

# Valproic acid inhibits corticotropin-releasing factor synthesis and release from the rat hypothalamus in vitro: evidence for the involvement of GABAergic neurotransmission

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**Objective:** Corticotropin-releasing factor (CRF), the major adrenocorticotrophic hormone (ACTH) secretagogue, acts within the brain to integrate the stress responses of the central nervous, endocrine and immune systems. The involvement of this peptide in the origin and pathophysiology of various endocrine, neurologic, inflammatory and psychiatric diseases, particularly affective disorders, has also been suggested. The antiepileptic drug valproic acid is frequently used as a mood-stabilizing agent in patients with bipolar disorders; however, its mechanism of action for the latter indication is still poorly characterized. We investigated whether valproic acid can directly modulate CRF production by using the incubation of rat hypothalamic explants as an in-vitro model. We then studied the involvement of the  $\gamma$ -aminobutyric acid (GABA) system as a putative mediator of the effects of valproic acid on CRF production. **Methods:** Rat hypothalamic explants were incubated in a 24-well plate (2 hypothalami per well) at 37°C in a humidified atmosphere (5% CO<sub>2</sub> and 95% O<sub>2</sub>) in incubation medium, 700  $\mu$ L, then were treated with medium alone (control) or test substances, namely, valproic acid, KCl, bicuculline methiodide and muscimol. Released CRF was measured by radioimmunoassay. CRF mRNA was measured by RNase protection analysis. **Results:** Incubation of the hypothalamic fragments with valproic acid, 100  $\mu$ mol/L, resulted in a reduction of basal CRF secretion after 3 hours' treatment. The drug was also able to inhibit KCl-stimulated CRF release. Moreover, valproic acid, 100  $\mu$ mol/L, significantly decreased CRF mRNA levels after 3 hours. A specific GABA<sub>A</sub> receptor antagonist, bicuculline methiodide, completely reversed the inhibition of CRF gene expression and peptide release induced by valproic acid; in this paradigm, the GABA<sub>A</sub>-specific agonist muscimol inhibited both CRF gene expression and peptide release in a concentration-dependent manner. **Conclusions:** These results suggest that valproic acid may exert part of its therapeutic effect as a mood-stabilizing drug via the modulation of CRF secretion from the hypothalamus. This action may be mediated in part by the activation of GABAergic neurotransmission.

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**Objectif :** La corticolibérine, principal sécrétagogue de la corticotrophine (ACTH), agit à l'intérieur du cerveau pour intégrer les réactions au stress des systèmes nerveux central, endocrinien et immunitaire. On a aussi indiqué que ce peptide pourrait avoir un effet sur l'origine et la pathophysiologie de diverses maladies endocriniennes, neurologiques, inflammatoires et psychiatriques, et en particulier les troubles affectifs. On utilise souvent l'acide valproïque, antiépileptique, comme agent thymorégulateur chez les patients qui ont des troubles bipolaires, mais son mécanisme d'action dans ce dernier cas demeure mal caractérisé. Nous avons cherché à déterminer si l'acide valproïque peut moduler directement la production de corticolibérine en utilisant l'incubation d'explants d'hypothalamus de rat comme modèle *in vitro*. Nous avons ensuite étudié le rôle du système de l'acide  $\gamma$ -aminobutyrique (GABA) comme médiateur hypothétique des effets de l'acide valproïque sur la production de corticolibérine. **Méthodes :** On a incubé des explants d'hypothalamus de rat sur une plaque à 24 puits (2 hypothalamus par puits) à 37 °C dans une atmosphère humidifiée (5 % de CO<sub>2</sub> et 95 % d'O<sub>2</sub>) dans un milieu d'incubation, 700  $\mu$ L, et on les a traités ensuite avec du milieu seulement (témoin) ou des substances d'essai, soit l'acide valproïque, le KCl, la bicuculline methiodide et le muscimol. On a mesuré la corticolibérine produite par dosage radio-immunologique. On a mesuré l'ARNm de la corticolibérine par analyse de protection de la RNase. **Résultats :** L'incubation des fragments d'hypothalamus dans 100  $\mu$ mol/L d'acide valproïque a entraîné une baisse de la sécrétion basale de corticolibérine après trois heures de traitement. Le médicament a aussi pu inhiber la production de corticolibérine stimulée par le KCl. De plus, l'acide valproïque à 100  $\mu$ mol/L a réduit considérablement les concentrations d'ARNm de corticolibérine après trois heures. La bicuculline methiodide, antagoniste spécifique des récepteurs de GABA<sub>A</sub>, a inversé complètement l'inhibition de l'expression génique de la corticolibérine et la production de peptide déclenchée par l'acide valproïque. Dans ce paradigme, l'agoniste spécifique de GABA<sub>A</sub>, le muscimol, a inhibé à la fois l'expression génique de la corticolibérine et la production de peptide en fonction de la concentration. **Conclusions :** Ces résultats indiquent que l'acide valproïque peut exercer une partie de son effet thérapeutique comme médicament thymorégulateur en modulant la sécrétion de corticolibérine par l'hypothalamus. Cet effet peut être produit en partie par l'activation de la neurotransmission par le GABA.

