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Transient forebrain over-expression of CRF induces plasma corticosterone and mild behavioural changes in adult conditional CRF transgenic mice

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ABSTRACT

Background: Converging findings support a role for extra-hypothalamic CRF in the mediation of the stress response. The influence of CRF in the amygdala is well established, while less is known of its role in other areas of the forebrain where CRF and CRF_1 receptors are also expressed. In the present study CRF was genetically induced to allow forebrain-restricted expression in a temporally-defined manner at any time during the mouse lifespan. This mouse model may offer the possibility to establish a model of the pathogenesis of recurrent episodes of depression.
Methods: Mice were engineered to carry both the rtTA transcription factor driven by the CamKIIα promoter and the doxycycline-regulated operator (tetO) upstream of the CRF coding sequence. Molecular, biochemical and behavioural characterisation of this mouse is described.
Results: Following a three-week period of transcriptional induction, double transgenic mice showed approximately 2-fold increased expression of CRF mRNA in the hippocampus and cortex, but not hypothalamus. These changes were associated with 2-fold increase in morning corticosterone levels, although responses to the dexamethasone suppression test or acute stress were unaffected. In contrast, induced mice displayed modestly altered behaviour in the Light and Dark test and Forced Swim test.
Conclusions: Transient induction of CRF expression in mouse forebrain was associated with endocrine and mild anxiety-like behavioural changes consistent with enhanced central CRF neurotransmission. This mouse allows the implementation of regimens with longer or repeated periods of induction which may model the initial stages of the pathology underlying recurrent depressive disorders.

1. Introduction

Repeated or prolonged exposure to stress is proposed to lead to the development of mood disorders such as unipolar or bipolar depression. Once established, mood disorders are generally characterised by cyclic or recurrent episodes, with periods of well-being alternating with periods of severe symptoms. There is increasing evidence that these pathologies are most frequent in subpopulations of genetically predisposed individuals and this susceptibility may arise from genetic variants that can lead to exaggerated or maladaptive response to mild environmental stressors (DeKloet et al., 2005; Monroe et al., 2006). In order to reduce the risk for these subjects, it is crucial to understand how genetic variants alter the response to stress and are influenced by intervening environmental factors.

An important component mediating the response to stress is the corticotropin-releasing factor (CRF), a 41 amino acid neuropeptide expressed in several telencephalic, limbic and hypothalamic brain structures (Vale et al., 1981), acting via CRF_1 and CRF_2 G-protein coupled receptors (Reul and Holsboer, 2002). Convergent findings indicate that CRF participates in anatomically-defined regulatory networks supporting different aspects of the response to stress, including the physiological functions necessary to prepare or sustain the stress response. Typically, exposure to a stressful event increases CRF release from the median eminence into the portal vessels to stimulate the CRF_1 receptor-dependent release of ACTH from the pituitary (Plotsky, 1988; Pich et al., 1993). In addition, CRF_1 receptor activation in the amygdala (Merlo Pich et al., 1995; Cook, 2002) drives the behavioural, autonomic and endocrine response to stress (Rassnick et al., 1993; Pelleymounter et al., 2000).

In the present study we investigated whether transient forebrain CRF over-expression might be one of the mechanisms underlying the susceptibility to stress in subjects predisposed to recurrent mood

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disorders. Specifically, we hypothesised that a transient episode of increased CRF release, lasting a few weeks, might enhance the impact of external stressors. If repeated, these episodes might lead to a progressive loss of stress-coping capability, long-term impairment of HPA axis function, and eventually precipitation into a depressive-like episode.

In this study we focused on the forebrain CRF system, since the targeted deletion of CRF_1 receptors in the hippocampus and cortex results in mice with lower anxiety-like behaviour and attenuated HPA axis response to stress (Muller et al., 2003). Furthermore, the importance of this anatomical area is highlighted by the observation that deletion of the glucocorticoid receptor type I (GR) gene in the hippocampus, leading to increased circulating glucocorticoids, impairs HPA axis feedback and produces a depression-like behavioural response to stress which is reversed by imipramine treatment (Boyle et al., 2005). On the basis of these results, a possible glucocorticoid–CRF interaction at the level of the forebrain has been suggested to contribute to the genesis of some aspects of human mood disorders (Holsboer, 1999; Arborelius et al., 1999).

In the present study, the conditional transgenic technology was used to develop a mouse in which CRF expression is regulated by an inducible promoter specifically restricted to the central nervous system. This approach is novel, since studies examining the effects of CRF over-expression reported so far described mice constitutively over-expressing the peptide from birth (Stenzel-Poore et al., 1992). These mice exhibit an exaggerated endocrine, Cushing-like syndrome, probably related to hypothalamic CRF over-expression, and behavioural inhibition, possibly resulting from the convergent effects of central CRF over-expression and peripheral increase of glucocorticoids (Stenzel-Poore et al., 1992; Dirks et al., 2001, 2002). In our study, transgenic mice were engineered to avoid the development of a severe endocrine syndrome by restricting the expression of CRF using the CamKIIα promoter, as previously described (Mansuy et al., 1998). This has the added advantage of addressing the over-expression in forebrain areas such as the hippocampus, as discussed earlier. In addition, the rtTA/tetO+ inducible system controlled by doxycycline (DOX) (Rose and MacDonald, 1997) allows conditional expression of CRF upon exogenous induction. Consequently, the expression of CRF can be switched on/off when desired only in the forebrain regions and at any moment of the postnatal life of the mouse. This article describes the phenotypic consequences of the first activation of the CRF transgene for a period of 3 weeks.

2. Methods and materials

2.1. Generation of inducible CRF transgenic mice

The rat genomic CRF sequence (EcoRI–Asp781) was ligated into the BamHI site of the pTRE2 plasmid between the tetO sequence and the rabbit β-globin polyadenylation site. The CamKIIα-rtTA transgenic line was generated by Mansuy et al. (1998). Double transgenic mice were identified by multiplex PCR with specific primers complementary to the rabbit β-globin gene to screen for tetO-CRF construct (right 5′-ATT ACC CAG AAG TCA GAT GCT C-3′ and left 5′-GAA GAT GTC CCT TGT ATC ACCA-3′) and to the rtTA sequence (right 5′-AGG CCG AAT AAG AAG GCT-3′ and left 5′-TCG ACG CCT TAG CCA TTG A-3′).

