

Corticotropin-releasing factor (CRF) over-expression down-regulates hippocampal dopamine receptor protein expression and CREB activation in mice

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Abstract

BACKGROUND: Stress results in hyperactivity of the hypothalamic-pituitary-adrenal (HPA) axis, characterized by increased central CRF activity, elevated circulating glucocorticoid levels, impaired glucocorticoid-mediated negative feedback and abnormal hippocampal functions, possibly contributing to the development of behavioral pathologies, such as depression. The hippocampus is critically involved in the control of the HPA axis as well as in explicit memory, contextual aspects of fear, organization of the behavioral response to environmental novelty and in habituation. We have previously shown that mice that over-express CRF in the brain exhibit impaired novelty detection and altered psychophysiological and behavioral habituation, functions linked to dopamine receptor-dependent hippocampal plasticity.

OBJECTIVE AND METHODS: Therefore, the aim of the present study was to measure D1 and D2 dopamine receptor expression and related signaling, such as CREB and ERK protein levels and phosphorylation, in the hippocampus and other brain regions of mice with post-natal CRF over-expression (CRF-OE mice).

RESULTS: We found a region-specific down-regulation of both D1 and D2 protein expression, without altered CRF receptor protein expression, in the hippocampus in CRF-OE mice. This was accompanied by an impaired phosphorylation of hippocampal CREB, but not ERK1 and ERK2, in the same animals.

CONCLUSIONS: These results suggest that post-natal onset CRF over-expression results in an impairment of dopamine signaling in the hippocampus, which may underlie cognitive and motivational aspects of stress-related, CRF-driven mood disorders.

INTRODUCTION

Novelty detection, habituation and contextual learning, all involved in the organized adaptive behavioral response to changing environment and adversity (stress), depend on the intact functioning of the hippocampus (Eichenbaum 2006). Hippocampus has also been shown to regulate the activity of the HPA axis and plays a major role in the limitation of the neuroendocrine stress response (Herman & Cullinan 1997). Hippocampal afferents are involved in modulating the activity of other brain regions, such as prefrontal cortex and ventral striatum, which contribute with cognitive and motivational content to the stress response (Del Arco & Mora 2009; Grace 2010). Conversely, stress has been shown to affect the structural and functional integrity of the hippocampus and other brain regions, including the prefrontal cortex and the amygdala. For example, chronic stress results in dendritic remodeling in the hippocampus, amygdala and in the prefrontal cortex, decreased neurogenesis in the dentate gyrus of the hippocampus, and hippocampal volume reduction (Lupien *et al.* 2009).

CRF is the main neurobiological substrate to mediate effects of stress on behavioral, hormonal and physiological adaptation of the organism. Besides its neuroendocrine role to activate the HPA axis, CRF acts on a number of forebrain regions, including the hippocampus, amygdala and prefrontal cortex, to organize the behavioral response to stress (Lupien *et al.* 2009). Several lines of evidence from preclinical studies and clinical research have shown that brain CRF hyperactivity induced by stress plays a major role in the long-term behavioral and neuroendocrine consequences of early adverse environment and in the pathophysiology of anxiety disorders and depression (Binder & Nemeroff 2010). For example, early postnatal CRF hypersecretion has been shown to cause derangement of dendritic spine dynamics in the hippocampus, which may contribute in the development of abnormal hippocampal plasticity that underlies pathological, anxiety- and depression-like behaviors in later life (Chen *et al.* 2008). Clinically, childhood trauma has been associated with increased central CRF activity, reduced hippocampal volume and depression in later life (Heim *et al.* 2008).

In order to investigate the role of early-onset CRF hypersecretion in chronic stress in adulthood, we have recently developed a transgenic mouse model in which CRF hyperactivity starts at 4 to 8 days after birth, continues throughout adulthood and is restricted to the central nervous system due to the promoter and regulatory regions used (Dirks *et al.* 2002a). These mice show impaired novelty detection and altered psychophysiological and behavioral habituation to novel environmental stimuli (Dirks *et al.* 2002b; Kasahara *et al.* 2007). Such behavioral endophenotype may reflect abnormal dopamine-dependent hippocampal plasticity (Lisman & Atmakhova 2001).