## Introduction

In addition to its effect on pituitary adrenocorticotrophic hormone (ACTH) release, the 41-amino-acid peptide corticotropin-releasing factor (CRF) plays a key role in the regulation of endocrine, autonomic and behavioural responses to stress, as well as the communication among the immune, central nervous and endocrine systems.<sup>1,2</sup> Clinical and experimental evidence also suggest the involvement of this peptide in the origin and pathophysiology of several endocrine, neurologic, inflammatory and, more recently, psychiatric diseases. As far as the latter disorders are concerned, several studies suggest that increased CRF secretion may contribute to the development and symptoms of affective and anxiety-related disorders.<sup>3</sup>

The antiepileptic drug valproic acid is used as a mood-stabilizing agent in patients with bipolar disorders.<sup>4</sup> In spite of its widespread use for this clinical condition, the mechanism(s) of action underlying the favourable therapeutic activity of valproic acid is poorly understood at present. It is possible that valproic acid acts via the control of CRF neurons in the brain. Indeed, it has been shown that valproic acid can alter the hypothalamic–pituitary–adrenal (HPA) axis in humans.<sup>5,6</sup> In the rat, it has recently been reported that

this drug can modulate negatively the CRF neuronal system *in vivo*.<sup>7,8</sup>

In the present study, we used a well-established *in vitro* model, namely, the incubation of rat hypothalamic explants,<sup>9</sup> to investigate the molecular mechanism(s) through which valproic acid regulates CRF production in the brain. Because it has been claimed that an increased  $\gamma$ -aminobutyric acid (GABA)-ergic tone accounts, at least in part, for the antiepileptic action of valproic acid,<sup>10</sup> we also investigated GABAergic neurotransmission as a putative mediator of the effect of valproic acid on CRF production and release.

## Methods

Male Wistar rats (200–250 g) were decapitated between 9 am and 10 am, and the brains were rapidly removed. The use of animals for this experimental work was approved by the Italian Ministry of Health (licensed authorization to P.N.). Hypothalami were dissected with the following limits: the posterior border of the optic chiasm, the anterior border of the mamillary bodies and the lateral hypothalamic sulci, with a depth of about 2 mm. The explants were then divided longitudinally in 2 halves<sup>11</sup> to aid diffusion of medium. Total dissection time was less than 2 minutes from decapitation.

This procedure maintained the anatomical integrity of the CRF neuron pathway, which ranges from the paraventricular nuclei of the hypothalamus to the median eminence. This enabled the observation of the possible effects of test substances mediated via interactions at the level of cell bodies, as well as those on nerve terminals at the median eminence.

The hypothalami were incubated in a 24-well plate (2 hypothalami per well) at 37°C in a humidified atmosphere consisting of 5% CO<sub>2</sub> and 95% O<sub>2</sub> in incubation medium, Minimum Essential Medium (MEM) with Earle's Salts, 700 µL, supplemented with 0.2% human serum albumin; glutamine, 2 mmol/L; ascorbic acid, 60 µg/mL; and aprotinin, 100 IU/mL, of pH 7.4. In this experimental model, hypothalamic neurons remained viable and functional during the time frame of the experiments, as assessed by the lactate dehydrogenase (LDH) assay for cellular toxicity, which showed no statistical difference between control and treated hypothalami (data not shown). Thus, variation in CRF gene expression or peptide release did not appear to be correlated with toxic damage to the tissues.

After a 60-minute preincubation period (during which the medium was changed every 20 minutes), the medium was aspirated and replaced with fresh medium alone (control) or the test substances at appropriate concentrations. In experiments with KCl, hypothalami were treated with medium alone (control) or valproic acid, 10–100 µmol/L, for 1 hour; this was followed by a second incubation with medium alone (control), 56 mmol/L KCl alone or 56 mmol/L KCl in the presence of valproic acid at the above concentrations, for 30 minutes. Whenever KCl was used, MEM was replaced by a medium consisting of KCl, 56 mmol/L, and NaCl, 67 mmol/L, with the same concentration of the other ions as found in MEM. In experiments with the GABA<sub>A</sub> antagonist bicuculline methiodide, the latter was added to the incubation medium 20 minutes before and then during the incubation in the presence of valproic acid.

