Regulation of corticosteroid receptor gene expression in depression and antidepressant action

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Based on the Heinz Lehmann Award Lecture presented by Dr. Barden at the 20th Annual Meeting of the Canadian College of Neuropsychopharmacology, July 13–17, 1997, in Cambridge, England.

Objective: Major alterations of the hypothalamic–pituitary–adrenocortical (HPA) system are often seen in patients with depression, and can be reversed by successful antidepressant therapy. Persuasive evidence points to the involvement of a dysfunctional glucocorticoid receptor system in these changes. The authors developed a transgenic mouse to determine the mechanism for these changes. **Design:** In vivo and in vitro animal experiments. **Animals:** Transgenic mice expressing glucocorticoid receptor antisense RNA and control mice. **Interventions:** In vivo: hormone assays and dexamethasone suppression tests; in vitro: cell transfection, chloramphenicol acetyl transferase assay, Northern blot analysis, binding assays of cytosolic receptor. **Outcome measures:** Indicators of depressive disorder in transgenic mice, effect of antidepressant therapy on dexamethasone binding in transgenic mouse hippocampus, mouse behaviour, and glucocorticoid receptor activity. **Results:** Transgenic mice showed no suppression of corticosterone with a dose of 2 mg per 100 g body weight dexamethasone. Treatment with amitriptyline reduced levels of corticotropin and corticosterone, increased glucocorticoid receptor mRNA concentrations and glucocorticoid binding capacity of several brain areas, and reversed behavioural changes. In vitro experiments also showed that desipramine increased glucocorticoid receptor mRNA. **Conclusion:** These transgenic mice have numerous neuroendocrine characteristics of human depression as well as altered behaviour. Many of these neuroendocrinologic and behavioural characteristics are reversed by antidepressants. The antidepressant-induced increase in glucocorticoid receptor activity may render the HPA axis more sensitive to glucocorticoid feedback. This new insight into antidepressant drug action suggests a novel approach to the development of new antidepressant drugs.

Objectif : On constate souvent, chez des patients atteints de dépression, des altérations majeures du système hypothalamo–hypophyso–surrénalien qu'il est possible d'inverser par une thérapie aux antidépresseurs. Des preuves convaincantes indiquent qu'une dysfonction des récepteurs des glucocorticoïdes joue un rôle dans ces changements. Les auteurs ont créé une souris transgénique pour déterminer le mécanisme de ces changements. **Conception :** Études sur des animaux in vivo et in vitro. **Animaux :** Souris

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Medical subject headings: amitriptyline; antidepressive agents; corticotropin-releasing hormone; depressive disorder; dexamethasone; disease models, animal; hypothalamic hormones; mice, transgenic; receptors, glucocorticoid

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transgéniques exprimant l'ARN antisens des récepteurs des glucocorticoïdes et souris témoins. **Interventions :** In vivo : dosages hormonaux et épreuves de freinage à la dexaméthasone. In vitro : transfection de cellules, épreuve à l'acétyltransférase du chloramphénicol, technique de Northern, épreuve de fixation des récepteurs cytosoliques. **Mesures de résultats :** Indicateurs des troubles dépressifs chez des souris transgéniques, effet d'une thérapie aux antidépresseurs sur la fixation de la dexaméthasone dans l'hippocampe de souris transgéniques, comportement des souris et activité des récepteurs des glucocorticoïdes. **Résultats :** Les souris transgéniques n'ont montré aucune suppression de la corticostérone avec une dose de dexaméthasone de 2 mg par 100 g de masse corporelle. Le traitement à l'amitriptyline a réduit les taux de corticotrophine et de corticostérone, augmenté les concentrations d'ARNm récepteurs des glucocorticoïdes et la capacité de fixation des glucocorticoïdes de plusieurs zones du cerveau, et inversé les changements de comportement. Les expériences in vitro ont aussi montré que la désipramine augmente le taux d'ARNm récepteurs des glucocorticoïdes. **Conclusion :** Ces souris transgéniques ont présenté de nombreuses caractéristiques neuro-endocriniennes de la dépression chez les êtres humains, ainsi que des changements de comportement. Un grand nombre de ces caractéristiques neuro-endocrinologiques et comportementales sont inversées par des antidépresseurs. L'augmentation de l'activité des récepteurs des glucocorticoïdes produite par les antidépresseurs peut sensibiliser davantage l'axe hypothalamo–hypophyso–surrénalien à la rétroaction des glucocorticoïdes. Cette nouvelle connaissance de l'action des antidépresseurs évoque une nouvelle façon d'aborder la mise au point de nouveaux antidépresseurs.

Introduction

Hypothalamic–pituitary–adrenocortical (HPA) system regulation

The driving force for HPA activation is hypothalamic corticotropin-releasing hormone (CRH), acting in synergy with vasopressin (produced in either the same or distinct neurons of the paraventricular nucleus [PVN]) to enhance release of pituitary pro-opiomelanocortin (POMC)–derived peptides (corticotropin [ACTH] and endorphins). Adrenal glucocorticoid hormone secretion (corticosterone in rodents, cortisol in humans) is stimulated mainly by ACTH, although the adrenocortical sensitivity to ACTH may be modified by sympathetic innervation of the adrenal gland.[1] Different regulatory forces are superimposed on this system to coordinate adrenal secretions during periods of inactivity and stress. The first of these is a circadian rhythm of basal activity derived from the suprachiasmatic nucleus.[2] Stress-induced responses of the HPA system involve afferent inputs from numerous other brain regions, including noradrenergic innervation from the brain stem A1 and A2 cell groups, the pontine locus ceruleus,[3] the amygdala,[4,5] the cerebral cortex and the hippocampus.[6] In general, the septum and hippocampus have inhibitory actions on HPA activity, while the effect of the amygdala is largely permissive. Another regulatory force on HPA system activity is provided by feedback inhibitory actions of adrenal steroids exerted through corticosteroid receptors located in various brain areas.