2.2. Experimental mice

CamKIIα-rtTA/tetO-CRF double transgenic male and female mice (>eighth generation, C57BL6/N backcrossed), equally represented, were used in all the experiments and data were grouped, unless differently specified. Mice were individually housed under standard laboratory conditions (21 ± 1 °C; lights on 06:00–18:00 h) and administered a diet containing doxycycline (DOX, 600 mg/100 g dry food; Bio-Serv, US & Canada, Frenchtown, NJ) starting at 8–11 weeks of age, for three weeks before the initiation of any evaluation. Three weeks of DOX supplemented chow were sufficient to reach the maximal CRF induction (data not shown). At the time of tests, all the cohorts were 11–14 weeks of age and gender/age-matched wild type littermates, receiving the same dietary conditions as transgenic mice, were used as controls. Because of the very demanding breeding procedure required by this specific genetic switch which leads to a very low rate of production of mice transgenic for both constructs (approximately 10 mice/100 newborns), the double transgenic group non-DOX treated could not be included as additional negative control in each experiment. However, it was initially characterised to ensure that no leakage of the transcriptional system was detected. Experiments were carried out in accordance with the Italian law (art. 7, Legislative Decree no. 116, 27 January 1992), which acknowledges the European Directive 86/609/EEC, and were fully compliant with GlaxoSmithKline policy on the care and use of laboratory animals and related codes of practice.

2.3. rtTA in situ hybridization

Coronal brain sections (14 μm thick) were cut at −20 °C and mounted on Superfrost Plus microscope slides (BDH ITALIA, Milan, Italy). The 45 bp-long antisense oligodeoxynucleotide probe 5′-GAT CTT CCA ATA CGC AAC CTA AAG TAA AAT GCC CCA CAG CGC TGA-3′, complementary to the rtTA mRNA sequence, was 3′-end labelled with [^{33}P]α-dATP, using Terminal Deoxynucleotidyl Transferase (Amersham Pharmacia Biotech Italia, Cologno Monzese, Italy) and used for *in situ* hybridization as described previously (Mugnaini et al., 2002). Quantitative analysis of autoradiograms was performed by computer-assisted microdensitometry (AIS, Imaging Research, St. Catharines, Ontario, Canada).

2.4. Real-time quantitative RT-PCR

Reverse transcription (RT) reactions were performed on 1 μg total RNA using oligo(dT)12–18 primers and Superscript II reverse transcriptase (Invitrogen, Milan, Italy). The amplification reaction was performed using TaqMan probes, TaqMan Universal PCR Master Mix and ABI Prism 7700 Sequence Detector (Applied Biosystems Italy, Monza, Italy). Primer sequences and probes were as follows: rat CRF (GeneBank Accession number: M54987): right 5′-CAG GGC AGA GCA GTT AGC TCA-3′ and left 5′-CGC AAC ATT TCA TTT CCC G-3′, probe: 5′-CAA GCT CAC AGC AAC AGG AAA CTG ATG G-3′; rat GAPDH (GeneBank Accession number: AF106860): right 5′-CAA GGT CAT CCA TGA CAA CTT TG-3′ and left 5′-CCA GGT AAG AAA AGC TTC GCC-3′, probe: 5′-ACC ACA GTC CAT GCC ATC ACT GCC A-3′. Cycle threshold (Ct) values were calculated by the SDS software v1.9 (Applied Biosystems, Monza, Italy) from fluorescent readings and values were converted into copy number/ng total RNA input by using a standard curve of known amounts of a vector containing the tetO-CRF microinjected fragment for CRF quantification and of rat genomic DNA for GAPDH quantification. Due to the homology identity between rat and mouse CRF sequence, all read-outs represent the sum of the induced rat CRF mRNA and the endogenous mouse CRF sequence.

2.5. Hormonal assays

All hormonal assays were performed on samples collected between 8.00 and 10.00 am in individual cohorts of mice, unless otherwise specified. Plasma corticosterone was measured using Immuchem TM Double Antibody Corticosterone ^{125}I RIA kit (MP Biomedicals, New York, U.S.A.). The intra- and interassay coefficients of variation were 7.3% and 7% respectively. Plasma ACTH was measured using a Radioisotopic Kit for the Quantitative Determination of ACTH Levels in Human Plasma (Nichols Institute Diagnostics, San Juan Capistrano, California, U.S.A.). The intra- and interassay coefficients of variation were 3.1% and 7.5% respectively.

2.6. Dexamethasone suppression test

Mice ($n = 10$–12/group) were injected subcutaneously (10 mL/kg) with either saline or 0.1 mg/kg dexamethasone (MP Biomedicals, New York, U.S.A.) at 9.30 am (Groenink et al., 2002). Six hours later they were rapidly decapitated for blood collection.

2.7. HPA axis response to acute stress

Mice ($n = 7$/group) were exposed to 5-min Forced Swim test (FST) to elicit an acute stress response. The animals were sacrificed by rapid decapitation at 15, 45, and 95 min from the onset of FST exposure. Unstressed transgenic and wild type mice were used as controls ($t = 0$).

2.8. Analysis of spontaneous locomotor behaviour

Spontaneous locomotor activity was measured by using the Digiscan Animal Activity Monitors (Model RXYZCM-8; Ominitech Electronics, Inc. Columbus, OHIO). Each monitor was made of clear Plexiglas 40 × 40 × 30.5 cm in size covered with a perforated Plexiglas lid. Infrared monitoring sensors were located around the perimeter walls (horizontal sensors). Two additional sensors were located 4 cm above the floor on opposite sides (vertical sensors). Data were collected and analysed ($n = 10$/group) by a computer-assisted Digiscan Analyzer (Model DCM-4; Ominitech Electronics, Inc. Columbus, OHIO). Horizontal activity and vertical activity were recorded during the 60-min test period.

2.9. Forced Swim test

Mice were placed individually into glass cylinders (height 25 cm, diameter 10 cm) containing 10 cm water maintained at 25 ± 1 °C as described by Porsolt (Porsolt et al., 1977). A mouse was judged immobile when it floated in an upright position and made only small movements to keep its head above water. The duration of immobility was recorded during the last 4 min of the 6 min testing period.

2.10. Light and Dark box test

The test apparatus was composed of Perspex box 45 × 27 × 27 cm in size divided into two compartments communicating via an opening in the wall. One third of the box was coloured black and the remaining two thirds were coloured white, the floor marked into 9 cm squares. The white compartment was illuminated by a 75 W bulb and the black compartment by a 60 W red bulb. Fifteen minutes before test, one cohort of male transgenic and wild type mice ($n = 8$/group) was exposed to 5-min restraint stress whereas another cohort was left undisturbed in their cage. Mice were placed individually into the centre of the dark area and allowed to explore the test box for 5 min. Mouse behaviour was filmed and the latency to enter into the light compartment, the time spent in light area and the number of line crossings in the light compartment was scored.