Valproic acid, bicuculline methiodide and muscimol hydrobromide were purchased from Sigma Chemicals (St. Louis, Mo.). Stock solutions of the chemicals were prepared in distilled water and then further diluted to working concentrations in incubation medium or KCl medium, 56 mmol/L, when appropriate. None of these substances interfered with CRF assay.

At the end of the experiments, incubation media were collected and stored at -35°C until assay for CRF

immunoreactivity. For RNA analysis, hypothalami were embedded in 2 mL of RNA Later solution (Ambion, Austin, Tex.) and kept at -20°C until RNA extraction.

#### *CRF radioimmunoassay*

CRF was measured by radioimmunoassay,<sup>11</sup> with the following modifications: a CRF antiserum (kindly donated by Prof. R. Bernardini) and (2-[<sup>125</sup>I]-iodohistidyl<sup>32</sup>)-CRF were used. The detection limit of the assay was 1 pg per tube (200-µL sample volume for incubation media), with intra-assay and interassay coefficients of variation of 5% and 10%, respectively. Released amounts of CRF were expressed as picograms per millilitre.

#### *RNA extraction*

Total RNA was extracted by the guanidine thiocyanate lysis method described by Chomczynski and Sacchi.<sup>12</sup> The average yield of RNA was 45–55 µg per hypothalamus.

#### *RNase protection assay*

RNase protection analyses were performed as described elsewhere.<sup>13</sup> Briefly, to measure CRF mRNA expression, a 702-nucleotide riboprobe containing a 642-nucleotide antisense sequence specific to rat CRF (554–1195 bp) was generated using T3 RNA polymerase in the presence of [<sup>32</sup>P] UTP (800 Ci/mmol) (1 Ci = 3.7 × 10<sup>10</sup> Bq) and a plasmid (pBCRF), containing the full-length rat CRF cDNA, as template. Rat glyceraldehyde-3-phosphate dehydrogenase (rGAPDH) was used as an internal loading control to allow assessments of total RNA levels for normalizing samples. A 244-nucleotide-specific antisense riboprobe resulting in a protected fragment of 134 nucleotides was synthesized with SP6 RNA polymerase and [<sup>32</sup>P] UTP (400 Ci/mmol) using the rGAPDH plasmid as template (pTRI, Ambion, Austin, Tex.).

RNase protection assays were performed<sup>14</sup> by hybridizing 25 µg of total RNA in 24 µL deionized formamide plus 6 µL hybridization buffer containing 3.5 × 10<sup>5</sup> cpm rCRF and 8000 cpm rGAPDH riboprobes. After heating to 80°C, the samples were hybridized at 45°C for 15 hours and subsequently digested by RNase (RNase A, 200 µg/mL, and RNase T1, 350 U/mL) at room temperature for 60 minutes. The samples were

resolved on 5% polyacrylamide–8M urea gels. Quantitative analysis was performed using the ImageMaster Video Documentation System for gel electrophoresis and the Imagesystem software package (Amersham-Pharmacia Biotech, San Francisco, Calif.). The intensity of the protected CRF fragments was normalized to the intensity of the protected GAPDH fragment of the same sample, and the results were reported as corrected arbitrary units.