Glucocorticoid hormones terminate the stress response by negative feedback action at the level of the pituitary, hypothalamus and limbic brain areas, including hippocampus, amygdala and septum. This action is mediated by 2 types of corticosteroid receptors, which have been identified[7] and their complementary DNA cloned.[8,9] The type I or mineralocorticoid receptor (MR) has a higher affinity (dissociation constant [Kd] ~ 0.2 nmol/L) and lower capacity for glucocorticoids than the type II or glucocorticoid receptor (GR) (Kd ~ 2–5 nmol/L). MR is expressed either alone or together with GR mainly in hippocampal neurons, while GR is distributed more ubiquitously in the brain,[10] particularly in neurons. This dual system may be necessary to cope with corticosteroid concentrations ranging from 0.5 to 50 nmol/L during the diurnal cycle and up to 100 nmol/L or more in response to stress.[11] An adequate physiologic response to such a wide hormone concentration range can be achieved by these 2 receptors, since the MR mediates the effects of, and possibly controls, low basal circadian levels of circulating glucocorticoids[7,12] and the GR appears to mediate the effects of high stress levels of glucocorticoids and to be responsible for the negative feedback effects of glucocorticoids on the HPA system.[7,13–15] At basal levels of corticosterone, the MR is approximately 80% occupied. The GR becomes extensively occupied only with stress levels of corticosterone or during diurnal peaks.

HPA disturbances in affective disorders

Disturbed regulation of the hypothalamic–pituitary–adrenocortical (HPA) system is often demonstrated by alterations of neuroendocrine function in patients suffering from severe depression. Features of affective disorders secondary to Cushing's syndrome are frequently indistinguishable from those of primary major depressive disorder.[16] In certain patients with depression, cortisol secretion may be increased and may show an abnormal 24-hour secretory pattern. Unless exogenous CRH is administered, this pattern may be resistant to suppression by exogenous steroids.[17–19] Somewhat surprisingly, very similar changes are also seen during the manic phase of bipolar illness.[20] Lack of cortisol suppression by dexamethasone in patients with depression could indicate primary hyperactivity of the adrenal glands rather than a central defect, but recent studies using CRH have eliminated this possibility.[21–24] A key role of elevated CRH secretion in depression is indicated by both clinical[25] and preclinical[26,27] studies, and the ability of alprazolam to inhibit CRH secretion may explain why this benzodiazepine derivative has unusual antidepressant activity.[28] In patients with depression, the following findings have been observed: elevated concentrations of CRH in the cerebrospinal fluid,[29] increased numbers of CRH-containing cells in the PVN,[30] decreased CRH binding in the frontal cortex[31] and blunted ACTH response to an intravenously administered test dose of CRH.[23,32] These findings are believed to reflect the desensitization of CRH receptors at corticotropic cells or a restricted secretory response of ACTH to CRH, caused by elevated basal cortisol levels. The latter mechanism is probably the most important and is underscored by a normalized net ACTH output in patients with depression pretreated with metyrapone.[33,34] Despite blunted ACTH response to CRH, the associated cortisol response is unchanged because continuing HPA overactivity gradually produces adrenocortical hyperplasia, rendering the gland hypersensitive to ACTH. Other non-ACTH mechanisms, such as neural sympathetic factors or humoral factors from the immune system, may also contribute to the dissociation between ACTH and cortisol in depression.

Is disturbed glucocorticoid feedback a trait of affective disorders?

Although a genetic predisposition may be necessary for affective disorders to develop, stressful life events often trigger their onset. To avoid detrimental consequences, the cascade of effects that constitutes the adaptive response to this stress must be effectively terminated. The mechanisms for this appear to be defective in patients with depression.[35–39]

Elimination of primary adrenal gland hyperactivity[21–24] now points to a key role for elevated CRH secretion[25–27] in the mechanism leading to the failure of dexamethasone to suppress cortisol in patients with depression. Diminished corticosteroid receptor concentrations, caused by a malfunctioning of the systems involved in the regulation of their gene expression, could explain many of the findings concerning the altered HPA system in depression. Although there is no direct evidence of reduced receptor concentrations in the brain of patients with depression (because of degradation, it has proved impossible to measure corticosteroid receptors in postmortem brain), many circumstantial findings point to defective feedback action of cortisol in depression.[35–40] A decreased concentration of GR in the lymphocytes of patients with depression underlines this possibility.[40] What remains to be elucidated is whether a primary disturbance occurs at the level of corticosteroid receptors in the limbic brain (MR and GR in hippocampus, GR in hypothalamus), thus modifying the fine-tuning of ACTH secretion via CRH, vasopressin and POMC regulation. Alternatively, other factors that drive the expression of CRH and vasopressin and their release into hypophyseal portal blood could result in an excess of ACTH and cortisol, which secondarily decreases corticosteroid receptor capacity and function. Whether the corticosteroid-receptor disturbance is the cause or the consequence of a "CRH hyperdrive" of HPA system activity is not yet known.

To help answer these questions, we have developed a transgenic mouse that has a disturbed glucocorticoid receptor function as a primary disturbance. Several characteristics of this mouse, including a hyperactive HPA system and behavioural changes sensitive to correction by antidepressant therapy, underscore the critical role of a disturbance of corticosteroid receptor regulation in the etiology of depressive illness. Since the abnormal (nonsuppression) response of serum cortisol levels to dexamethasone returns to normal (suppression) during antidepressant therapy,[41,42] and since antidepressants can render the HPA axis more sensitive to inhibition by dexamethasone,[43] we postulated that antidepressant drugs could modify the glucocorticoid re-

ceptor system of brain areas involved in control of the HPA axis.[44,45]

Methods

In vivo experiments

Animals

Transgenic mice expressing glucocorticoid receptor antisense RNA were produced in our laboratory, as described elsewhere,[46] and bred as an homozygous line (line 1.3). Control, B6C3F1 hybrid mice were obtained from Charles River Canada. All experiments were performed with 8- to 12-week-old animals weighing between 25 and 30 g. Although transgenic mice tend to have increased fat deposition, young adult mice show no significant difference in body weight compared with age-matched controls. The mice were housed 6 per cage under standard conditions with a 12-hour light/12-hour dark cycle (lights on from 7:30 am to 7:30 pm) and received food and water ad libitum. One day before the experiment, the animals were separated and placed 2 to each cage to avoid uncontrolled stress reactions. Amitriptyline, if administered, was given at a dosage of 10 mg per kg body weight every day for 3 weeks.

Hormone assays

Trunk blood was collected into ice-cold EDTA-coated tubes and plasma prepared and stored at either -80°C or -20°C before radioimmunoassay of ACTH and corticosterone, respectively, using kits provided by ICN Biomedicals (Costa Mesa, Calif.). Inter- and intra-assay coefficients of variability were 7% and 5% for ACTH and 7% and 4% for corticosterone, respectively. The limits of detection were 2 pg/mL for ACTH and 1.5 ng/mL for corticosterone.

Dexamethasone suppression tests

For the dexamethasone suppression tests, animals were injected intraperitoneally with dexamethasone (2 g per 100 g body weight) in 0.3 mL of 0.9% saline solution containing 0.5% ethanol. Controls received the vehicle alone. The injections were performed between 8:00 am and 8:30 am, and animals were killed 1 to 6 hours later by decapitation after rapid anaesthesia with halothane (a procedure known not to change plasma hormone levels[47]).