2.11. Data analysis

Data obtained from real-time quantitative PCR were analysed using analysis of covariance (ANCOVA) or 2-way analysis of variance (ANOVA) with genotype and treatment as fixed factors. Corticosterone and ACTH measurements collected at baseline and Forced Swim test data were analysed using the unpaired Student's *t*-test. Thymus weight/body weight ratio was considered a dependent variable and was analysed by a two-way analysis of variance (ANOVA) with genotype (wild type and transgenic) and gender as fixed factors. Body weight was analysed by repeated measure and ANOVA with genotype and time-points as fixed factors. Dexamethasone suppression test was analysed by a two-way ANOVA, with genotype and drug or genotype and time-points as fixed factors, and corticosterone level as dependent variable. Two-way ANOVA was used also for the analysis of the HPA axis response to stress experiment. Since each time-point was an independent group of mice, it was not possible to analyse each single level in respect to the basal (difference from baseline). Cumulative locomotor activity data gathered over 60 min were analysed by using the paired Student's *t*-test while the analysis of 10-min intervals was performed by repeated measure two-way ANOVA with genotype and time-points as fixed factors and number of beam interruptions as dependent variable followed by Newman–Keuls *post-hoc* test. Light and Dark test data were analysed by using a two-way ANOVA with genotype and stress exposure as fixed factors. ANOVA analysis was followed by Duncan's *post-hoc* test if not differently described. The level of significance was $p \leq 0.05$ for all tests. All data are presented as means ± s.e.m. The statistical software Statistica 6.1 (Statsoft Inc., Tulsa, U.S.A.) was used for all the analysis.

3. Results

3.1. Conditional induction of CRF expression

The limited number of models reported in the literature using the TET-ON transcriptional system described here has historically shown that the kinetic of the switch is rather fast and occurs within a few days. Mansuy et al. characterised and published the inducer strain we used, reporting that maximum induction is reached within the first week of DOX administration (3–6 days from administration) and can be reversed in two weeks by removal of doxycycline. All the experiments shown in this paper were performed after 3 weeks of DOX administration, to guarantee maximum induction for at least 2 weeks. Sporadic checks run on experimental mice confirmed that in our condition (3 up to 5 weeks) we were always at the maximal level of induction.

The anatomical localisation for the transcriptional regulation of CRF was provided by the CaMKIIα promoter driving the constitutive expression of the reverse form of the tetracycline-sensitive transcription factor (transactivator or rtTA, Fig. 1a) predominantly in the forebrain areas, as assessed by ISH using a probe specific of the rtTA molecule (Fig. 1c). The CamKIIα-driven rtTA transgene expression was localised in the telencephalic regions, namely hippocampus and cortex, but not in the hypothalamus, as previously described by Mayford et al. (1996). RT-PCR with primers specific to the rtTA molecule in homogenates derived from the thymus and adrenal glands failed to detect any ectopic expression of the transactivator (data not shown). The responder mouse line was generated by microinjecting the rat CRF coding sequence under the control of the rtTA operator (tetO-CRF, Fig. 1a). Inter-breeding of these two lines produced in the progeny double transgenic mice positive for both the CamKIIα-rtTA and the tetO-CRF transgenes (Fig. 1b). The modulation of this system was studied in the hippocampus where CRF mRNA level was significantly increased by doxycycline treatment when compared with either untreated transgenic mice or wild type littermates, with and without doxycycline treatment [genotype: $F\ (1,\ 29) = 16.00$, $p < 0.001$; doxycycline treatment: $F\ (1, 29) = 22.65$, $p < 0.001$; genotype × treatment interaction $F\ (1, 28) = 5.31$, $p < 0.05$]. Due to the very low rate of production of double transgenic mice (12%/gender) it was not possible to include in every experiment CamKIIα-rtTA mice non-DOX treated as additional negative control. However, also this group was initially characterised at a molecular level, confirming that, as expected, basal levels of CRF mRNA expression (Fig. 1d) and peripheral corticosterone (data not shown) were comparable to those observed in wild type controls and in mice transgenic for each single parental construct.

The increase of the CRF in induced double transgenic mice was 1.8-fold vs. controls, indicating a modest but significant up-regulation. A