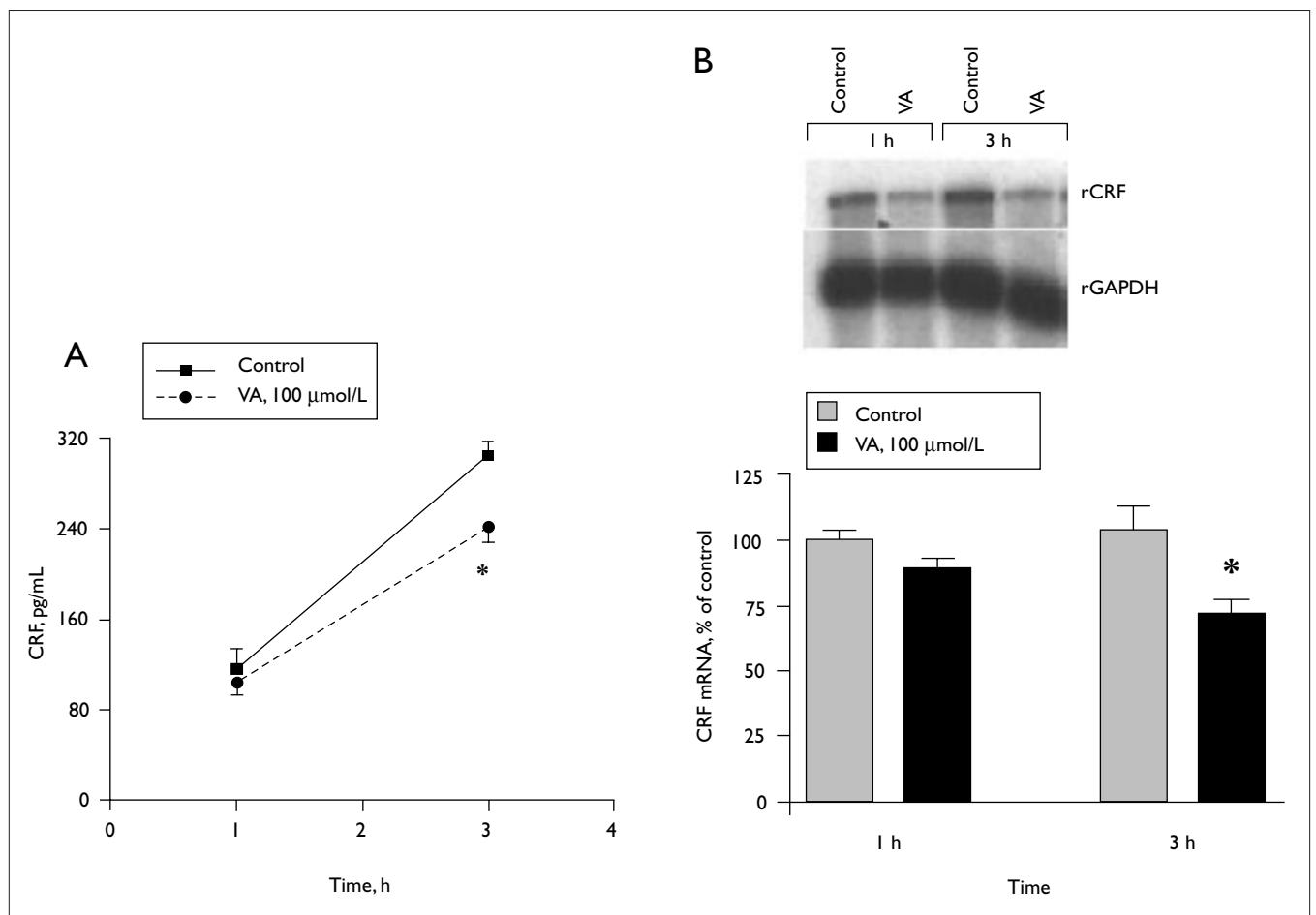
#### Statistical analysis

All results are presented as the mean (and standard error of the mean [SEM]) of at least 3 different experiments

performed in triplicate, unless otherwise specified. Data were analyzed by 1-way analysis of variance (ANOVA), the post hoc Newman–Keuls test for comparison between group means and Student's *t* test; differences were considered statistically significant if  $p < 0.05$ .

#### Results

The effect of valproic acid on CRF production was determined by incubating the hypothalamic explants with graded doses (1–100  $\mu\text{mol/L}$ ) of the drug. Although a concentration-dependent *trend* toward inhibition of basal CRF release was observed after 1 hour of treatment, reduction did not reach significant levels