In vitro experiments

Cell cultures

Mouse fibroblast (Ltk-) and neuroblastoma (Neuro 2A) cell lines obtained from American Type Culture Collection (ATCC, Manassas, Va.) were used in these studies. Ltk- cells were grown in minimum essential α medium and Neuro 2A cells were grown in minimum essential medium, supplemented with 10% fetal calf serum (Gibco BRL, Grand Island, NY). All transfected cultures were maintained at 37°C in a 5% carbon dioxide–95% air atmosphere. Before transfection, cells were washed with an isotonic buffer (phosphate-buffered saline [PBS]) and then incubated in tissue culture medium, with or without test substances.

Cell transfection

Reporter plasmids were expressed either transiently or after stable integration in the host cell genome.[48] For transient expression assays, the reporter plasmids pMMTVCAT (5 g) or pHGR2.7CAT (1 g) were cotransfected with 2.5 g of pRSV-*lacZ* (*Escherichia coli lacZ* gene, which encodes for β-galactosidase, fused to the Rous sarcoma virus long terminal repeat) by the calcium phosphate technique.[49] The reporter plasmid pMMTVCAT consists of the chloramphenicol acetyl transferase (CAT) gene fused to mouse mammary tumour virus long terminal repeat (MMTV LTR) and confers glucocorticoid inducibility in Ltk- and Neuro 2A cells. The reporter plasmid pHGR2.7CAT consists of the same CAT gene under control of a 2.7-kb fragment of the glucocorticoid receptor gene promoter region, constructed as described elsewhere.[50] Cells were treated with 10^{-6} mol/L desipramine for 1 to 4 days and then transfected with plasmid. Cells transfected with pMMTVCAT were incubated with dexamethasone (10^{-6} mol/L) for 24 hours before harvesting for assay of β-galactosidase and CAT activities.

Stable transfectants were produced by coprecipitating the reporter plasmid (20 g pMMTVCAT or pHGR2.7CAT) and a neomycin resistance vector (pRSVNEO, 0.4 g) with calcium phosphate[49] on 0.5 × 106 Ltk- or Neuro 2A host cells.[51] After 24 hours, and every 3 days thereafter, the medium was replaced with

fresh medium containing the neomycin analogue G418 (155 g/mL for Ltk- cells and 180 g/mL for Neuro 2A cells) to select for neomycin-resistant clones. Cells that had stably integrated either pMMTVCAT or pHGR2.7CAT into their genome were cloned. After 2 weeks, cell colonies were pooled and grown until used for treatment with 10^{-6} mol/L desipramine for 1 to 4 days.

CAT assay

Cells were harvested in 40 mmol/L tris-hydrochloric acid (pH 7.4), 1 mmol/L EDTA, 0.15 mol/L sodium chloride, pelleted and resuspended in 0.25 M tris-hydrochloric acid (pH 7.8). They were subsequently lysed by 3 cycles of freezing-thawing, centrifuged and the supernatants taken for protein, β-galactosidase and CAT assays. β-galactosidase activity was measured in an aliquot of the extract containing 50 mg of protein[52] by colorimetry at 420 nm after hydrolysis of 0.8 mg of 0-nitrophenyl-β-D-galactoside in 1 mL of 0.1 mmol/L sodium phosphate (pH 7.0), 10 mmol/L potassium chloride, 1 mmol/L magnesium sulfate, 50 mmol/L β-mercaptoethanol and was used to normalize the quantity of supernatant subsequently taken for the CAT assay. In transiently transfected cells, CAT activity was measured in a final volume of 150 μL (0.25 mol/L tris-hydrochloric acid, pH 7.8, 0.2 μCi of ^{14}C-chloramphenicol [Amersham], 0.5 mmol/L acetyl coenzyme A) incubated at 37°C for 45 minutes. Acetylated forms of ^{14}C-chloramphenicol were separated by thin-layer chromatography on Whatman LK6D plates and quantified by liquid -scintillation spectroscopy. CAT activity measurements in stable transfectants were performed with cell extracts containing equal amounts of protein.[53]

Northern (RNA) blot analysis

RNA was prepared from cells (15 to 20 × 10^6) or tissue by the guanidium isothiocyanate method,[54] separated on 0.8% agarose-formaldehyde denaturing gels and blotted onto nylon filters (Hybond N, Amersham) prior to hybridization with glucocorticoid receptor or CRH cRNA antisense probes. Measurement of β-actin mRNA on the same filters was used to control for variations in total RNA amounts between samples. Glucocorticoid receptor cRNA antisense probe was produced by T7 polymerase runoff transcription with [^{32}P]-UTP of a 1815-bp glucocorticoid receptor cDNA fragment[8] subcloned into plasmid pGEM-1. The β-actin cRNA probe was generated from a 1500-bp β-actin cDNA Pst 1 fragment[55] inserted into pGEM-1. The CRH cRNA probe was transcribed from a Pst1 fragment of exon II of the mouse CRH gene subcloned in pGEM4Z. Filters were prehybridized for 4 hours at 42°C in a mixture of 50% formamide, 5 × SSC (0.15 mol/L sodium chloride, 0.015 mol/L sodium citrate), 6× Denhardt solution, 0.1% sodium dodecyl sulfate (SDS), 50 mmol/L sodium phosphate, 200 μg of yeast t-RNA/mL and 200 μg of denatured salmon sperm DNA/mL and hybridized at 65°C for 20 hours. After hybridization, the filters were washed twice for 30 minutes each in 2 × SSC containing 0.1% SDS at room temperature and twice (1 hour each) in 0.1 × SSC–0.1% SDS at 70°C, dried slightly and exposed to Kodak X-Omat film. The autoradiograms were evaluated by combined area and density measurement using a digital video image analyzer, and final results were expressed as a quotient of integrated grey values taken from experimental band (GR on CRH) and its corresponding β-actin autoradiograms.

In vitro binding assays of cytosolic receptor

Glucocorticoid binding was measured with [^{3}H]-dexamethasone as ligand. Cells (1.5 × 106 cells per flask) or brain tissue was rinsed with an isotonic buffer (PBS) and disrupted in 30 mmol/L tris, 1 mmol/L EDTA, 10 mmol/L molybdate, 10% (v/v) glycerol and 1 mmol/L dithiothreitol (TEDGM, pH 7.4). After centrifugation at 55 000 rpm for 15 minutes at 4°C, an aliquot of the cytosol was incubated with 10 nmol/L [^{3}H]-dexamethasone (spec. act. 44.7 Ci/mmol, New England Nuclear, Boston) for 20 to 24 hours at 4°C and the nonspecific binding determined in parallel incubations containing a 200-fold excess of the unlabeled type II glucocorticoid-receptor-specific agonist RU 28362. Sephadex LH20 (Pharmacia) columns (7 × 1 cm; equilibrated with TEDGM buffer) were used to separate bound from unbound steroid before quantification by liquid scintillation spectroscopy.