We, therefore, hypothesized that mice with early-onset, chronic CRF overexpression may have altered dopamine receptor expression in the hippocampus. Furthermore, we also hypothesized that these mice may show impairments in the stress-induced activation of intracellular signaling downstream of both CRF and dopamine receptors. Such changes may contribute in cognitive dysfunctions and hippocampal structural alterations in chronic stress states and associated psychopathology, such as depression, driven by long-term over-activity of the brain CRF system. To test these hypotheses, we measured dopamine D1 and D2 receptor protein expression and activation of dopamine-related intracellular signaling, such as cAMP-response element binding protein (CREB) and extracellular signal regulated kinase 1&2 (ERK1/2), in the hippocampus and in other brain regions that have been found to show high expression of the CRF transgene in these mice (Dirks *et al.* 2002a) and to contribute in the organization of behavioral stress response, such as amygdala and frontal cortex, of mice over-expressing CRF and in wild-type littermates.

METHODS

CRF-OE mice were generated as previously described by Dirks *et al.* (2002a). Male CRF-OE (n=6 per group, aged 27–30 wks) and their wild-type littermates (C57BL/6J WT) without any history of previous behavioral experiments were used. The animals were group-housed in plastic cages (12 × 22 × 15 cm; Techniplast, Buggiate Italy) enriched with bedding (EnviroDri®, BMI, Helmond, The Netherlands), a piece of PVC-tubing (diameter 5 cm) and nesting material at constant room temperature (r.t.p., 21±2°C) and relative humidity (40–50%). Standard rodent food pellets (Special Diet Services Ltd., Essex, UK) and tap water was freely available. Mice were maintained on a 12h light / 12h dark cycle (lights on from 6:00 a.m. until 6:00 p.m.). All experimental procedures were conducted during the light phase of the cycle, between 9:00 a.m. and 4:30 p.m. and were approved by the ethical committee on animal experiments of the Faculties of Pharmacy, Biology, and Chemistry of Utrecht University, The Netherlands.

To obtain samples for the analysis of D1, D2 and CRF-R1/2 receptor protein expression animals were decapitated and frontal cortices, striata, hippocampi and amygdalae were immediately dissected out on ice. For the measurement of the signaling molecules CREB and ERK and their activated (phosphorylated) forms, CRF-OE and WT mice were subjected to a mild transportation/novel environment stress for about 15 min (Shen *et al.* 2004), after which they were sacrificed by decapitation and their hippocampi were dissected out on dry ice. The tissues were individually placed in vials and were frozen in liquid nitrogen and were kept at –20°C until homogenization.

For Western blotting, the tissues were homogenized and centrifuged for 30 min at 4°C. Then the samples