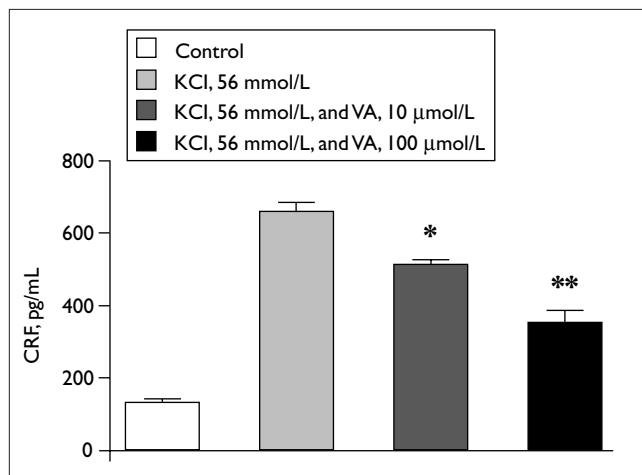


**Fig. 1A:** The effect of valproic acid (VA) on the release of corticotropin-releasing factor (CRF) in the rat hypothalamus. Hypothalamic explants were incubated with medium alone (control) or with medium containing VA, 100  $\mu\text{mol/L}$ , for the indicated times. Results are presented as the mean (and standard error of the mean [SEM]) of 5 independent experiments performed in duplicate. \*Significantly different from the control group ( $p < 0.01$ ). **B:** The effect of valproic acid on CRF mRNA levels in the rat hypothalamus. Hypothalamic explants were incubated as in panel A, and total RNA was extracted. The results are from 5 independent experiments performed in duplicate. \*Significantly different from the control group ( $p < 0.05$ ). A representative 5% polyacrylamide–8M urea gel is shown above the bar graph. rCRF = rat CRF, rGAPDH = rat glyceraldehyde-3-phosphate dehydrogenase.

(data not shown). However, valproic acid, 100  $\mu\text{mol/L}$ , significantly decreased CRF release to 79.2% of control value after 3 hours ( $p < 0.01$ , Fig. 1A). The effect on CRF mRNA expression was investigated by incubating hypothalamic fragments for 1 hour and 3 hours in the presence of valproic acid, 100  $\mu\text{mol/L}$ . RNase protection analyses of total RNA isolated from the explants showed that the drug produced a significant decrease in CRF mRNA levels (to 69.4% of control,  $p < 0.05$ ) after 3 hours (Fig. 1B). Moreover, the drug was also able to inhibit KCl-stimulated CRF release at concentrations of 10 and 100  $\mu\text{mol/L}$  ( $p < 0.05$  and  $p < 0.01$  v. control, respectively [Fig. 2]).

To determine whether the decrease in CRF production induced by valproic acid was mediated by the activation of GABAergic neurotransmission within the hypothalamus, incubation of the explants was performed with valproic acid in the presence of 100  $\mu\text{mol/L}$  of bicuculline methiodide, a GABA<sub>A</sub>-selective antagonist, for 3 hours. As shown in Figure 3, bicuculline methiodide completely abolished the action of valproic acid.

The effect of the GABA<sub>A</sub>-selective agonist muscimol on CRF production was determined by incubating the hypothalamic fragments with graded doses of the drug (1–100  $\mu\text{mol/L}$ ) for 3 hours. As shown in Figure 4, muscimol inhibited, in a concentration-dependent manner, both CRF release (to 59% of control, at the concentration



**Fig. 2: The effect of valproic acid on KCl-stimulated CRF release.** Hypothalamic explants were incubated with medium alone (control) or with medium containing KCl, 56 mmol/L, or with medium containing KCl, 56 mmol/L, and the indicated doses of valproic acid. The results are presented as the mean (and SEM) of 5 independent experiments performed in duplicate. \*Significantly different from KCl alone ( $p < 0.05$ ). \*\*Significantly different from KCl alone ( $p < 0.01$ ).

of 100  $\mu\text{mol/L}$ ,  $p < 0.05$ ) and mRNA accumulation (to 60.6%,  $p < 0.05$ , and 53.7% of control,  $p < 0.01$ , at concentrations of 10  $\mu\text{mol/L}$  and 100  $\mu\text{mol/L}$ , respectively).

## Discussion

It is now generally agreed that abnormally increased CRF secretion resulting in hyperactivity of central CRF pathways might be related to the development of several psychiatric disorders, such as major depression, anxiety-related disorders and anorexia nervosa.<sup>15–17</sup> Indeed, patients with affective disorders show hyperactivity of the CRF system with consequent disturbances in the HPA axis.

Anticonvulsant drugs, such as valproic acid, are widely used as mood-stabilizing agents, and patients with bipolar disorder have been successfully treated by long-term administration of this drug.<sup>4</sup> Although valproic acid is often used in affective disorders and has also been studied in animal models, relatively little is known about its mechanism of action in these pathologies. Reports have been published with regard to its ability to modulate the CRF system in vivo,<sup>7,8</sup> and it has been suggested that the therapeutic effects of valproic acid in patients with affective disorders may be mediated, at least in part, via the interaction with this pathway. Although these observations clearly establish that valproic acid can decrease the production of CRF within the brain, the precise mechanism(s) of action at the molecular level that underlies the effect of the drug had not been reported before the present study. Stout et al<sup>7</sup> observed an increase in plasma corticosterone levels after both single and subchronic (7 d) parenteral administration of the drug. This raised the possibility that a negative feedback exerted by the increase in corticosterone levels induced by valproic acid might be responsible for the changes in CRF production, although this effect could be related to the route of administration of the drug. Indeed, in a subsequent study, in which nonstressful procedures were used (i.e., long-term administration of valproic acid with food), corticosterone levels did not differ significantly between control and treated rats.<sup>8</sup> In our study, we found that valproic acid caused a reduction in basal and stimulated CRF release, as well as in CRF mRNA accumulation in vitro. It should also be noted that the maximal effective dose that we used, 100  $\mu\text{mol/L}$ , corresponds to a drug concentration of 16.6  $\mu\text{g/mL}$ , which is below the therapeutic range in humans.<sup>4,18</sup>

