The Effects of Chronic Treatment with the Mood Stabilizers Valproic Acid and Lithium on Corticotropin-Releasing Factor Neuronal Systems

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Running Title: VPA and Lithium Alter CRF Neuronal Systems

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Number of text pages: 
Number of tables: 0
Number of figures: 3
Number of words – abstract: 257
Number of words – introduction: 732
Number of words – discussion: 1182

Nonstandard abbreviations: VPA, valproic acid

Recommended section assignment: Neuropharmacology

List of Abbreviations: ACTH, adrenocorticotropic hormone; BLA, basolateral amygdaloid nucleus; BNST, bed nucleus of the stria terminalis; CeA, central amygdaloid nucleus; CNS, central nervous system; CRF, corticotropin-releasing factor; EW, Edinger-Westphal nucleus; F/P CTX, frontal/parietal cortex; HPA axis, hypothalamic-pituitary-adrenal axis; LS, lateral septum; mRNA, messenger ribonucleic acid; PVN, paraventricular nucleus of hypothalamus; VMH, ventromedial hypothalamus; VPA valproic acid.
ABSTRACT

Corticotropin-releasing factor (CRF) plays a preeminent role in coordinating the endocrine, autonomic, and behavioral responses to stress. Dysregulation of both hypothalamic and extrahypothalamic CRF systems have been reported in patients with major depression and post-traumatic stress disorder. Moreover, effective treatment of these conditions leads to normalization of these CRF systems. Although there is virtually no data concerning alterations of CRF systems in bipolar disorder (manic depressive illness), previous work indicates that valproic acid, an anticonvulsant also effective in the treatment of acute mania, alters central CRF neuronal systems. In the current studies, we chronically administered valproic acid and lithium, two clinically effective mood stabilizers, in non-stressed rats to extend our previous findings. Chronic valproic acid administration decreased CRF mRNA expression in the paraventricular nucleus of the hypothalamus; lithium administration increased CRF mRNA expression in the central nucleus of the amygdala. Although valproic acid increased CRF₁ receptor mRNA expression in the cortex, CRF₁ receptor binding was decreased in both the basolateral amygdala and cortex, suggesting that chronic valproate treatment may in fact dampen the overall tone in this central stress pathway. Valproate treatment decreased CRF₂A mRNA expression in both the lateral septum and hypothalamus, though CRF₂A receptor binding was unchanged. Lithium administration decreased CRF₁ mRNA expression in both the amygdala and frontal cortex, but CRF₁ receptor binding also remained unchanged. These results suggest that the therapeutic actions of these mood stabilizers may, in part, result from their actions on central CRF neuronal systems. The distinct actions of each drug on CRF systems may underlie their synergistic clinical effects.
The neuropeptide corticotropin-releasing factor (CRF) acts as a neurotransmitter in the central nervous system (CNS) to regulate the autonomic and behavioral responses to stress in addition to its role as a secretagogue in the hypothalamic-pituitary-adrenal (HPA) axis where it regulates the neuroendocrine stress response (Owens and Nemeroff, 1991). CRF-containing neurons are distributed in cortical, limbic, and brain stem nuclei in the rat (Swanson et al., 1983) and primate (Foote and Cha, 1988; Lewis et al., 1989; Bassett and Foote, 1992), with those CRF neurons in the amygdala and hypothalamus projecting to the medullary noradrenergic nuclei implicated in the CNS response to stress. The two CRF receptors in the CNS, CRF₁ and CRF₂, are heterogeneously located, perhaps indicating unique roles for each in modulating neuronal systems (Chalmers et al., 1996). Indeed, the discovery of urocortin, a second endogenous ligand for these receptors possessing a ten-fold greater affinity for CRF₂, and several congeners, as well as a distribution to neuroanatomical sites preferentially expressing CRF2 mRNA, suggests a division into CRF-CRF₁ and urocortin-CRF₂ systems (Skelton et al., 2000b).