were heated at 70°C for 10 min and then loaded onto 12-well NuPAGE® Novex 4–12% Bis-Tris gel (Invitrogen, California, USA). The electrophoresis was carried out with XCell II™ Blot Module (Invitrogen, California, USA) and was run at 200 volts for 45 min. Magic Mark™XP Western Protein Standard (Invitrogen, California, USA) was also loaded onto each gel and was used as the protein size marker. Transfer membranes (Immunobilon-P, Millipore, Massachusetts, USA) were pre-soaked in methanol for 2 min and washed with transfer buffer. The membrane transfer was carried out in the XCell II™ Blot Module (Invitrogen, California, USA) at 30 volts for 1 h. The membranes with transferred proteins were washed three times, 7 min each with Tris-buffered saline with 0.1% Tween-20 (TBS-T) and were incubated in blocking solution (TBS-T containing 1% albumin bovine serum, BSA) overnight at 4°C. On the following day, the membranes were washed and then incubated with a primary antibody solution. D1 dopamine receptor (H-109, sc-14001; rabbit polyclonal IgG, epitope corresponding to amino acids 338–446 mapping at the C-terminus of dopamine receptor type 1 of human origin), D2 dopamine receptor (H-50, sc-9113; rabbit polyclonal IgG, epitope corresponding to the amino acids 1–50 at the N-terminal extracellular region of the human dopamine receptor type 2 to detect both long and short forms of D2 receptors) and CRF-R1/2 (C-20, sc-1757; goat polyclonal IgG, epitope mapping at the C-terminus of the CRF-R1 of human origin to detect both R1 and R2 CRF receptors) primary antibodies were obtained from Santa Cruz Biotechnology Inc. CREB (48H2; No. 9197; rabbit monoclonal antibody to detect endogenous levels of total CREB protein), phospho-CREB-Ser133 (87G3, No. 9198; rabbit monoclonal antibody to detect CREB when phosphorylated at Ser133), ERK 1/2 (No. 9102; rabbit polyclonal antibody to detect ERK1/2 [p44/42 MAPK]) and phospho-ERK1/2 (No. 9106; mouse monoclonal antibody, detects p44 and p42 MAP Kinase [Erk1 and Erk2] when dually phosphorylated at Thr202 and Tyr204 of Erk1 [Thr185 and Tyr187 of Erk2], and singly phosphorylated at Tyr204) primary antibodies were obtained from Cell Signaling Technology. After imaging and stripping, the membranes were incubated with β -actin primary antibody (AC-15, Sigma-Aldrich) for 24 h at 4°C to control for the quantity of proteins each sample contained. After incubating with the primary antibody the membranes were washed three times, for 10 min each and then incubated with the appropriate secondary antibody solution for 2 h at 4°C and washed with TBS-T. The membranes were then incubated with 4 ml of SuperSignal® West Pico Chemiluminescent Substrate (Pierce, Illinois, USA) for 5 min before imaging using GeneGnome HR (Syngene, Cambridge, UK). Gel analysis plug-in of the ImageJ software (National Institute of Health, Maryland, USA, available at <http://rsb.info.nih.gov/ij/>) was used to quantify the intensity of protein bands. The obtained figures for

each sample were normalized by dividing them with the figure for β -actin of the same sample. The experiment was repeated and the average figures from two experiments was calculated and used for statistical analysis. The data were analyzed by one-way analysis of variance (ANOVA) on genotype for each brain region or by t-test using STATVIEW (SAS Institute, Inc., North Carolina, USA). The level of significance was set at $p < 0.05$.

RESULTS

Dopamine D1 receptor protein expression was significantly decreased ($F_{1,8}=7.496$, $p=0.026$; –58%) in the hippocampus, but not in the in the frontal cortex ($F_{1,7}=0.238$, $p=0.641$), striatum ($F_{1,6}=0.0003$, $p=0.986$) and amygdala ($F_{1,6}=0.892$, $p=0.382$), of CRF over-expressing (CRF-OE) mice compared to wild-type (WT) mice (Figure 1A). Similarly, D2 receptor protein expression was also decreased ($F_{1,8}=6.094$, $p=0.039$; –82%) in the hippocampus,), but not in the in the frontal cortex ($F_{1,7}=1.742$, $p=0.228$), striatum ($F_{1,6}=0.022$, $p=0.888$) and amygdala ($F_{1,6}=0.323$, $p=0.591$), of CRF-OE mice compared to WT mice (Figure 1B). Dopamine and CRF receptor-related downstream signaling, e.g. levels of CREB and ERK as well as their activated, phosphorylated, forms (pCREB, pERK1, pERK2), was measured in response to a mild transportation/novelty stress in the hippocampus of CRF-OE and WT littermates. We found a significant decrease in pCREB levels in the hippocampus of CRF-OE mice compared to WT animals (t-test, $p=0.013$; –67%; Figure 1C), but no changes in CREB, ERK1 and ERK2 or pERK1 and pERK2 levels (Figure 1C).

As compensatory changes in CRF receptor protein levels could underlie the observed differences in dopamine receptor and pCREB expression in CRF-OE mice (Korosi *et al.* 2006) we measured CRF-R1/2 protein expression in these regions. We found an increased expression in the CRF-OE frontal cortex ($F_{1,7}=6.738$, $p=0.036$; +252 %) but no difference in the hippocampus, amygdala and in the striatum between CRF-OE and WT mice (Figure 2). β -actin protein levels were not different between WT and CRF mice in any regions measured (frontal cortex, $F_{1,6}=1.169$, $p=0.321$; hippocampus, $F_{1,6}=1.156$, $p=0.707$; striatum, $F_{1,6}=0.149$, $p=0.713$; amygdala, $F_{1,6}=0.266$, $p=0.624$).