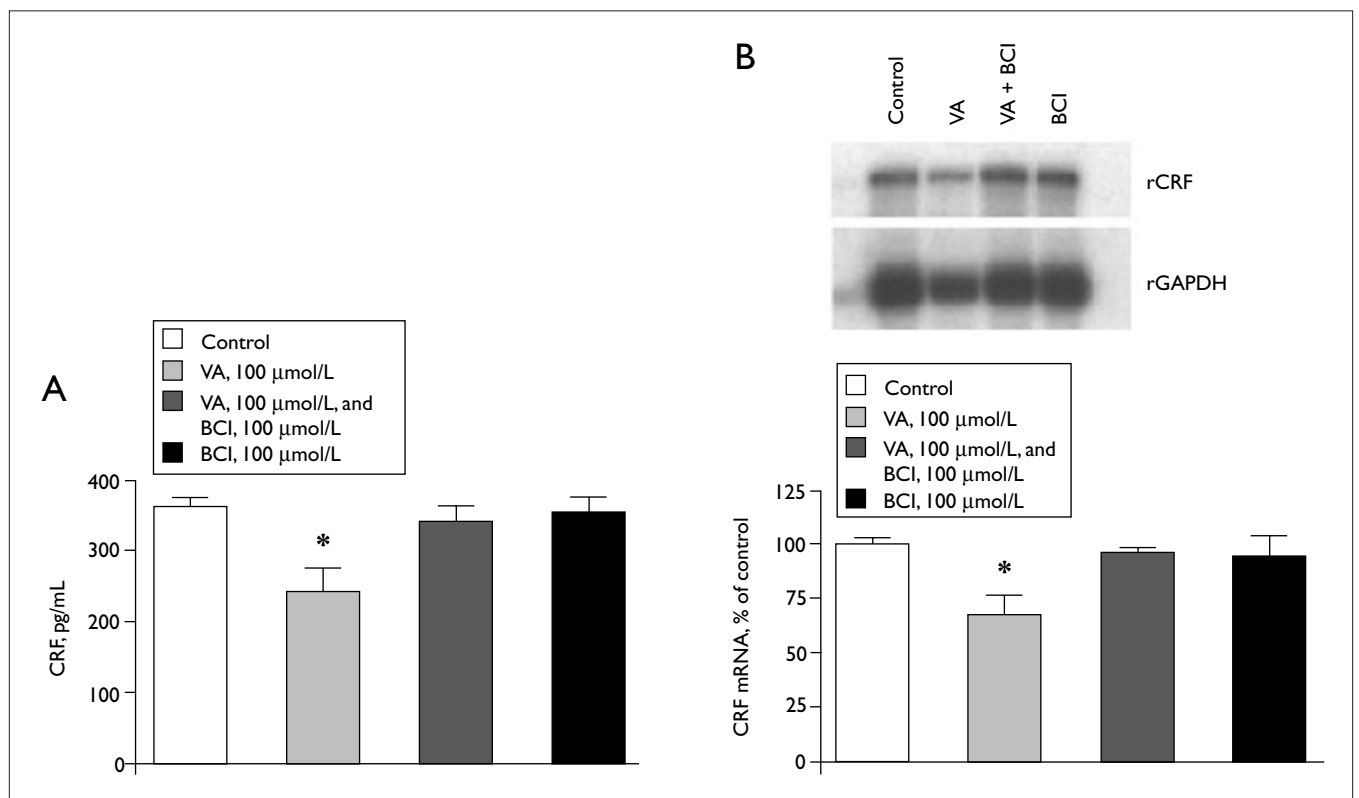


The results presented here further support the hypothesis that valproic acid may directly modulate CRF gene expression and peptide secretion in the hypothalamus and that the inhibition of mRNA accumulation may play a major role in the valproic acid-induced decrease in CRF.

