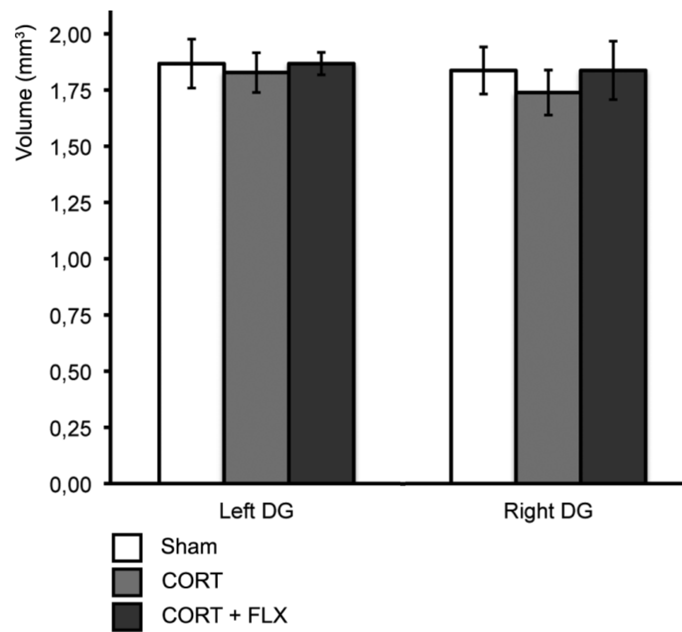
Identical patterns of hybridization signal distribution are obtained when using separately two different ^{33}P-labeled oligonucleotide probes specific for GIRK1 mRNA (**A1, A2**), GIRG2 mRNA (**B1, B2**) or GIRK3 mRNA (**C1, C2**). Hybridization signal shown is abolished when washing is performed at 80°C (**A3, B3, C3**). Hybridization of ^{33}P-labeled probe is prevented in the presence of an excess (50-fold) of the same unlabeled oligonucleotide probe in the incubation buffer (**A4, B4, C4**). Bar= 5 mm.



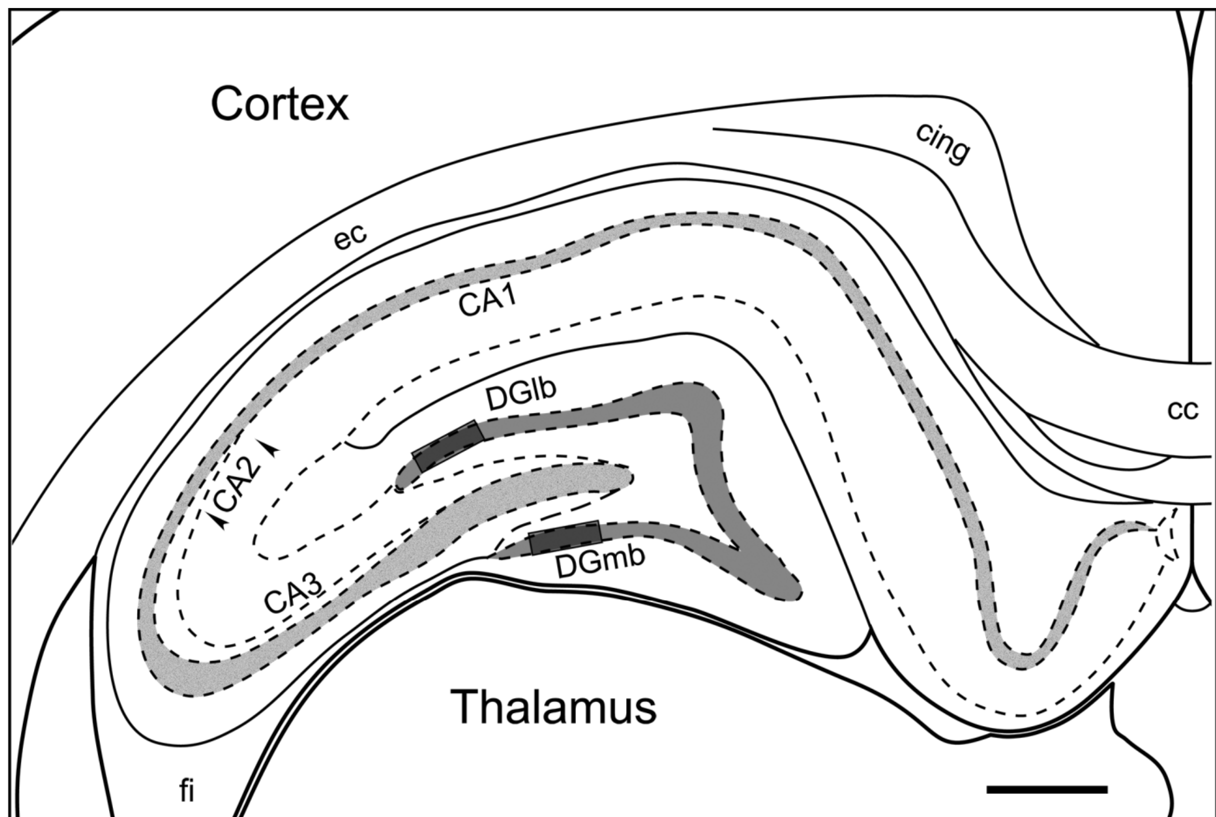
Supplementary Figure 3. Daily weight control of animals over chronic treatment with corticosterone pellets (200 mg). See text for details on treatment groups. Every animal was weighted daily at 9 am. Chronic treatment (sham n=10; treated n=10; fluoxetine-treated n=9). Note that all animal groups treated with corticosterone lost more weight after surgery than the corresponding controls. Data are mean±SEM.