DISCUSSION

Our present results show that brain-specific, early post-natal onset CRF over-expression is associated with decreased D1 and D2 dopamine receptor expression and with an impaired activation of CREB signaling selectively in the hippocampus of adult male mice. However, fine spatial analysis of receptor changes was not possible with the dissection method used in this study, therefore other methods, such as *in situ* hybrid-

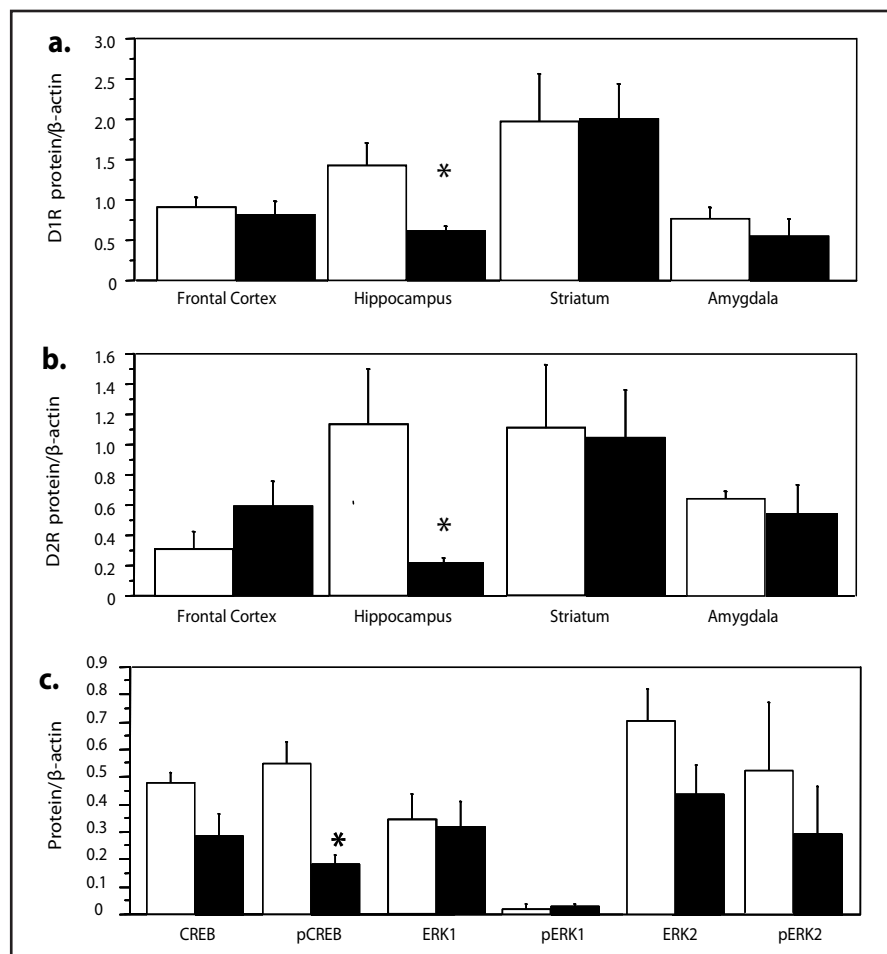


Fig. 1. D1 and D2 dopamine receptor expression and CREB, pCREB, ERK1, pERK1, ERK2 and pERK2 expression (mean \pm S.E.M.; expressed as ratio to β -actin) in the frontal cortex, hippocampus, striatum and amygdala brain regions of WT (open bars) and CRF-OE (closed bars) adult (27-35 weeks old) male mice. (a) D1 dopamine receptor protein (D1R) levels are significantly decreased in the hippocampus, but not in the in the frontal cortex, striatum and amygdala, of CRF-OE mice compared to WT mice. (b) D2 dopamine receptor protein (D2R) levels are significantly decreased in the hippocampus, but not in the in the frontal cortex, striatum and amygdala, of CRF-OE mice compared to WT mice. (c) Phosphorylated CREB (pCREB) levels are significantly lower in the hippocampus of CRF-OE mice compared to WT after a short transportation/novelty stress. No differences are found in CREB, ERK1/2 and phosphorylated ERK1/2 (pERK1/2) protein levels in the hippocampus. * $p < 0.05$.