Valproic acid was also able to inhibit significantly KCl-induced CRF release. This would suggest that, apart from the modulation of mRNA accumulation, nongenomic mechanism(s) at the level of neuronal membrane may also be involved. In fact, the actions of valproic acid within the central nervous system have been associated with its capacity to prolong the recovery of voltage-activated Na<sup>+</sup> channels from inactivation, as well as the stimulation of GABAergic neurotransmission.<sup>10</sup> The latter effect has been shown to modulate CRF release *in vitro*<sup>19</sup> and *in vivo* negatively.<sup>20</sup> Moreover, the blockade of GABA degradation

in the rat brain causes a decrease in CRF mRNA levels within the hypothalamus,<sup>21</sup> whereas the intracerebral injection of GABA receptor antagonists results in a marked increase in hypothalamic CRF gene expression.<sup>22</sup> In our study, incubation of the explants with the GABA<sub>A</sub> receptor antagonist bicuculline methiodide, given at a concentration blocking the GABA<sub>A</sub> receptor *in vitro*,<sup>23</sup> completely abolished the actions of valproic acid. In addition, the selective GABA<sub>A</sub> agonist muscimol inhibited, in a dose-dependent manner, the expression of CRF both at the RNA and peptide levels, thus mimicking the effects of valproic acid. These findings suggest that GABA may play a major role in mediating the inhibitory effect of valproic acid on CRF production.

We observed a decrease in CRF production after 3 hours' treatment with valproic acid, whereas at least a few days of treatment with the drug are required to