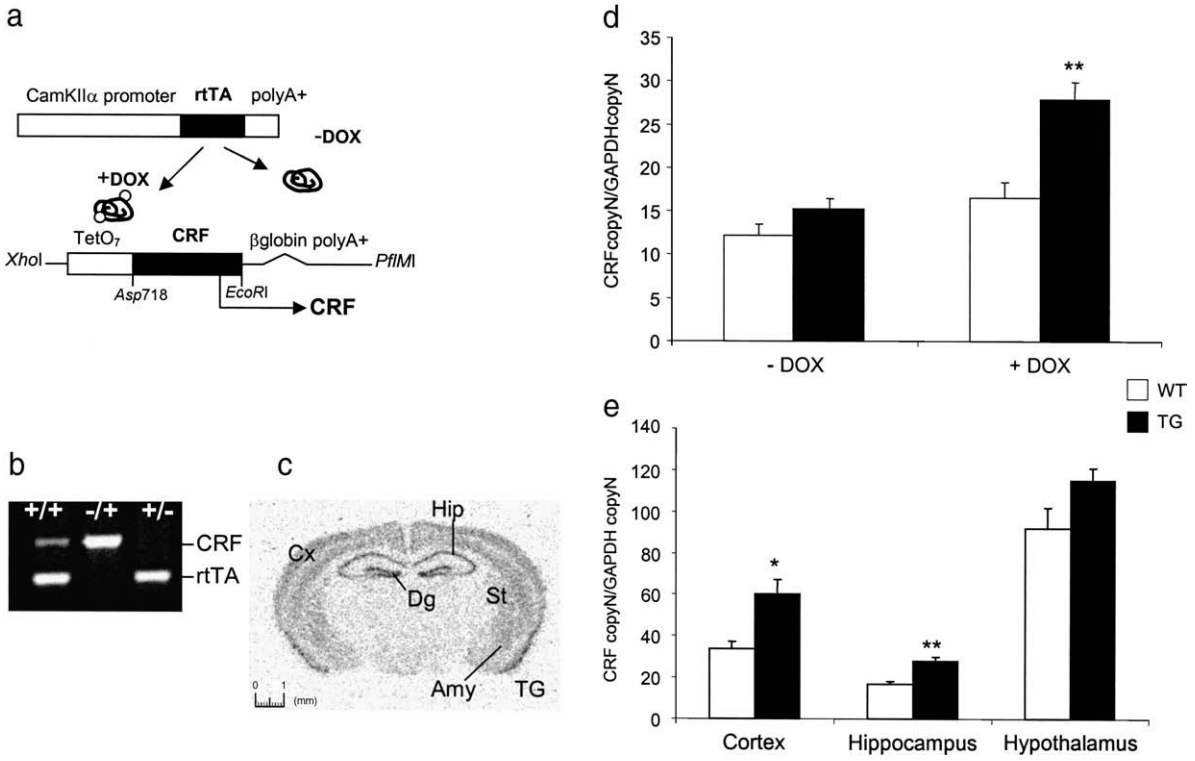

Fig. 1. Generation of CRF inducible mouse line and *in vivo* evidence of the transcriptional efficiency of the construct. (a) Schematic representation of the strategy to obtain doxycycline-dependent expression of CRF in forebrain. Transgenic mice carrying the rtTA gene driven by the CamKIIα promoter (18) were crossed with mice carrying the tetO-CRF construct. Double transgenic mice expressed CRF when fed with doxycycline-complemented food. In the absence of doxycycline, rtTA does not bind to tetO operator and the CRF transgenic transcription is inhibited. (Key: poliA+: polyadenylation signal sequence). (b) PCR analysis on genomic DNA from offsprings derived from the crossing between the two parental lines (Key: +/+ double transgenic mice, −/+ tetO-CRF transgenic mice, and +/− CamKIIα-rtTA transgenic mice). (c) *In situ* hybridization of the CamKIIα-rtTA transgene expression in double transgenic mice indicating the telencephalic distribution. The section was taken at approximately − 1.58 mm from Bregma (Paxinos and Franklin, 2001). Signal was undetectable in wild type mice (not shown). Key: Amy, amygdaloid nuclei; CA, cornu Ammonis; Cx, cerebral cortex; DG, dentate gyrus; CPu, caudate putamen. The calibration bar corresponds to 1 mm. (d) Doxycycline-induced CRF expression (mRNA) in the hippocampus of wild type and transgenic mice in the non-induced (−DOX) and induced states (+DOX) ($n=6$–10, $^{**}p<0.01$ vs. wild type). (e) Effects of doxycycline induction on CRF expression levels in few brain areas of CRF-induced vs. wild type mice ($n=9$–10, $^{*}p<0.05$ and $^{**}p<0.01$ vs. wild type).

comparable increase of CRF messenger RNA was also observed in cortex [1.4-fold change vs. control, $F\ (1, 17)=5.36$, $p<0.05$], but not in hypothalamus, where only a modest increase was observed [1.2-fold change vs. control, $F\ (1, 16)=4.0$, $p=0.063$] (Fig. 1e). Although these messenger RNA levels are only an indirect evidence of the restricted anatomical distribution of CRF peptide in these mice, both ISH and quantitative PCR confirmed that the inducer molecule is expressed and localised in accordance to the spatial transcriptional activity of the CamKIIα promoter and as previously described by Mansuy et al. (1998). Direct immunohistochemical quantification, which would have allowed also a more detailed anatomical localisation of the CRF molecule, could not be performed with these levels of induction and the CRF antibodies which are currently available.

Prior and after CRF induction, all transgenic mice showed normal development and no differences in either male or female body weights were detected (data not shown).

3.2. Neuroendocrine effects of transient CRF induction

Three-week induction of CRF expression in young adult transgenic mice (8–11 weeks of age at the beginning of the doxycycline treatment) was associated to 2-fold increased plasma corticosterone levels measured at 8.00–10.00 am (Fig. 2a; $p<0.05$). This elevation was statistically significant and was consistently observed in several mice cohorts. Corticosterone levels in non-DOX treated double transgenic mice were comparable to those measured in wild type mice where DOX had no induction effect (data not shown). Plasma ACTH levels were also measured as a single-point assessment, showing no difference when compared with wild type mice (Fig. 2b; $p=0.69$).

Post-mortem assessment in CRF-induced female mice, but not in male mice, showed that the thymus was smaller compared to wild type controls. Expressed as thymus weight/body weight, control thymus was $3.13\pm0.11\times10^{-3}$ and transgenic thymus was $2.44\pm0.10\times10^{-3}$ [genotype $F\ (1, 16)=7.13$, $p<0.05$; gender $F\ (1, 16)=87.87$, $p<0.01$; interaction genotype × gender $F\ (1, 16)=8.56$, $p<0.01$]. Adrenal gland analysis indicated that no abnormalities were present in terms of size, weight or histology (data not shown).

The dexamethasone suppression test was performed to assess the HPA axis feedback in transgenic and wild type mice exposed to the same diet for 3 weeks. Dexamethasone (0.1 mg/kg s.c.) produced a significant corticosterone plasma level suppression in both groups 6 h later, showing no difference in the magnitude of the suppression between the transgenic and wild type mice group [genotype $F\ (1, 39)=0.93$, $p=0.34$; treatment $F\ (1, 39)=20.14$, $p<0.001$; genotype × treatment interaction $F\ (1, 38)=0.52$] (Table 1).

In a different cohort of mice the response of the HPA axis to acute stress was measured using the Forced Swim Stress as stressor (Fig. 2c). Five minutes of swim stress elicited the same peak response of corticosterone secretion in both CRF-induced and wild type mice, showing no significant differences at any of the time-points analysed over the 90-min sampling period [genotype $F\ (1, 49)=0.0022$, $p=0.96$; time $F\ (3, 49)=34.58$, $p<0.01$; genotype × time interaction $F\ (3, 49)=1.15$, $p=0.34$)]. However, transgenic mice were characterised by higher baseline of corticosterone and by a trend for a faster