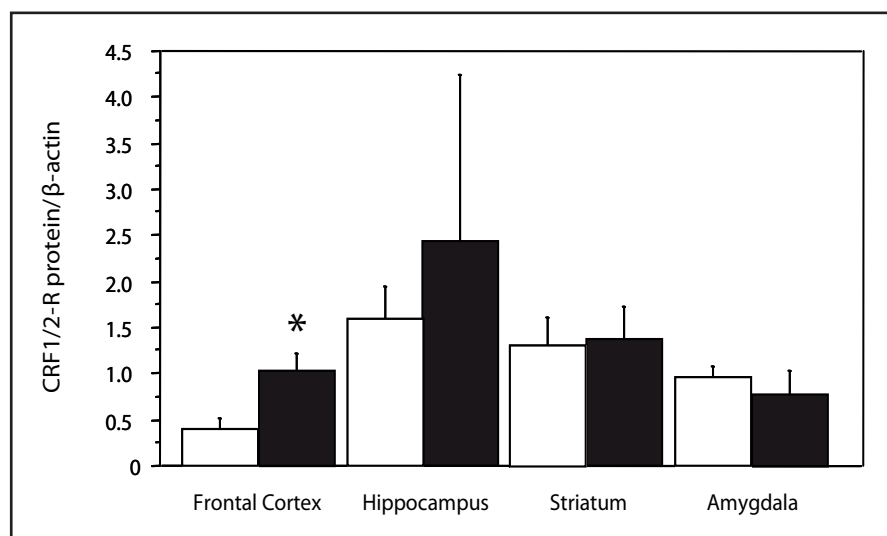


Fig. 2. CRF type-1/2 receptor (CRF-R1/2) protein expression (mean \pm S.E.M.; expressed as ratio to β -actin) in the frontal cortex, hippocampus, striatum and amygdala of WT (open bars) and CRF-OE (closed bars) mice. * $p < 0.05$.

ization or receptor autoradiography, will be needed to reveal sub-region specific alterations of D1 and D2 receptor mRNA or protein expression, respectively.

It has repeatedly been shown that stress alters dopaminergic functions in the brain, including dopamine release (Cabib & Puglisi-Allegra 1994) and dopamine receptor binding (Kram *et al.* 2002). However, to the

best of our knowledge, this study is the first to demonstrate that chronically elevated CRF tone in the brain down-regulates dopamine receptors in the hippocampus. One possible explanation is that elevated circulating corticosterone, which has been found in the same line of CRF-OE mice (Groenink *et al.* 2002) may mediate the observed changes. Although corticos-

terone-induced alterations of dopamine release (Piazza *et al.* 1996), receptor binding (Biron, *et al.* 1992) and dopamine transporter binding (Sarnyai *et al.* 1998) have been shown in other terminal areas of the ventral tegmental area dopaminergic projections, such as the nucleus accumbens and cerebral cortex, no direct evidence indicate changes in hippocampal dopamine receptors. It is somewhat unlikely that the elevated corticosterone levels in the CRF-OE mice play a major role, since the deficit in prepulse inhibition of startle, a dopamine-related sensorimotor gating abnormality observed in the same line of transgenic mice, can be normalized by blocking CRF receptors but not by glucocorticoid antagonists and adrenalectomy (Groenink *et al.* 2008). However, in the CRF-OE mice CRF over-expression starts in early postnatal life and persists throughout adulthood (Groenink *et al.* 2002). It is, therefore, most likely that changes observed in this study are not the direct consequence of elevated CRF and/or corticosterone levels themselves but they rather constitute long-term adaptive changes in the hippocampus in response to the life-long elevation of brain CRF. Further studies using adrenalectomy or the administration of a CRF antagonist will be needed to clarify the relative contribution of circulating corticosterone versus elevated brain CRF.