There is ample evidence from animal models that modulation of central CRF systems play a role in stress responses. For example, central administration of CRF in rats produces behaviors associated with anxiety (Sutton et al., 1982; Dunn and Berridge, 1990) while CRF antagonists act as anxiolytics (Heinrichs et al., 1992; Rassnick et al., 1993). Similarly, in non-human primates, central CRF administration produces symptoms of behavioral despair (Kalin, 1990). Adverse early experience paradigms, developed to model mood and anxiety disorders in rodents and primates, produce altered CRF neuronal systems with changes persistent into adulthood (Sanchez et al., 2001).
Alterations in CRF systems have also been found in patients diagnosed with mood and anxiety disorders. Patients with major depression (Nemeroff et al., 1984) and post-traumatic stress disorder (Bremner et al., 1997; Baker et al., 1999) exhibit elevated concentrations of CRF in cerebrospinal fluid. Similar to animal models of adverse early experience, childhood stressors such as abuse appear to cause persistent CRF system alterations that may contribute to psychopathology in adulthood (Heim et al., 2000; Kaufman et al., 2000). Moreover, elevated cerebrospinal fluid CRF concentrations are normalized following treatment with antidepressants (Debellis et al., 1993; Veith et al., 1993) or ECT (Nemeroff et al., 1991). These results have lead to the investigation of CRF receptor antagonists as potential novel therapeutic agents (Owens and Nemeroff, 1991).

There is definitive evidence of HPA axis dysregulation in various bipolar disorders (Manji and Lenox, 2000), and though there is no evidence to support changes in CSF CRF concentrations in euphoric mania (Berrettini et al., 1987; Banki et al., 1992; Risch et al., 1992), central CRF systems are likely hyperactive in dysphoric or mixed mania as such patients exhibit marked HPA axis hyperactivity. Moreover, a strong relationship exists between bipolar disorders and childhood abuse (Pribor and Dinwiddie, 1992; Levitan et al., 1998; Agid et al., 1999; Hyun et al., 2000), which as noted above, lead to lasting changes in CRF neuronal systems. Therefore, there are multiple lines of investigation to study CRF systems in bipolar disorder and its treatment.

Lithium is still considered the first line of treatment for manic depressive illness, and is approved by the US Food and Drug Administration for both acute and maintenance treatment. Divalproex sodium, a common formulation of valproic acid (VPA), is often effective in the significant population of patients non-responsive to lithium or unable to tolerate its side-effects (Nemeroff,
2000), and though approved for acute treatment only may be more effective in duration of mania prophylaxis and posses greater antidepressant effects than originally thought (Bowden et al., 1994; Bowden et al., 2000). Moreover, VPA appears to be more effective in treating specific subsets of bipolar disorder including rapid-cycling, mixed bipolar disorder, and bipolar II disorder (Calabrese and Delucchi, 1990; Calabrese et al., 1992; McElroy et al., 1992; Bowden, 1998a; Keck and McElroy, 1998).

Preliminary experiments from our laboratory suggest that subchronic (7 days) treatment with VPA alters CRF neuronal systems (Stout et al., 2001). In the present studies, we sought to more thoroughly test the hypothesis that the mood altering effects of chronic (≥2 weeks) VPA may be partially attributed to its effects on CRF neuronal systems, and to compare these effects to those of lithium. The current results support and extend our previous findings that mood stabilizers do alter neuronal CRF systems, albeit perhaps via unique mechanisms.
METHODS

Animals and Dosing Paradigm

Male Sprague-Dawley rats (150-175 g on arrival; Charles River Laboratories, Raleigh, NC) were housed two per cage with food and water available ad libitum in an environmentally controlled animal facility with a 12 hr light/dark cycle. Animals were weighed and handled every other day throughout the time course of the experiment. Two days after arrival, animals in the drug groups were switched from standard rat chow to formulated chow containing either 22.5 g/kg valproic acid for 2 wks or 1.2 g/kg lithium carbonate for 1 week followed by 2.4 g/kg lithium carbonate for 2 weeks. Because step-up dosing over 3 weeks was required for lithium treatment compared to 2 weeks at a single dose for valproic acid, unique control groups were maintained for either two or three weeks. Animals in the lithium control and treatment groups were also provided with 0.9% saline ad libitum. Doses were chosen following a series of dose finding experiments in which serum drug concentrations were determined following 1 week treatment (data not shown).

At the end of the dosing period, the rats were killed by decapitation between 0900 and 1030. Trunk blood was collected for measurement of ACTH and corticosterone as well as drug concentrations; the brains were rapidly removed, frozen on dry ice, and stored at –80 °C.