Statistical analysis

The statistical significance of any difference between groups was analyzed with the Duncan-Kramer multiple range test[56] after an analysis of variance (ANOVA).

Results

Transgenic mouse model of the neuroendocrine changes associated with affective disorders

Introduction of genes into the germ line of mice offers the possibility of generating animal models of human genetic disease and of verifying whether a defective glucocorticoid receptor system could induce the neuroendocrinologic characteristics of depressive disorders. We produced a transgenic mouse with decreased GR in the brain[46] by inserting into the mouse genome an inverted 1815-bp fragment of the 3′ noncoding region of the glucocorticoid receptor cDNA downstream from a 2.3-kb EcoR1/Hind III human neurofilament gene promoter element (Fig. 1). We had previously verified in cell cultures the ability of this antisense RNA-generating construction to block normal GR mRNA formation or processing and translation.[57] Transgenic mice that express GR antisense RNA have HPA system changes characteristic of those seen in depression, including increased HPA activity, a resistance to suppression of adrenocortical secretions by dexamethasone,[46,58] feeding disturbances,[59] and behavioural[60,61] and cognitive[62] deficits. While normal mice show a circadian cycle in blood corticosterone and ACTH levels, with low levels in the morning and higher values in the evening, transgenic mice showed a flattening of this circadian cycle of secretion. This sort of phase shift is also a characteristic of patients with depression.[63] Two hours after an intraperitoneal injection of 2 g or more of dexamethasone per 100 g body weight, the plasma corticosterone concentrations of normal mice were suppressed to almost nondetectable levels. This effect was maintained for up to at least 6 hours (Fig. 2). In the transgenic mice, however, no suppression of corticosterone was seen with a dose of 2 g of dexamethasone per 100 g body weight, and a 10-fold higher dose (20 g per 100 g) was needed before a significant corticosterone sup-

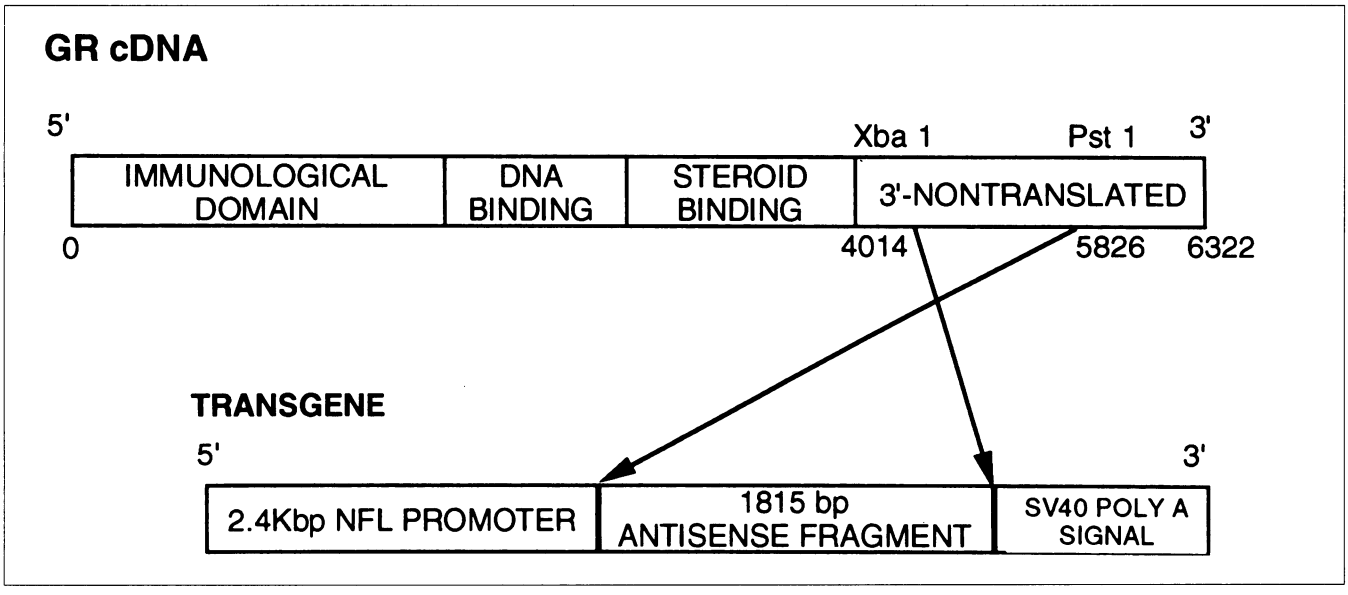

Fig. 1. Structure of the neurofilament promoter–glucocorticoid receptor (GR) antisense RNA construct. This construct directed the transcription of antisense RNA with a sequence complementary to that of regions of the mouse glucocorticoid mRNA. Plasmid NFL-ASGR was constructed by cloning a 1815-bp fragment of the 3′ noncoding region of the rat glucocorticoid receptor cDNA in the reverse orientation downstream from the human neurofilament gene promoter. The plasmid containing the human neurofilament L gene promoter (pNF-L, gift of Dr. J.P. Julien) was linearized by digestion with Hind III, which cleaves at a sequence at the 3′ end of the promoter. Extruding sequences were filled in with the Klenow fragment of DNA polymerase and a blunted Xba 1-Pst 1 fragment of the pSG-1 plasmid (a derivative of pRM16{42}, a gift of Dr. R. Miesfeld) was inserted into pNF-L in a reverse orientation. Finally, we added a Pst 1-Bam H1 fragment of the VP1 gene of SV40, which contains a polyadenylation signal. cDNA sequences containing the least degree of homology with other steroid receptors (3′ nontranslated region for glucocorticoid receptor) were used to generate antisense RNA and thus avoid cross-inhibition of other steroid receptor activity.

pression was attained.[58,64] In addition, the adrenal glands of the transgenic mice were slightly hypertrophic, as judged from their higher protein content.[65] These findings in transgenic mice thus support the hypothesis that a dysfunctional glucocorticoid receptor system is involved in the neuroendocrine components of affective disorders and suggest that the transgenic mouse is an appropriate model to study antidepressant response.