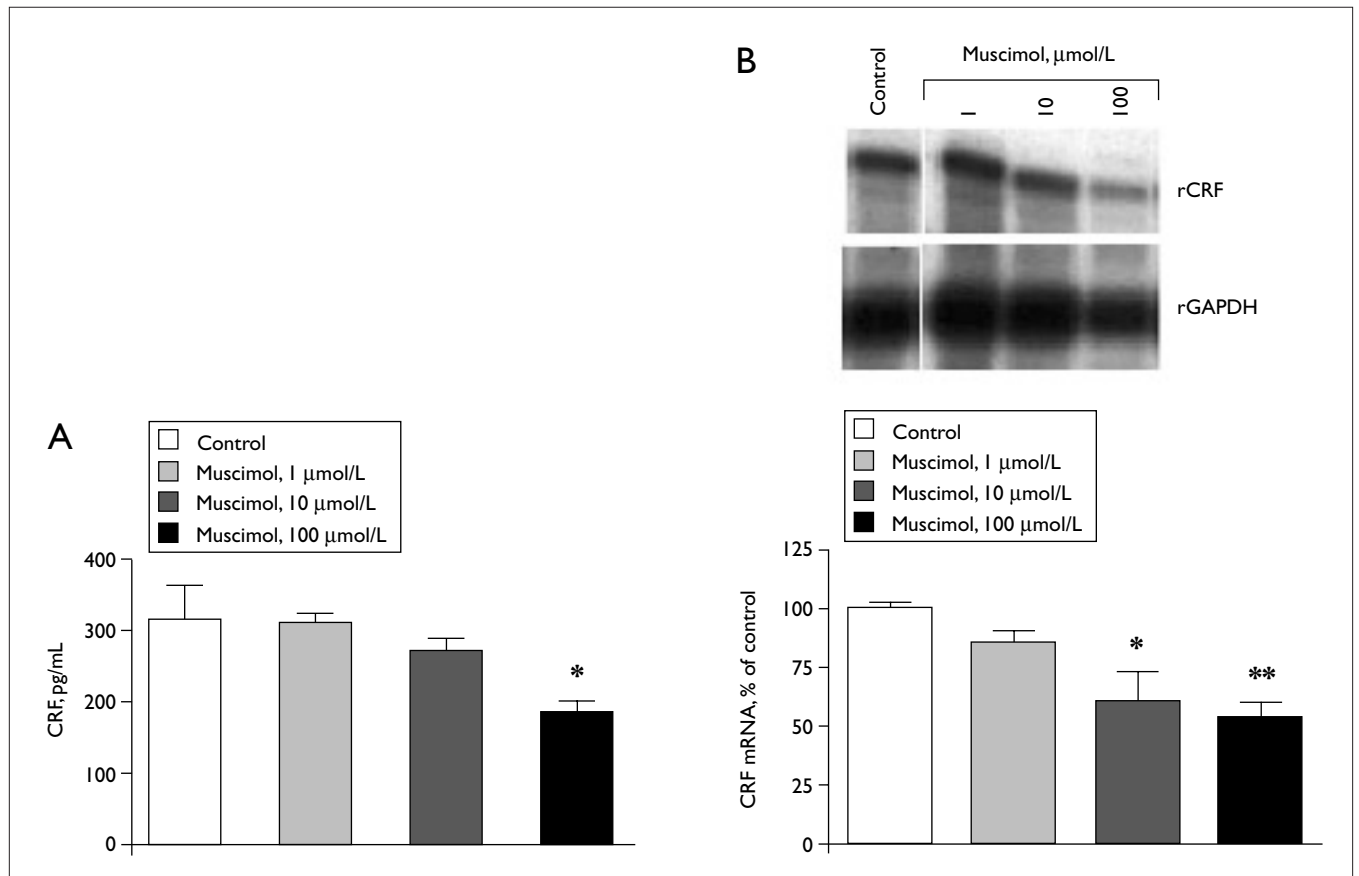


**Fig. 3A:** The effect of bicuculline methiodide (BCI) on valproic acid-induced inhibition of CRF release in the rat hypothalamus. Hypothalamic explants were incubated with medium alone (control) or with medium containing valproic acid, 100 μmol/L, or BCI, 100 μmol/L, or both, for 3 hours. The results are presented as the mean (and SEM) of 3 independent experiments performed in triplicate. \*Significantly different from the control group ( $p < 0.05$ ). **B:** The effect of BCI on valproic acid-induced inhibition of CRF mRNA accumulation in the rat hypothalamus. Hypothalamic explants were incubated as in panel A and total RNA extracted. Results are presented as the mean (and SEM) of 3 independent experiments performed in triplicate. \*Significantly different from the control group ( $p < 0.05$ ). A representative 5% polyacrylamide-8M urea gel is shown above the bar graph.

observe favourable therapeutic effects in patients with affective disorders. Such a discrepancy between in-vitro and in-vivo observations, which is common to most antidepressive agents, might be explained in the case of valproic acid by the fact that early changes in CRF synthesis and release might be followed by more prolonged variations in other neurotransmitters' function, in particular those belonging to the aminergic group. In support of this hypothesis are the recent findings of Price and Lucki,<sup>24</sup> who showed that intracerebroventricular administration of CRF is followed by long-term changes in aminergic neurotransmission in brain regions that are involved in the development of affective disorders, particularly depression and anxiety, such as the dorsal raphe nucleus and the lateral septum.

In the last few years, a number of novel biochemical actions of valproic acid have been described that might

account for its pharmacologic and clinical effects.<sup>25</sup> In particular, the actions of valproic acid have been related to both inositol depletion<sup>26</sup> and inhibition of histone deacetylase. The latter can cause gene hyperacetylation, and the degree of histone acetylation is one of the most important factors to influence the access of transcription factors to their target DNA segments.<sup>27</sup> We have recently shown that the blockade of histone deacetylase induced by valproic acid in endometrial and breast cancer human cell lines leads to the overexpression of the estrogen receptor- $\alpha$ , thereby causing an increased proliferative response to estradiol.<sup>28</sup> Indeed, using the cDNA microarray technique, more than 12% of 15 627 genes expressed in normal human theca cells were shown to be upregulated or downregulated by 48-hour treatment with valproic acid, 20  $\mu\text{mol/L}$ .<sup>29</sup> Valproic acid is also able to stimulate the extracellular signal-



**Fig. 4A:** The effect of muscimol on CRF release in the rat hypothalamus. Hypothalamic explants were incubated with medium alone (control) or with medium containing increasing concentrations of muscimol for 3 hours. Results are presented as the mean (and SEM) of 3 independent experiments performed in triplicate. \*Significantly different from the control group ( $p < 0.05$ ). **B:** The effect of muscimol on CRF mRNA levels in the rat hypothalamus. Hypothalamic explants were incubated as in panel A and total RNA extracted. Results are presented as the mean (and SEM) of 3 independent experiments performed in triplicate. \*Significantly different from the control group ( $p < 0.05$ ). \*\*Significantly different from the control group ( $p < 0.01$ ). A representative 5% polyacrylamide-8M urea gel is shown above the bar graph.

regulated kinase (ERK) pathway in the rat hippocampus and frontal cortex, and it has been suggested that this effect might mediate the antimanic action of the drug.<sup>30</sup> We can speculate that one or more of the pathways listed here could be involved in mediating the effects of valproic acid on CRF mRNA expression.

In conclusion, our results and the current evidence, taken together, suggest that the modulation of CRF mRNA levels and peptide secretion in the brain may play a part in the action of valproic acid as a mood-stabilizing drug. In addition, the effects of valproic acid on CRF production appear to be related, at least in part, to the activation of GABAergic neurotransmission.

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**Competing interests:** None declared.

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