Radioimmunoassays

ACTH radioimmunoassay. Trunk blood was collected on ice in EDTA-containing glass tubes and centrifuged for 10 min at 1500 x g at 4°C. ACTH was measured in duplicate plasma samples by a two-site immunoassay (Nichols Diagnostics, San Juan Capistrano, CA) with a coefficient of variation of 5% and sensitivity (blank + 2 SD) of 1 pg/ml.

Corticosterone radioimmunoassay. Trunk blood was collected on ice in polycarbonate tubes and centrifuged for 10 min at 1500 x g at 4°C. Corticosterone was assayed in duplicate serum.
samples by double antibody RIA (ICN Biochemicals, Costa Mesa, CA) with a coefficient of variation of 6% and sensitivity (blank + 2 SD) of 1.2 ng/ml.

Drug concentrations

Serum drug concentrations were determined using radioimmunoassay for valproic acid and a Beckman ISE instrument with an ion-specific electrode for Li⁺ by the Emory Medical Laboratories.

**In situ Hybridization**

Serial coronal sections (20 µm) of the rat brains were prepared on a cryostat at −18 °C, thaw mounted onto SuperFrost Plus slides under RNAse-free conditions, and stored with Humi-Cap desiccant capsules at −80 °C until assayed. CRF, urocortin, CRF₁, and CRF₂A mRNA *in situ* hybridization was performed as previously described in detail (Skelton et al., 2000a).

**CRF Receptor Autoradiography**

Serial coronal sections (20 µm) of the rat brains were prepared on a cryostat at −18 °C, thaw mounted onto SuperFrost Plus slides under RNAse-free conditions, and stored with Humi-Cap desiccant capsules at −80 °C until the assays. CRF₁ and CRF₂A receptor binding autoradiography were performed as previously detailed (Skelton et al., 2000a).

**Image Analysis**

Images on film from *in situ* hybridization and receptor autoradiography assays were digitized with a DAGE-MTI CCD-72 (Michigan City, IN) image analysis system equipped with a Nikon camera using MCID (Imaging Research) software. Optical densities were calibrated against ¹⁴C-standards for in situ hybridization or [¹²⁵I]-standards for receptor autoradiography. Messenger
RNA expression levels were calculated for distinct anatomical regions as defined by Paxinos and Watson (Paxinos and Watson, 1986) in each brain slice by subtracting the neutral background density from the specific signal. The density of CRF receptor binding was calculated for distinct anatomical regions (Paxinos and Watson, 1986) as follows: CRF$ _1$ receptor-specific binding = total binding – CRF$_{2A}$; CRF$_{2A}$ receptor-specific binding = CRF$_{2A}$ receptor-specific binding – nonspecific binding. For each animal, brain region, and assay four to eight individual measurements were averaged to produce a single value for that animal. Measurements made by two independent observers on slides coded to blind them to the dosing paradigm for each animal were indistinguishable in the final results.

Data analysis

3 animals were eliminated from the control VPA group for all analysis because their ACTH concentrations at the time of sacrifice were greater than 3 standard deviations from the mean for unknown reasons. Therefore, the number of animals analyzed in each group was as follows: VPA control, n = 7; VPA, n = 10; lithium control, n = 10; lithium, n = 12. For each brain region examined, the mean treatment group desitometric value was compared to the appropriate mean control group densitometric value via a two-tailed t test. The mean treatment group densitometric value and SEM were then expressed as a percent of the mean control group densitometric value, and are represented as vertical bars ± SEM in order to allow straightforward comparison across different brain regions and assays.
RESULTS

Weight Gain/Plasma Drug Levels

Animals in the VPA treatment group gained 56% as much weight as the control group (69 ± 6.3 g vs 121 ± 8.3 g) over the two-week treatment period. Animals in the lithium treatment group gained 42% as much as the control group (73 ± 5.8 g vs 173 ± 2.8 g) over the three-week treatment period. Animals in all groups gained weight continuously.