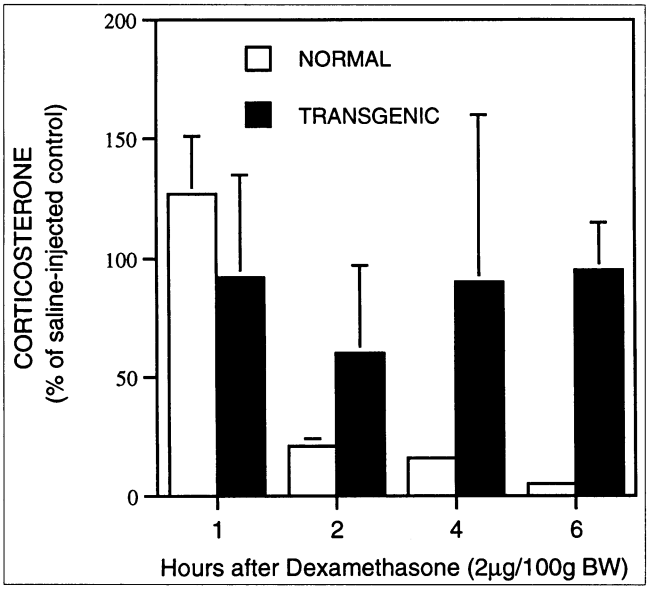

Fig. 2. Dexamethasone suppression test. Control and transgenic mice were killed 1 to 6 hours after an injection of dexamethasone (2 g per 100 g body weight), and trunk blood was collected for corticosterone assay in EDTA-coated tubes. The susceptibility of transgenic and normal mice to the suppressive effect of dexamethasone on corticosterone differed greatly. Almost complete suppression was obtained in normal (white bars) but not in transgenic (black bars) mice between 2 hours ($p < 0.05$) and 6 hours ($p < 0.01$) after dexamethasone administration. The results are shown as means and standard errors ($n = 5$). Statistical differences between means was calculated by the Duncan-Kramer test after an analysis of variance.

Reversal of the neuroendocrine and behavioural changes in transgenic mice by antidepressants

Treatment of transgenic mice with the antidepressant amitriptyline for 14 days reduced the concentrations of both ACTH and corticosterone (Table 1). Somewhat surprisingly, in view of a constant antisense RNA expression,[66] this treatment with the same or a different[66] antidepressant increased the GR mRNA concentrations and glucocorticoid binding capacity of several brain areas, including the hippocampus (Fig. 3) and hypothalamus. These changes coincided with a decrease in hypothalamic CRH mRNA concentrations (Table 1), suggesting a mechanism for the restoration of the hyperactive HPA system to normal levels.

With the exception of performance in the Morris water maze,[60,67] all other behavioural changes in the transgenic mice, including performance in the elevated plus maze,[61,62,68] acoustic startle paradigm[61] and Porsolt forced swim test[62,69] (Fig.4) were reversed by long-term antidepressant treatment.

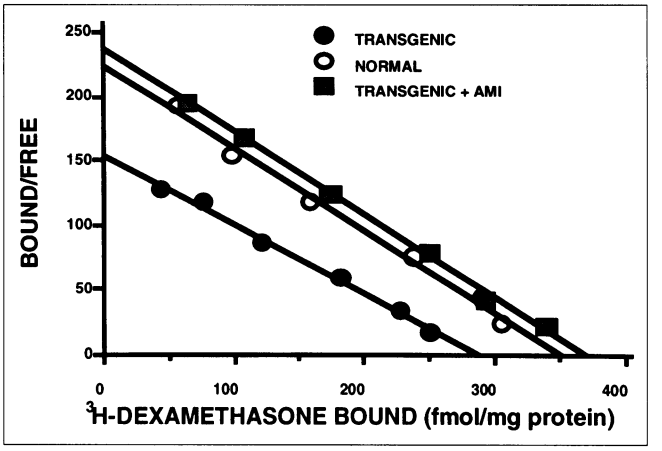

Fig. 3. Effect of amitriptyline on [³H]-dexamethasone binding in transgenic mouse hippocampus. Scatchard plots of saturation binding data for normal, transgenic and amitriptyline-treated transgenic mice are shown.

Table 1: Reversal of transgenic mouse hypothalamic–pituitary–adrenocortical system hyperactivity by amitriptyline

	Peptide/hormone level or ratio, mean (standard error of the mean) [*n*]		
Transgenic mouse group	Corticotropin (ACTH), pg/mL	Corticosterone, ng/mL	Ratio of corticotropin-releasing hormone mRNA to ß-actin
Control (vehicle)	198.3 (21.3) [29]	55.4 (13.3) [29]	0.50 (0.07)
Treated with amitriptyline	130.0 (15.8) [25]	26.9 (3.3) [28]	0.38 (0.01)

Antidepressant stimulation of glucocorticoid receptor gene expression

To investigate the hypothesis that antidepressants modulate glucocorticoid receptors, we examined their effects on glucocorticoid receptor gene expression in mouse fibroblast Ltk- cells and in mouse Neuro 2A neuroblastoma cells. Northern blot analysis of total RNA extracted from Neuro 2A cell cultures showed that treatment of cells with 10^{-6} mol/L desipramine for 24 hours produced an increase in glucocorticoid receptor mRNA concentrations (Fig. 5A). We have noted similar changes in glucocorticoid receptor mRNA concentrations induced by antidepressants in primary neuronal cultures derived from fetal-rat brain areas.[44] Lower concentrations of desipramine, equivalent to the levels of antidepressant drugs achieved in vivo during therapy, have been shown to produce the same effects on corticosteroid receptors.[50] Glucocorticoid binding activity was also increased by treatment of Neuro 2A cells with desipramine (Fig. 5B). To determine whether the antidepressant-induced increase in glucocorticoid receptor mRNA concentrations and glucocorticoid binding activity was actually associated with an increase in functional glucocorticoid receptors, we used the MMTV LTR fused to the CAT structural gene as reporter plasmid (pMMTVCAT) to measure the cellular response to dexamethasone. Glucocorticoid receptor, when bound to glucocorticoids, will interact with the glucocorticoid response elements localized in the MMTV LTR 70 and induce transcription of the CAT gene. We have previously demonstrated, by transfection of CV1 cells (cells that have no detectable glucocorticoid receptor and cannot mediate the dexamethasone induction of CAT activity) with different amounts of a glucocorticoid receptor expression vector (RSV glucocorticoid receptor), that MMTV-CAT promoter activity is proportional to the amount of functional glucocorticoid receptor present in the cell.[57] With the pMMTV-CAT chimeric construct, we observed a twofold increase in glucocorticoid-stimulated CAT activity when the cells were treated with desipramine (Fig. 5C). This increase in CAT activity in response to dexamethasone is comparable to the increase in glucocorticoid binding capacity of cells treated with antidepressants. Finally, to investigate more directly the site of antidepressant action, we have used a chimeric gene construct consisting of the glucocorticoid receptor gene promoter region fused to the CAT gene (pHGR2.7CAT). Up to a threefold increase in CAT activity was seen when cells stably transfected with pHGR2.7CAT were treated with desipramine (Fig. 5D). Desipramine had a dose-dependent effect on CAT activity directed by the 2.7-kbp glucocorticoid receptor gene promoter element, and maximal stimulation of CAT activity was achieved at a desipramine concentration of 10^{-8} mol/L,[50] which correlates well with the minimum plasma concentration necessary for clinical efficacy.[71] Antidepressant effects on glucocorticoid receptors are also evident after long-term (but not short-term) treatment of rodents, and regardless of the specificity of antidepressants on monoamine reuptake.[44] Together, these results support the hypothesis that antidepressants can increase glucocorticoid receptor gene expression and that this action could be the basis for antidepressant-induced restoration of HPA axis sensitivity to circulating glucocorticoid hormones in depressive illness.