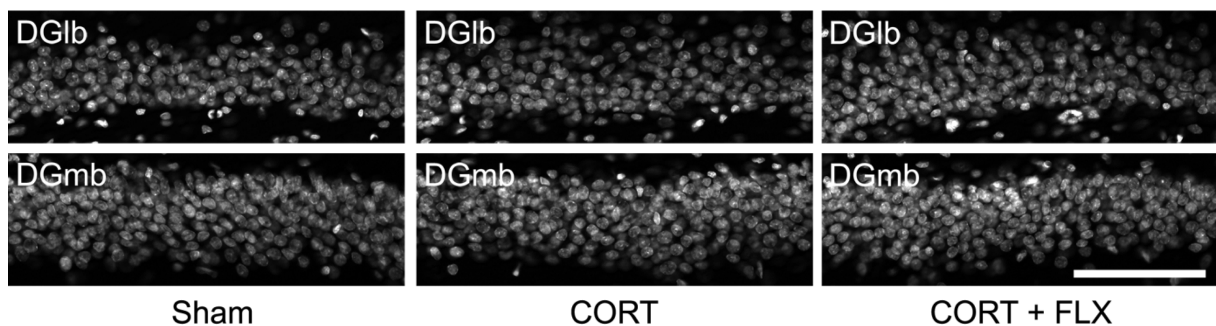


Supplementary Figure 4. Steps of the image processing to measure the volume of the granular layer of the rat dentate gyrus. **a**: Micrograph of a Nissl-counterstained coronal section of the rat hippocampus; **b**: Binary image resulting from threshold operation; **c**: Binary image generated after removing particles outside the granular layer of the dentate gyrus. Scale bar=1 mm.

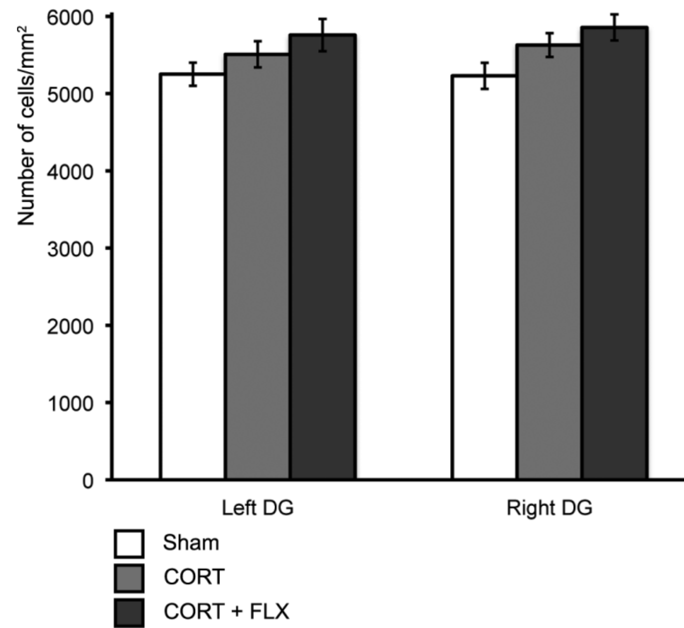


Supplementary Figure 5. Volume of granular layer of the rat dentate gyrus. The bar graph shows mean values for the total volume of the granular layer of the left and right dentate gyri in sham control, chronic corticosterone and chronic corticosterone+fluoxetine-treated groups. Data are mean±SEM (n=5 in all groups). No significant differences were observed among groups.





Supplementary Figure 6. Depiction of counting boxes used for quantification of cell nuclei in the granular layer of the rat dentate gyrus. **Upper panel**: Schematic drawing of a coronal section of the rat hippocampus to depict counting boxes used for quantification of cell nuclei in the granular layer of the left and right dentate gyri in sham control, chronic corticosterone and chronic corticosterone+ fluoxetine-treated groups. **Lower panel**: Representa-tive ApoTome images of Hoechst-stained nuclei of the lateral and medial blades of the granular layer of the rat dentate gyrus used for quantification. CA1-3, fields CA1-3 of the Ammmon's horn; cc, corpus callosum; cing, cingulum bundle; DGlb, lateral blade of dentate gyrus; DGmb, medial blade of dentate gyrus; ec, external capsule. Scale bars: upper 500 µm; lower 100 µm.



Supplementary Figure 7. Counting of Hoechst-stained nuclei in the granular layer of the rat dentate gyrus. The bar graph shows mean values for Hoechst-stained nuclei in the granular layer of the left and right dentate gyri in sham control, chronic corticosterone and chronic corticosterone + fluoxetine-treated groups. Data are expressed as number of cells per mm^2. Data are mean±SEM (n=5 in all groups). No significant differences were observed among groups.