The mean serum VPA concentration at the time of sacrifice was 27.4 ± 0.8 µg/ml (range 23.5 – 31.8). These concentrations represent the lower half of concentrations seen throughout the day (data not shown). Valproate concentrations at 1600 hour are twice those observed at the time of sacrifice (unpublished observation). The average serum lithium level was 1.0 ± 0.01 mmol/L (range 0.94 – 1.07 mmol/L).

HPA Axis Activity

Chronic VPA treatment resulted in a mean ACTH concentration of 90.4 ± 24.7 pg/ml that was not significantly different than the VPA control group (54.3 ± 15.6 pg/ml; Figure 1A). Similarly, the mean corticosterone concentration in the VPA treatment group was 22.0 ± 5.7 ng/ml, not significantly different than the control group (41.0 ± 20.9; Figure 1A).

Chronic lithium treatment also did not alter ACTH concentrations, 103.4 ± 30.7 pg/ml versus 53.3 ± 10.1 pg/ml in the lithium treatment versus lithium control group (Figure 1B). Corticosterone concentrations (85.3 ± 20.4 ng/ml) were unchanged versus the lithium control group (28.6 ± 8.9).
CRF/Urocortin mRNA Expression

CRF mRNA expression measured in the bed nucleus of the stria terminalis (BNST), central nucleus of the amygdala (CeA), and paraventricular nucleus of the hypothalamus (PVN, Figure 2A). VPA significantly decreased CRF mRNA expression in the PVN (17%), but did not significantly alter CRF mRNA in the BNST or CeA. Lithium significantly increased CRF mRNA expression in the CeA (28%), but did not significantly alter CRF mRNA in the BNST or PVN.

Urocortin mRNA expression was measured in the Edinger-Westphal nucleus (EW, Figure 2B). Neither VPA nor lithium significantly altered urocortin mRNA expression.

CRF receptor mRNA expression and binding

CRF$_1$ mRNA expression and receptor binding were examined in the basolateral amygdala (BLA) and frontal/parietal cortices (F/P CTX). Chronic VPA treatment significantly increased CRF$_1$ mRNA expression in the cortex (26%), but did not in the BLA (Figure 3A). In contrast, chronic VPA treatment significantly decreased CRF$_1$ receptor binding in the BLA (18%) and in the cortex (40%; Figure 3B).

Chronic lithium treatment decreased CRF$_1$ mRNA expression in the BLA (27%), and in the cortex by (40%; Figure 3A), but did not alter CRF$_1$ receptor binding in either region (Figure 3B).

CRF$_2$ mRNA expression and receptor binding were measured in the lateral septum (LS) and ventromedial hypothalamus (VMH). Chronic VPA treatment significantly decreased CRF$_2$ mRNA expression in the LS (24%) and in the VMH (23%; Figure 3C), but did not significantly alter CRF$_2$ receptor binding in either region (Figure 3D).
Chronic lithium treatment did not significantly alter either CRF$_2$ mRNA expression or receptor binding (Figures 3C and 3D).
DISCUSSION

We have shown that chronic administration of the mood stabilizers valproic acid and lithium alters CRF neuronal systems, though the effects of VPA appear more widespread. The precise mechanism(s) of action of these drugs in producing their therapeutic actions remain obscure, but it is likely that effects on various pathways combine to produce their therapeutic effects. Placing the current data in the framework of the growing body of evidence implicating CRF system pathology in a number of psychiatric conditions, and the stabilization of these systems following successful psychopharmacological treatment, clearly suggests that mood stabilizers, and VPA in particular, may act in part through their effects on CRF neuronal systems.

Eliminating stressful procedures is a primary concern when designing studies measuring indices of CRF neuronal activity. Using rat chow as the means of drug administration eliminated possible activation of CRF systems through repetitive injections or implantation surgery and subsequent manipulation of minipumps. Based on the administration of divalproex sodium in patients via sprinkle caps (Keck and McElroy, 1998) and the delivery of lithium in rat chow (Lambert et al., 1999; Yuan et al., 1999), preliminary trials were successfully conducted to test the feasibility of administering VPA in rat chow. Furthermore, initial concerns that a non-metered dosing paradigm would result in a wide range of drug serum levels both between animals and diurnally proved to be unfounded as the range was actually quite limited (see Results).