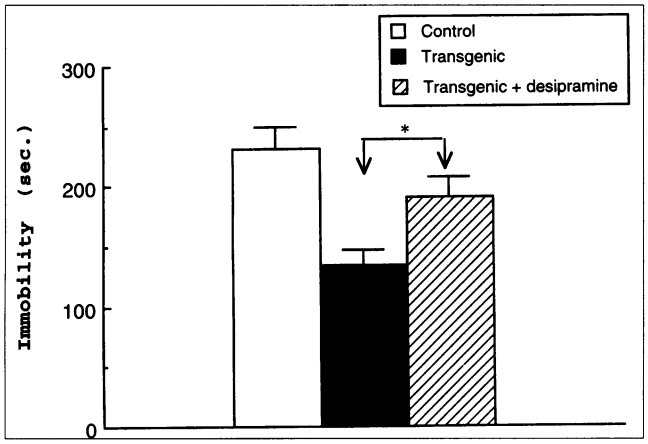

Fig. 4. Effect of long-term antidepressant therapy on transgenic mouse behaviour in the Porsolt forced swim test. Duration of immobility (in seconds) during a 6-minute observation period for normal, transgenic and desipramine-treated (10 mg per kg per day for 3 weeks) transgenic mice. * $p < 0.05$.

Discussion

A malfunctioning of systems involved in the regulation of corticosteroid receptor gene expression could explain the defective feedback action of cortisol and the hyperactive HPA system seen in patients suffering from severe depression. A primary disturbance in limbic brain corticosteroid receptors (MR and GR in the hippocampus and GR in the hypothalamus) could modify ACTH secretion via CRH and vasopressin. Al-

ternatively, an increased drive of CRH and vasopressin expression, and their release into hypophyseal portal blood, could result in excess levels of ACTH and cortisol, which secondarily decrease corticosteroid receptor capacity and function. We have designed a transgenic mouse with a primary defect in corticosteroid receptor function to test the first of the above alternatives, which would suggest that the corticosteroid receptor disturbance is the cause of the hyperactive HPA system seen in depression.

These GR-deficient transgenic mice exhibit neuroendocrine changes reminiscent of those seen in depression.[46] Their HPA system is activated at a time when, in normal mice, it is quiescent. This observation is comparable to the increased pulsatile ACTH and corticosterone secretions[72-74] accompanied by a phase shift of peak values seen in patients with depression.[63] The mice also show resistance to suppression of adrenocortical secretions by dexamethasone, which has also been noted in severe depression.[75,76] Despite blunted ACTH response to CRH, the associated cortisol response of patients with depression is unchanged because continuing HPA system overactivity gradually produces adrenocortical hypertrophy, rendering this gland hypersensitive to ACTH.[77-79] Our GR antisense transgenic mice do not, however, fulfil all the criteria for a perfect model of the neuroendocrine changes associated with affective disorders. For example, while in affective dis-