Early life stress has been shown to influence stress response at cellular and systems level as well as hippocampal functions in adulthood (Lupien *et al.* 2009). Stress has been shown to activate CREB and ERK signaling in the hippocampus (Shen *et al.* 2004). Therefore, we measured CREB and ERK1/2 protein levels as well as levels of their activated (phosphorylated) forms in the hippocampus in response to a mild transportation/novelty stress. We found decreased levels pCREB in CRF-OE mice compared to WT, suggesting a possible impairment in the stress-induced activation of intracellular signaling, associated with CRF over-expression. Activation (phosphorylation) of CREB is downstream of many receptors mediating effects neurotransmitters and neuromodulators responsive to stress, including both CRF and dopamine (Lonze & Ginty 2002). Therefore, down-regulation of these receptors may underlie the observed impairment in CREB phosphorylation. While CRF receptor expression remains intact in the hippocampus of the CRF-OE mice (see below), dopamine receptor expression is decreased, possibly implicating the down-regulation of dopamine receptors in the functional changes of intracellular signaling in the hippocampus of CRF-OE mice.

Long-term over-expression of CRF may lead to adaptive changes in CRF receptor expression in the brain (Korosi *et al.* 2006), which can underlie associated molecular and functional alterations in these mice. However, we found no difference in CRF receptor protein expression using an antibody that recognizes both R1 and R2 types of CRF receptors in the hippocampus, the region where dopamine receptor and pCREB

changes were found. This is in line with results of a recent study using the same line of CRF-OE mice showing no changes in CRF-R1 mRNA expression in the CA1, CA3 and dentate gyrus sub-regions of the hippocampus in CRF-OE mice (Korosi *et al.* 2006). On the other hand, in the present study, we found an increase in CRF-R1/2 expression in the frontal cortex of CRF-OE mice, where no changes in dopamine receptor expression were identified. Together, these findings rule out the possibility that adaptive changes at the level of CRF receptors may underlie the observed decrease in dopamine receptors and in CREB signaling in CRF-OE mice. The observed up-regulation of CRF receptor protein expression in the frontal cortex is somewhat unexpected, as hypersecretion of CRF by stress or increased circulation of corticosterone often results in down-regulation of CRF receptors (Bale & Vale 2004). The frontal cortex was not described in the previous CRF-R mRNA *in situ* hybridisation study on CRF-OE mice by Korosi *et al.* (2006), which concluded that the central CRF over-expression results in down-regulation of CRF-R1 mRNA and up-regulation of CRF-R2 mRNA in a brain-region-specific way. This present study may add to their finding and suggest that the up- and down-regulations of CRF-Rs by chronic central CRF over-expression may be more complex than previously described. Since reduction in functional CRF-R1 in the frontal cortex reduces anxiety (Heinrichs *et al.* 1997; Nguyen *et al.* 2006), the up-regulation of CRF receptor protein expression may predict heightened anxiety-like behavior in response to stress. In line with this, we have previously shown that CRF-OE mice exhibit anxiety-like behavior in a novel open-field (Kasahara *et al.* 2007).

Intrahippocampal dopamine has been shown to be involved in memory encoding, novelty detection and habituation (Lisman & Atmakhova 2001). Deficits in hippocampal functioning were found during verbal declarative memory encoding in major depression (Bremner *et al.* 2004). Furthermore, D2 dopamine receptor binding in the hippocampus positively correlates with hippocampal memory functions in healthy volunteers (Takahashi *et al.* 2007). Heightened brain CRF neurotransmission has been associated with impaired habituation to novelty and attention (Kasahara *et al.* 2007; van Gaalen *et al.* 2003), impaired spatial learning (Heinrichs *et al.* 1996) and sensorimotor gating (Dirks *et al.* 2002b), functions related to hippocampus. Therefore, it is possible that impaired dopamine signaling in the hippocampus observed in the present study may underlie some of the cognitive, emotional and motivational abnormalities associated with the hyperactivity of brain CRF systems.

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CONFLICT OF INTEREST

None declared.

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