In addition to utilizing non-stressful methodology, a primary goal of this dosing paradigm was to achieve drug plasma concentrations that fell within an expected therapeutic range for these drugs in psychiatric patients. Preliminary trials and previous work (Stout et al, 2001) indicated that rather high doses of valproic acid were needed to achieve such levels due to the extremely short half-life of valproate in the rat (~17 minutes). Both valproate and lithium administration were
associated with decreased weight gain compared to control groups over the course of the experiment. This is distinct from the weight gain currently experienced by patients treated with these agents and raises the questions as to whether the drug treatment rendered the animals ill. However, drug treated rats did exhibit weight gain albeit less than controls, and all animals appeared overtly healthy.

The mean serum VPA concentration of 27.4 \( \mu \text{g/ml} \) is below the reported therapeutic range of 45 - 100 \( \mu \text{g/ml} \) required for response in mania (Bowden et al., 1996) and as an anticonvulsant. However, serum concentrations ranging from 20-45 \( \mu \text{g/ml} \) have been reported to be effective in treating cyclothymia and bipolar II disorder (Jacobsen, 1993), subtypes of bipolar disorder reported to respond well to VPA treatment. Unpublished observations showed that the serum concentrations reported here for rats killed in mid-morning were ~2-2.5-fold higher in the late afternoon (~1600 hours). The average lithium concentration of 1.0 mmol/L was precisely within the recommended therapeutic serum concentration of 0.8-1.2 mmol/L (Lenox and Manji, 1998).

The effects of VPA and lithium on CRF neuronal pathways can be interpreted within an “antiparallel” stress system hypothesis, supported by several groups (Liebsch et al., 1999; Skelton et al., 2000a), which posits that there are two central CRF stress systems with opposing effects. CRF released from neurons in the CeA and PVN acts upon CRF1 receptors in the cortices, BLA, locus coeruleus (LC) and pituitary to coordinate the central nervous system response to stress. In contrast, urocortin released by terminals of neurons located in the EW nucleus is thought to act on CRF2 receptors in regions such as the LS and VMH to regulate stress-coping behaviors. In addition to more classic pharmacological (Radulovic et al., 1999) and maternal deprivation experiments (Ladd et al., 1996; Eghbal-Ahmadi et al., 1997), recent
evidence from transgenic mice strongly supports this hypothesis. For example, mice
overexpressing CRF exhibit anxiogenic behaviors (Heinrichs et al., 1997) while those lacking
the CRF₁ receptor exhibit decreased basal and stress-induced anxiety (Timpl et al., 1998). In
contrast, animals lacking the CRF₂ receptor exhibit increased anxiety-like behavior and decreased
stress-coping behaviors (Bale et al., 2000; Coste et al., 2000; Kishimoto et al., 2000).

Placing our results in this framework, the major effects of VPA appear to be in the CRF-CRF₁
pathway as opposed to the urocortin-CRF₂ pathway. VPA decreased CRF mRNA expression in
the PVN and ultimately decreased CRF₁ receptor binding. This suggests that VPA may mediate
its therapeutic effects in part by ultimately dampening the overall tone of the CRF-CRF₁ pathway.
Interpreted in this way, the effects of VPA would be similar to those of the benzodiazepine
alprazolam. Previous results show that alprazolam alters CRF neuronal systems in accordance
with the working hypothesis outlined above (Owens et al., 1989; Owens et al., 1991; Skelton
et al., 2000a), that alprazolam and VPA both increase GABAergic neurotransmission (Illig et
al., 2000), and are useful in the treatment of mania (Bowden, 1998b). Therefore, one might
have predicted that the effects of VPA on CRF systems would be similar to those of alprazolam.
Although we failed to detect an upregulation of the urocortin-CRF₂ pathway as observed with
alprazolam, and in fact found a decrease in CRF₂ mRNA, this does not preclude the possibility
that the effects of VPA on the CRF-CRF₁ pathway are therapeutically relevant.

The effects of lithium on CRF neuronal systems were less pronounced than those of VPA. There
was a significant increase in CRF mRNA expression in the CeA and significant decreases in
CRF₁ mRNA expression in the BLA and cortices. However, no changes in binding for either
receptor were observed. Although mania and anxiety are not necessarily regulated through the
same neuronal pathways, an increase in the CeA, an important anxiogenic center for CRF, could
be interpreted as increasing the tone of the CRF-CRF$_1$ pathway. Changes in receptor mRNA did not appear to translate into actual changes in receptor binding, obfuscating a clear interpretation of these data.