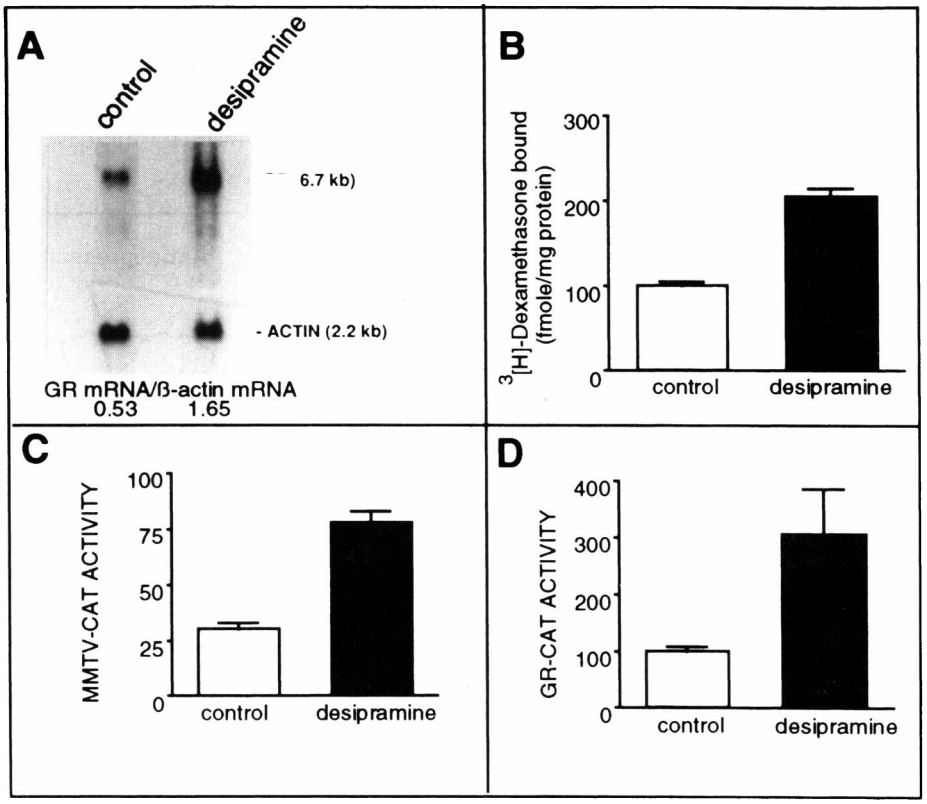

Fig. 5. Antidepressant effects on GR. A. Desipramine (10^{-6} mol/L for 1 to 4 days) increased the expression of the GR gene in Neuro 2A cells, as shown by increased GR mRNA levels. Total RNA was analysed by Northern blot hybridization with a GR cRNA and β-actin cRNA anti-sense. B. Desipramine elevated glucocorticoid binding activity, as measured in cell extracts using [^{3}H]-dexamethasone as ligand. C. Desipramine increased the capacity to respond to glucocorticoids, as measured by the reporter plasmid pMMTVCAT. Ltk- cells were incubated with 10^{-6} mol/L desipramine for up to 4 days before transient transfection with the pMMTVCAT plasmid. Dexamethasone (10^{-6} mol/L) was added 24 hours after transfection and the cells were harvested 24 hours later for assay of CAT activity (at constant β-galactosidase activity for each CAT assay). D. Promoter activity of the reporter plasmid pHGR2.7CAT, stably transfected in Ltk- cells and incubated with 10^{-6} mol/L desipramine for 24 hours.

orders the pituitary gland is refractory to CRH stimulation, in the GR antisense transgenic mice the reverse is true. Although adrenal hypertrophy is evident in the transgenic mice, this gland is evidently somewhat refractory to stimulation by ACTH.[80] The reasons for this are not clear, but may be related to some limited expression of antisense RNA in peripheral tissues.

The GR-deficient transgenic mice have been enormously useful in defining what eventually leads to the neuroendocrine changes associated with affective disorders. It is clear that a primary decrease in functional corticosteroid receptors can produce HPA system activation.[58,64,81] The subsequent increase in glucocorticoid levels could lead to secondary activation (or inhibition) of glucocorticoid-sensitive neurotransmitter gene expression, which could lead, in turn, to neurotransmitter imbalance. The transgenic mice are thus an appropriate model for studying some of the neuroendocrine and, possibly, behavioural symptoms of depression. They lend support to the hypothesis that an imbalance in glucocorticoid receptor system regulation is involved in the etiology of the neuroendocrine changes associated with depression, including the apparent lack of sensitivity to glucocorticoids.

The fact that antidepressant therapy can normalize cortisol levels and restore the suppressibility of cortisol by dexamethasone[36,41,42] suggests that the negative feedback action of cortisol at the limbic–hypothalamic level (possibly acting on CRH and vasopression secretion) is less effective in patients with depression and is restored to full efficiency by antidepressant therapy. A possible explanation for this is that the neurons involved in the central control of CRH production have reduced glucocorticoid receptor concentrations and are therefore less able to curtail stress-evoked cortisol levels. This, in turn, would lead to a deficient negative feedback effect on secretion of CRH and vasopression. Antidepressant-induced increase in glucocorticoid re-

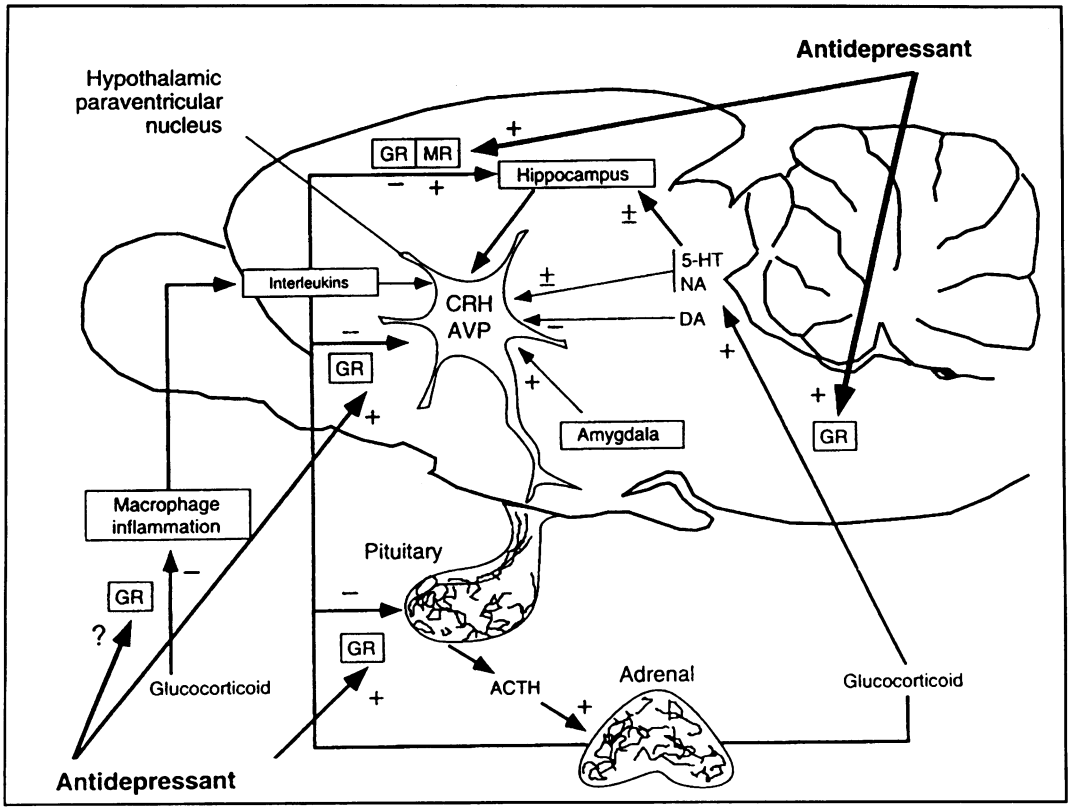

Fig. 6. Antidepressant-induced increases in GR and mineralocorticoid receptor (MR) gene expression suggest a novel mechanism of action of these drugs on the hypothalamic—pituitary—adrenocortical (HPA) system. The diagram shows stimulatory (+) and inhibitory (-) actions of neural inputs to brain regions involved in HPA system regulation and to sites of corticosteroid retroinhibition. Arrows indicate the sites at which antidepressants can have stimulatory actions on GR or MR.

ceptor gene expression could be a part of the mechanism whereby the HPA system becomes more sensitive to circulating glucocorticoid hormones, thereby reducing expression of the CRH gene, the over-reactivity of which has been implicated in depression.