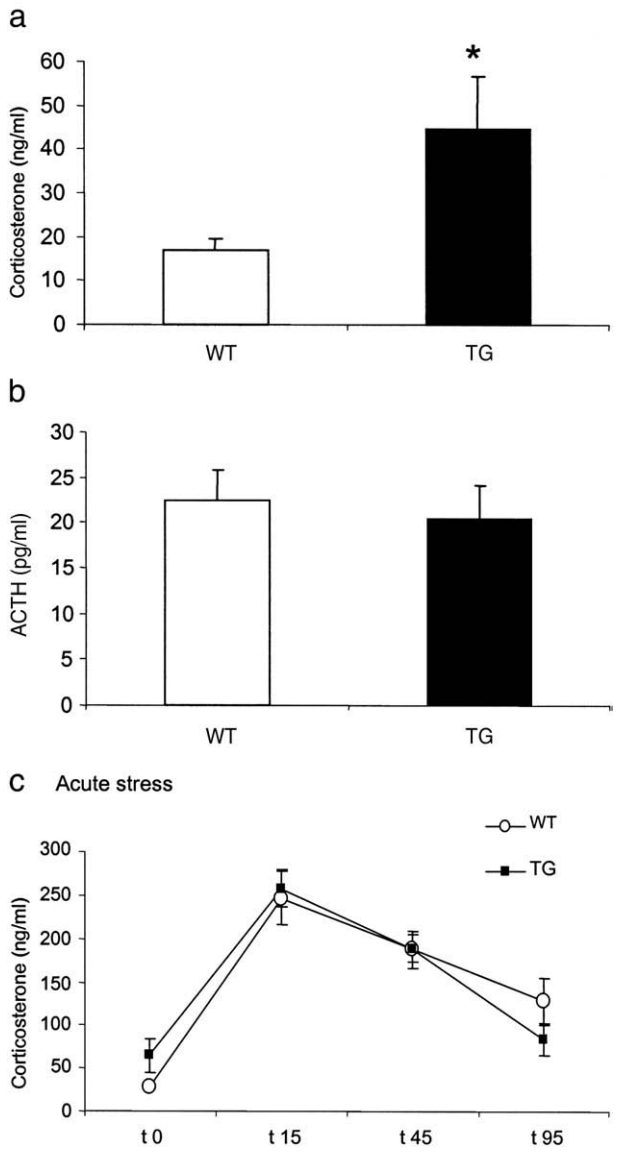

Fig. 2. Characterisation of the HPA axis endocrine phenotype in double transgenic mice. Plasma corticosterone (a) and ACTH (b) levels in double transgenic mice (TG, $n=15$) compared to wild type littermates (WT, $n=14$) measured in the morning (8:00–10:00 am) after 3-week CRF induction (*$p<0.05$). (c) Time course of plasma corticosterone level following 5 min of Forced Swim test used as acute stressor ($n=7$). Blood samples were taken in separate groups of mice at baseline (t0: unstressed mice), 15, 45 and 95 min from the onset of stress.

recovery to baseline values than wild type mice, possibly suggesting an over-efficient HPA axis feedback (Fig. 2c).

3.3. Effects of transient CRF induction on spontaneous locomotor behaviour

Locomotor activity was studied in both novel and familiar environments. In the novel environment, CRF-induced mice did not show any difference in exploratory locomotor activity when compared to their littermate controls (data not shown). The study in the familiar environment was performed by first exposing the mice to the apparatus before CRF induction and re-testing the mice after 3 weeks of doxycycline treatment (Fig. 3a). As expected, during the first exposure (non-induced state) no differences in locomotor activity were detected between transgenic and wild type mice. However, at the second exposure, after 3 weeks of CRF induction, the expected decrease of locomotor activity was observed only in the wild type mice (1-hour cumulative horizontal activity $p<0.05$, Fig. 3a), suggesting a reduced familiarisation of the CRF-induced mice to the environment compared to wild type mice. This difference was homogeneously distributed throughout each 10-min interval within 1-hour recording period (Fig. 3b) [genotype $F(1,16)=6.1$, $p<0.05$; time $F(5,80)=8.8$, $p<0.001$, genotype × time interaction $F(5,80)=3.38$, $p<0.01$]. No differences between wild type and transgenic mice were observed in vertical activity (data not shown).

3.4. Behavioural response to emotional challenge

Anxiety-like behaviour was assessed in the Light and Dark test. This test was conducted in CRF-induced transgenic mice and wild type littermates under basal conditions or following 5-min restraint stress to enhance the susceptibility to the behavioural inhibition. The number of line-crossing in the light compartment was significantly lower in CRF-induced transgenic mice showing a genotype main effect [genotype $F(1,27)=5.42$, $p<0.05$; stress exposure $F(1,27)=2.66$, $p=0.11$; genotype × stress exposure interaction $F(1,27)=0.05$, $p=0.82$] (Fig. 4a). However, the time spent in the lit compartment was slightly decreased (Fig. 4b) and the latency to enter the light compartment was only slightly increased (data not shown) suggesting

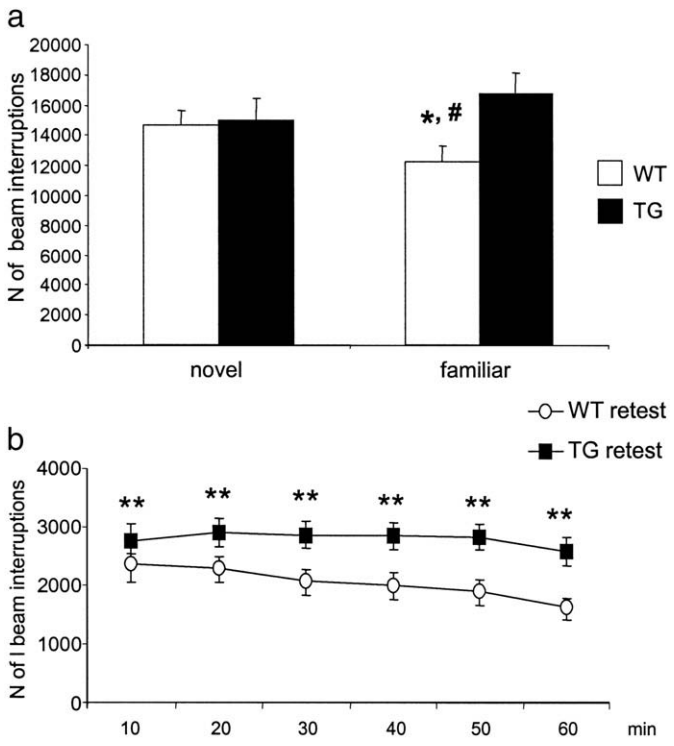

Fig. 3. Spontaneous locomotor behaviour. (a) The number of cumulative horizontal beam interruptions during 60 min obtained at the first test ("novel environment") and in the same animals at the re-test sessions ("familiar environment") after 3 weeks of CRF induction performed in double transgenic mice (TG) and wild type (WT) littermates (*$p<0.05$ vs. transgenic littermates at re-test session; $^{\#}p<0.05$ vs. wild type at test session). (b) Time course during the 60-min test of horizontal beam interruptions at 10-min time intervals in the re-test session ("familiar environment", $n=10$/group, **$p<0.01$ vs. wild type littermates).

Table 1
Dexamethasone suppression test.

Plasma corticosterone levels (ng/ml)		
	Vehicle	Dexamethasone
Wild type	114.8 ± 13.7	59.3 ± 15.2**
Double transgenic	110.2 ± 19.2	35.9 ± 9.3**

Plasma corticosterone levels in wild type and double transgenic CRF-induced mice 6 h following either dexamethasone or vehicle treatment ($n=10/12$, **$p<0.01$ vs. vehicle).