There are 2 major classes of antidepressant drugs: monoamine oxidase inhibitors and monoamine re-uptake inhibitors. The latter is the class most extensively used to treat unipolar affective disorders. It is a structurally heterogeneous group of drugs, which act by blocking the re-uptake transporter of either norepinephrine or serotonin or both.[5,82] Work from this laboratory was the first to show that different types of antidepressants, including tricyclic and atypical antidepressants, do in fact alter GR mRNA levels.[44,83] Subsequent work has indicated that the capacity of brain tissues to bind corticosteroids[47,84] and corticosteroid receptor immunoreactivity[85] are also increased by both classes of antidepressants. In transfected cells, GR gene promoter activity is stimulated by antidepressants[50] (Fig. 5), and several different antidepressants can modulate this receptor in vivo,[45] suggesting that they may have a common mechanism of action at the level of the corticosteroid receptor genes. Finally, long-term treatment of GR antisense transgenic mice with antidepressants resulted in a partial reversal of enhanced ACTH and corticosterone secretion (Table 1), concurrently with increased GR mRNA and steroid receptor binding activity.[66] Antidepressants thus clearly increase functional levels of GR and hormone binding activities.[44,47,84,86]

The time course of hippocampal and hypothalamic MR and GR concentration changes in rats treated with tricyclic antidepressants,[47] or with moclobemide, a reversible monoamine oxidase inhibitor,[84] show that both MR and GR are elevated 2 to 5 weeks after the start of treatment. This suggests that antidepressant-induced changes in brain corticosteroid receptor capacity may underlie the observed simultaneous decrease in basal circulating ACTH and corticosterone levels and the decreased adrenal size.[47,84] Furthermore, when challenged by a stressor, rats treated with antidepressants showed a decreased ACTH and corticosterone response, possibly because of enhanced effectiveness of negative corticosteroid feedback due to increased GR and MR capacity.[47,84] Some of these effects may be mediated through CRH, since in rats that were treated daily with imipramine (5 mg per kg intraperitoneally for 8 weeks)[87] or in amitriptyline-treated transgenic mice (Table 1), the CRH mRNA levels in the PVN were decreased by 26% to 37%. Although rapid (1 to 4 days) effects of antidepressants on corticosteroid receptors can be seen in vitro (Fig. 5), the time course of action in vivo (3 to 5 weeks) coincides closely with their long-term actions on HPA system activity and the clinical improvement of depression.[36,41,42,45,47,84]

While antidepressants produce changes in monoaminergic neurotransmitter systems, these changes do not correlate with therapeutic efficiencies or measures of hypothalamic–pituitary–adrenal function,[88] suggesting that other mechanisms of action may be operative. We found increased GR mRNA levels, irrespective of the preferential inhibitory action of antidepressants on the re-uptake of different classes of monoamine neurotransmitters. Furthermore, we showed increased GR gene transcription in the fibroblasts of mice treated with antidepressants,[50] although these cells do not possess monoamine re-uptake mechanisms. One action of antidepressants may be exerted at the genomic level (but not necessarily directly) to stimulate the transcription of the GR gene, and this action may not be limited to neurons.

Antidepressants also influence the HPA system by actions other than those mediated via corticosteroid receptors. Antidepressants can exert actions on monoamines, their receptors and transporters, and the secondary messenger systems involved in signal transduction. Increased HPA activity coincides with changes in the serotonin system, including increased serotonin turnover,[89] increased postsynaptic $(5\text{-HT})_2$ receptors[90] and decreased $(5\text{-HT})_{1A}$ receptors.[91–94] These same changes are noted in depression,[95] and tryptophan depletion can also have a mood-lowering effect.[96] Perhaps the most important aspect of the serotonin system is its relation to the negative feedback action of glucocorticoids.[97] The retroinhibitory action of corticosterone is exerted via receptor sites in neurons of the hypothalamus, hippocampus, septum, amygdala and reticular formation. Innervation of these areas is provided by noradrenergic efferents from the locus ceruleus and the serotoninergic raphe neurons. While adrenalectomy or corticosteroid administration does not alter norepinephrine metabolism, corticosterone elevates serotonin synthesis and concentrations and alters the dynamics of serotonin release/uptake by synaptosomes.[98] Lesion of the raphe nuclei by 5,7-dihydroxytryptamine decreases the GR and MR capacity of the hippocampus[99,100] by a mechanism distinct

from the down-regulation that can be caused by high glucocorticoid concentrations.[101] However, lesion of the locus ceruleus by 6-hydroxydopamine had no effect on GR.[102] The action of antidepressants on the HPA axis, other than that exerted directly on corticosteroid receptor genes, is thus most likely to be exerted via the serotoninergic system. I hypothesize that antidepressants can exert action on the HPA system via increased serotoninergic postsynaptic activation, leading to stimulation of corticosteroid receptor gene expression and increased sensitivity of the HPA system to retroinhibition by corticosteroids. A dual system for activation of corticosteroid receptor genes by antidepressants is thus likely: direct action via as yet unknown mechanisms, and indirect action via increased serotoninergic postsynaptic activation. Both actions would ultimately lead to stimulation of corticosteroid receptor gene expression, increased sensitivity to retroinhibiton by corticosteroids and more efficient shutdown of a hyperactive HPA system (Fig. 6).

Antidepressants clearly increase GR mRNA levels and functional corticosteroid hormone binding capacity.[44,47,84,86] In transfected cells, GR gene promoter activity is stimulated by antidepressants,[50] and several different antidepressants can modulate this glucocorticoid receptor in vivo.[45] Antidepressants could thus increase the capacity of neurons involved in the regulation of the HPA system to respond to feedback inhibition by glucocorticoids, and they may have a common mechanism of action at the level of the corticosteroid receptor genes. On this basis, we suggest that a primary action of antidepressants is the stimulation of corticosteroid receptor gene expression, with a resulting decrease in HPA system activity, including reduced CRH gene expression. Secondary effects, resulting from a reduction in glucocorticoid concentrations, are exerted on the expression of genes susceptible to glucocorticoid control, such as enzymes involved in neurotransmitter biosynthesis.[103] Neurotransmitter imbalance provoked by a hyperactive HPA system could thus be reversed; this suggests an additional mechanism through which antidepressants may modify mood. The need to normalize the HPA system to improve mood remains speculative, although it is supported by the fact that patients with hypercortisolemia (e.g., in Cushing's syndrome) or receiving glucocorticoid therapy often have symptoms of depression. The poor treatment response in patients with depression with persistent HPA alteration further supports this hypothesis. If additional evidence can be accumulated that neuroendocrine alterations are causally involved in affective disorders, as some preliminary data suggest,[19,104] this would provide a lead for the development of more efficacious drugs.

This work provides completely new insight into antidepressant drug action and suggests a novel approach to the development of new drugs by focusing on this action.

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