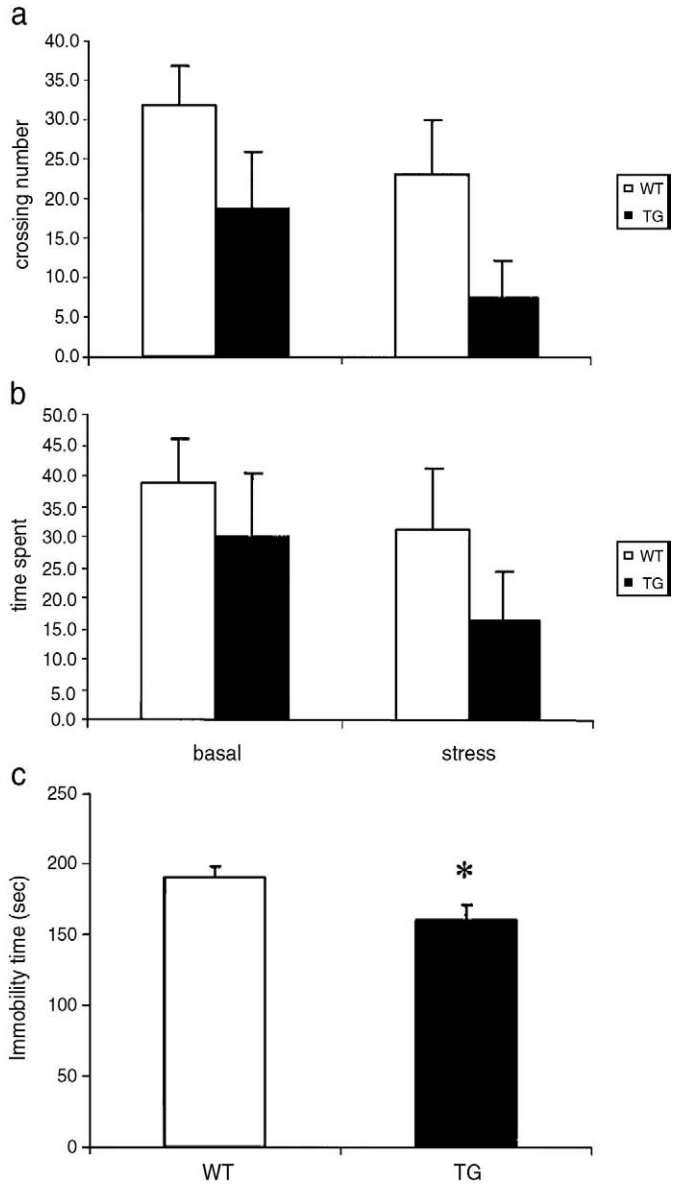

Fig. 4. Behavioural response to challenge. In the Light and Dark box test the number of line crossings in the light compartment ((a), showing significant genotype effect $p<0.05$) and the time spent in the illuminated compartment ((b), not significant effect) are shown under basal conditions (basal) and following 5 min of restraint stress (stress) in double transgenic CRF-induced mice (TG) and wild type (WT) littermates ($n=8$). In the Forced Swim test the mean values of immobility time (c) in double transgenic CRF-induced mice and wild type littermates are presented ($n=10$, $^*p<0.05$ vs. wild type littermates).

a mild behavioural inhibition produced by CRF over-expression. It is possible that higher significance could have been reached by increasing the number of mice/group.

The FST was also used to assess the behavioural "despair" response. A significant decrease in the immobility time was observed in CRF-induced mice with respect to wild type mice [Fig. 4c, $p<0.05$].

4. Discussion

The effect of transient (3 weeks) induction of forebrain CRF expression was assessed in a genetically-engineered mouse in which the over-expression of CRF was conditional to the presence of exogenous doxycycline acting on the rtTA/tetO-CRF system and restricted to the forebrain areas defined by the CamKIIα promoter. Double transgenic mice, including individuals belonging to the parental transgenic lines, were normal at birth and throughout development. In adult mice, following CRF transcriptional induction by doxycycline, a moderate but significant 1.4- and 1.8-fold increase of CRF mRNA expression was consistently measured in cortex and hippocampus supporting the anatomical selectivity of the CamKIIα promoter. The trend toward an increase in CRF mRNA also in the hypothalamus might not be in contrast with the intrinsic transcriptional activity of the promoter and may be determined by an increased level of the endogenous mouse CRF (homologous to the rat sequence) which is still subjected to the physiological HPA axis feedback system.

The level of CRF expression was lower than that reported in other constitutive CRF transgenic lines (Stenzel-Poore et al., 1992; Beckmann et al., 2001), but was closer to CRF levels induced in the PVN or amygdala following modulation of feedback in the HPA axis (Makino et al., 1994; Feldman et al., 1995). This time-restricted, forebrain-targeted, moderate CRF over-expression was found to be reliably associated with a significant increase in plasma corticosterone measured in the morning. This endocrine feature was not related to a dysfunctional HPA axis regulation, as may be indicated by the similarity in responses between transgenic and wild type mice when exposed to acute stressors or to the dexamethasone suppression test performed after a single-dose administration. Although multiple doses of dexamethasone might have revealed a different feedback process, we have applied the same procedure which was able to highlight a dysfunctional HPA axis in the constitutive CRF over-expressing mice, suggesting that any potential alteration in the system present in our model would be subtle. However, reduced thymus size was observed in CRF-induced transgenic females, a typical effect deriving from prolonged exposure to corticosterone and consistent with observations in female transgenic mice constitutively expressing CRF (Beckmann et al., 2001).

As a clear hypothalamic induction of messenger CRF was not observed in our transgenic mice, it is possible that the increased corticosterone levels could be due to an adjustment of the HPA axis feedback set-point, reflecting a higher central extra-hypothalamic CRF tone driving the hypothalamic CRF system. We propose that this effect is produced at the level of the hippocampus, since the cortex does not seem to be involved in glucocorticoid feedback (Feldman and Weidenfeld, 1999). A rich plexus of CRF-positive terminals in the hippocampus, as well as CRF-positive interneurons located in the pyramidal layer have been described (Chen et al., 2004). CRF_1 receptors appear to be located on pyramidal neurons in CA1 and CA3 (Chen et al., 2000), and exogenous CRF modulates their firing (Siggins et al., 1985). In stressed mice, the activation of a subset of hippocampal neurons is blocked by the CRF_1 receptor antagonist R191212 (Chen et al., 2004). The high circulating corticosterone level observed in CRF-induced transgenic mice is consistent with a change in the feedback set-point possibly produced by increased CRF_1-mediated excitatory input from the hippocampus to the hypothalamus driven by genetically-engineered CRF over-expression.

The effect of transient CRF induction on spontaneous locomotor activity was studied in both novel and familiar environments. No difference was observed between CRF-induced transgenic and wild type mice during exploration of a novel environment. However, increased locomotor activity of the CRF-induced mice when compared to controls was observed when they were re-exposed to the same environment. The difference in locomotor response to novelty may suggest aberrant behavioural adaptation induced by CRF. An altered relationship between behavioural reactivity to novelty and anxiety-related activity has also been described in mice constitutively over-expressing CRF (Van Gaalen et al., 2003; Heinrichs et al., 1996, Kasahara et al., 2007). Alternatively, the lack of habituation to the environment could be an indication of a learning impairment.

The effect of CRF induction was also determined in a series of stress challenge tests. Enhanced behavioural inhibition was observed in CRF-

induced transgenic mice in the Light and Dark test, following a brief exposure to restraint stress, as indicated by significant reduction in exploratory activity in the light compartment. However, these effects were considered mild, since the latency to enter the light compartment and the time spent in that compartment were not altered. In a second stress challenge model, the Forced Swim test, a decrease in immobility was observed. This paradoxical response is similar to that described with other CRF transgenic mice (Dirks et al., 2002; Van Gaalen et al., 2002) and in line with studies reported by Farrokhi et al. (2007) using the high selective CRF agonist cortagine. The two opposite responses may be explained by the intensity and measurement modalities of the two stressors (the restraint stress being mild and measured after its occurrence, the Forced Swim test being severe and measured during its occurrence), resulting in the recruitment of partially different neural systems to cope with the challenge (Butler et al., 1990; Forster et al., 2006). Alternatively, as suggested by Farrokhi et al., the increase in mobility observed in these genetically modified mice to over-express CRF or following selective CRF agonist administration rather represents an indication of an increase in escape attempts consequence of a generally elevated anxiety-like behaviour. Both activating and inhibiting behavioural stress effects were reported to be exaggerated in Thy1-driven CRF over-expressing mice (Dirks et al., 2002) or in rats following central CRF administration (Sherman and Kalin, 1988).

The present study suggests that spatially- and temporally-limited CRF induction has moderate effect on the animal response to stressors, with either behavioural inhibition or activation depending on the specific challenge. This interpretation is in line with the behavioural properties attributed to the central CRF system on the basis of findings accumulated over several years (Heinrichs and Koob, 2004).

In conclusion, 3-week induction of CRF over-expression in forebrain areas of transgenic mice was associated with higher circulating glucocorticoids and with mild phenotypic changes compatible with enhanced central CRF neurotransmission, possibly at the level of the hippocampus. The lack of gross behavioural effects following CRF induction in the present study is perhaps surprising given growing evidence of a key role for extra-hypothalamic CRF in stress-related behaviour and neuronal function. However, the low (1.4–1.8-fold) CRF increase and relatively short duration of induction (3 weeks) may not be sufficient to produce a more severe behavioural syndrome. A higher level of induction has been reported in constitutive CRF transgenic mice, but it is not clear that these levels of induction are relevant to human disorders. On the contrary, longer duration of induction or repeated periods of induction, alternating with periods of normal CRF expression may better model what happens in humans and, given the trends observed in the present study might be expected to produce greater phenotypic changes. Alternatively, since the transcriptional switch-on in the present study occurred at about 3 months of age, it is possible that CRF over-expression may have less impact in adult life than in early life, as early stressful events are well known to be a predisposing factor for mood disorders later in life. Thus it will be valuable to further explore this animal model for the impact of earlier CRF induction. These alternative regimens may result in a more significant syndrome, providing translational information about the role of forebrain CRF and are relevant to a better understanding of human affective disorders.

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References

Arborelius L, Owens MJ, Plotsky PM, Nemeroff CB. The role of corticotropin-releasing factor in depression and anxiety disorders. J Endocrinol 1999;160(1):1-12.
Beckmann N, Gentsch C, Baumann D, Bruttel K, Vassout A, Schoeffter P, et al. Non-invasive, quantitative assessment of the anatomical phenotype of the corticotrophin-releasing factor-overexpressing mice by NMR. NMR Biomed 2001;14:210–6.
Boyle MP, Brewer JA, Funatsu M, Wozniak DF, Tsien JZ, Izumi Y, et al. Acquired deficit of forebrain glucocorticoid receptor produces depression-like changes in adrenal axis regulation and behaviour. Proc Natl Acad Sci USA 2005;102(2):473–8.
Butler PD, Weiss JM, Stout JC, Nemeroff CB. Corticotropin-releasing factor produces fear-enhancing and behavioural activating effects following infusion in the locus ceruleus. J Neurosci 1990;10:176–83.
Chen Y, Brunson KL, Muller MB, Cariaga W, Baram TZ. Immunocytochemical distribution of corticotropin-releasing hormone receptor type-1 (CRF(1))-like immunoreactivity in the mouse brain: light microscopy analysis using an antibody directed against the C-terminus. J Comp Neurol 2000;420(3):305–23.
Chen Y, Brunson KL, Adelmann G, Bender RA, Frotscher M, Baram TZ. Hippocampal corticotropin releasing hormone: pre- and postsynaptic location and release by stress. Neuroscience 2004;126:540–54.
Cook CJ. Glucocorticoid feedback increases the sensitivity of the limbic system to stress. Physiol Behav 2002;75(4):455–64.
DeKloet ER, Joel M, Holsboer F. Stress and the brain: from adaptation to disease. Nat Rev 2005;6:463–72.
Dirks, A., Bouwknecht, J., Hijzen, T., Van der Gugten, J., Ronken, E., Verbeek, J. et al., Overexpression of corticotrophin-releasing hormone in transgenic mice and chronic stress-like autonomic and physiological alterations. In: Dirks A, editor. Corticotropin-releasing hormone, stress and anxiety — physiological, neuroendocrine and behavioral studies. PhD thesis, Utrecht University, The Netherlands 2001; pp 97–116.
Dirks A, Groenik L, Lutje Schipholt M, Van der Gugten J, Hijzen TH, Geyer MA, et al. Induced startle reactivity and plasticity in transgenic mice overexpressing corticotrophin-releasing hormone. Biol Psychiatry 2002;51:583–90.
Farrokhi CB, Tovote P, Blanchard RJ, Blanchard DC, Litvin Y, Spiess J. Cortagine: behavioral and autonomic function of the selective CRF receptor subtype1 agonist. CNS Drug Rev 2007;13:423–43.
Feldman S, Weidenfeld J. Glucocorticoid receptor antagonists in the hippocampus modify the negative feedback following neural stimuli. Brain Res 1999;821(1):33–7.
Feldman S, Conforti N, Weidenfeld J. Limbic pathways and hypothalamic neurotransmitters mediating adrenocortical response to neural stimuli. Neurosci Biobehav Rev 1995;19:235–40.
Forster GL, Feng N, Watt MJ, Korzan WJ, Mouw NJ, Summers CH, et al. Corticotropin-releasing factor in the dorsal raphe elicits temporally distinct serotonergic responses in the limbic system in relation to fear behavior. Neuroscience 2006;141(2):1047–55.
Groenink L, Dirks A, Verdouw PM, Schipholt M, Veening JG, Van der Gugten J, et al. HPA axis dysregulation in mice overexpressing corticotropin releasing hormone. Biol Psychiatry 2002;51(11):875–81.
Heinrichs SC, Koob GF. Corticotropin-releasing factor in brain: a role in activation, arousal, and affect regulation. J Pharmacol Exp Ther 2004;311(2):427–40.
Heinrichs SC, Stenzel-Poore MP, Gold LH, Battenberg E, Bloom FE, Koob GF, et al. Learning impairment in transgenic mice with central overexpression of corticotropin-releasing factor. Neuroscience 1996;74(2):303–11.
Holsboer F. The rationale for corticotrophin-releasing hormone receptor (CRH-R) antagonists to treat depression and anxiety. J Psychiatr Res 1999;33:181–214.
Kasahara M, Groenink L, Breuer M, Olivier B, Sarnyai Z. Altered behavioural adaptation in mice with neural corticotrophin-releasing factor overexpression. Genes Brain Behav 2007;6:598–607.
Makino S, Gold P, Schulkin J. Corticosterone effects on corticotropin-releasing hormone mRNA in the central nucleus of the amygdala and the parvocellular region of the paraventricular nucleus of the hypothalamus. Brain Res 1994;640:105–12.
Mansuy IM, Winder DG, Moallem TM, Osman M, Mayford M, Hawkins RD, et al. Inducible and reversible gene expression with the rtTA system for the study of memory. Neuron 1998;21:257–65.
Mayford M, Bach ME, Huang YY, Wang L, Hawkins RD, Kandel ER. Control of memory formation through regulated expression of a CamKII transgene. Science 1996;274 (5293):1678–83.
Merlo Pich E, Lorang M, Yaganeh M, Rodriguez de Fonseca F, Raber J, Koob GF, et al. Increase of extracellular corticotropin-releasing factor levels in the amygdala of awake rats during restraint stress and ethanol withdrawal as measured by microdialysis. J Neurosci 1995;15(8):5439–47.
Monroe SM, Torres LD, Guillaumot J, Harkness KL, Roberts JE, Frank E, et al. Life stress and the long-term treatment course of recurrent depression: III. Nonsevere life events predict recurrence for medicated patients over 3 years. J Consult Clin Psychol 2006;74(1):112–20.
Mugnaini M, Tessari M, Tarter G, Merlo PE, Chiamulera C, Bunnemann B. Upregulation of [3H]methyllycaconitine binding sites following continuous infusion of nicotine, without changes of alpha7 or alpha6 subunit mRNA: an autoradiography and in situ hybridization study in rat brain. Eur J Neurosci 2002;16:1633–46.
Muller MB, Zimmerman S, Sillaber I, Hagemeyer TP, Deussing JM, Timpl P, et al. Limbic corticotropin releasing-factor 1 receptor mediates anxiety-related behavior and adaptation to stress. Nat Neurosci 2003;6:1100–7.
Paxinos G, Franklin BJF. The mouse brain in stereotaxic coordinates. San Diego: Academic Press; 2001.
Pelleymounter MA, Joppa M, Carmouche M, Cullen MJ, Brown B, Murphy B, et al. Role of corticotrophin-releasing factor (CRF) receptors in the anorectic syndrome induced by CRF. J Pharmacol Exp Ther 2000;293(3):799–806.

Pich EM, Koob GF, Heilig M, Menzaghi F, Vale W, Weiss F. Corticotropin-releasing factor release from the mediobasal hypothalamus of the rat as measured by microdialysis. Neuroscience 1993;55(3):695–707.
Plotsky PM. Hypophysiotropic regulation of stress-induced ACTH secretion. Adv Exp Med Biol 1988;245:65–81.
Porsolt RD, Bertin A, Jalfre M. Behavioural despair in mice: a primary screening test for antidepressants. Arch Int Pharmacodyn Ther 1977;229:327–36.
Rassnick S, Heinrichs SC, Britton KT, Koob GF. Microinjection of a corticotropin-releasing factor antagonist into the central nucleus of the amygdala reverses anxiogenic-like effects of ethanol withdrawal. Brain Res 1993;605(1):25–32.
Reul JMHM, Holsboer F. Corticotropin-releasing factor receptors 1 and 2 in anxiety and depression. Curr Opin Pharmacol 2002;2(1):23–33.
Rose SD, MacDonald RJ. Integration of tetracycline regulation into a cell-specific transcriptional enhancer. J Biol Chem 1997;272(8):4735–9.
Sherman JE, Kalin NH. ICV-CRH alters stress-induced freezing behavior without affecting pain sensitivity. Pharmacol Biochem Behav 1988;30(4):801–7.
Siggins GR, Gruol D, Aldenhoff J, Pittman Q. Electrophysiological actions of corticotropin-releasing factor in the central nervous system. Fed Proc 1985;44(1 Pt 2):237–42.
Stenzel-Poore MP, Cameron VA, Vaughan J, Sawchenko PE, Vale WW. Development of Cushing's syndrome in corticotrophin-releasing factor transgenic mice. Endocrinology 1992;130:3378–86.
Vale W, Spiess J, Rivier C, Rivier J. Characterization of a 41-residue ovine hypothalamic peptide that stimulates secretion of corticotropin and beta-endorphin. Science 1981;213(4514):1394–7.
Van Gaalen MM, Stenzel-Poore MP, Holsboer F, Steckler T. Effects of transgenic overproduction of CRH on anxiety-like behaviour. Eur J Neurosci 2002;15(12):2007–15.
Van Gaalen MM, Stenzel-Poore M, Holsboer F, Steckler T. Reduced attention in mice overproducing corticotrophin-releasing hormone. Behav Brain Res 2003;142(1–2):69–79.