Bipolar disorders represent a group of complex disorders with noted changes in the CNS. Even within delineated bipolar disorder subtypes, there are subgroups of patients whose common clinical symptoms may be the result of distinct neuronal changes. This could explain, for example, why a significant subgroup of manic patients who are non-responsive to lithium treatment show marked remission of symptoms when treated with VPA (Nemeroff, 2000). Moreover, patients are likely in different physiological states in distinct phases of the illness, as demonstrated by the study of rapid cycling patients (Juckell et al., 2000). Therefore, it is quite probable that mood stabilizers exert their therapeutic effects through many different neural pathways depending on the state of the patient at a given time and the distinct pathways affected in that individual. We have shown that the mood stabilizers VPA and lithium do alter CRF neuronal systems in vivo, and based on these results posit that the therapeutic effects of VPA in particular may be mediated in part via these pathways. The continued development of tools to study CRF systems in vivo, either in animal models of affective disorders or the use of imaging techniques in bipolar patients (Soars et al., 2001), will greatly assist in delineating the complex effects of these pharmacological treatments for bipolar disorders.

ACKNOWLEDGEMENTS-

We would like to thank David Knight and Susan Plot for technical assistance.
REFERENCES


This work was supported by the National Institute of Mental Health Grant 42088, the Emory University Conte Center, and an unrestricted grant from Abbott Laboratories.
FIGURE LEGENDS

Fig. 1. Chronic valproic acid and lithium do not alter ACHT or corticosterone. Each vertical bar represents the mean ± SEM (VPA control, n=7; VPA, n=10; lithium control, n=10; lithium, n=12). Treatment groups were compared to their control group by a two-tailed t test. A. VPA (22.5 g/kg valproic acid formulated chow for 2 weeks) vs. control (standard rat chow for 2 weeks): ACHT, p = 0.28; corticosterone, p = 0.32. B. Li+ (1.2 g/kg lithium carbonate formulated rat chow for 1 week and 2.3 g/kg lithium carbonate formulated rat chow for 2 weeks) vs. control (standard rat chow for three weeks): ACTH, p = 0.21; corticosterone, p = 0.07.

Fig 2. Chronic valproic acid and lithium differentially alter CRF mRNA expression as measured by desitometric analysis subsequent to in situ hybridization. Effects of VPA treatment on CRF and urocortin mRNA expression are represented by solid black bars. Effects of lithium treatment on CRF and urocortin mRNA expression are represented by diagonal hatched bars. Each vertical bar represents the mean ± SEM (VPA control, n=7; VPA, n=10; lithium control, n=10; lithium, n=12). A. CRF mRNA expression is increased in the CeA after chronic valproic acid treatment and decreased in the PVN after chronic lithium treatment. B. Urocortin mRNA expression is not changed after chronic mood stabilizer treatment. *p < 0.05; ***p < 0.001.

Fig 3. Chronic valproic acid and lithium differentially alter CRF receptor mRNA expression and receptor binding. Effects of VPA treatment on CRF₁ and CRF₂ mRNA expression are represented by solid black bars. Effects of VPA treatment CRF₁ and CRF₂ receptor binding are represented by solid gray bars. Effects of lithium treatment on CRF₁ and CRF₂ mRNA expression are represented by diagonal hatched bars. Effects of lithium treatment on CRF₁ and CRF₂ receptor binding are represented by horizontal hatched bars. Each vertical bar represents the mean ± SEM (VPA control, n=7; VPA, n=10; lithium control, n=10; lithium, n=12). A. CRF₁
mRNA expression. B. CRF₁ receptor binding. C. CRF₂ mRNA expression. D. CRF₂ receptor binding. *p < 0.05; **p < 0.01; ***p < 0.001.
Fig. 1

A

Control  VPA

ACTH (pg/ml)

Corticosterone (ng/ml)

ACTH  Cort

B

Control  Li+

ACTH (pg/ml)

Corticosterone (ng/ml)

ACTH  Cort
Fig. 2

A

![Bar chart showing CRF mRNA expression in BNST, CeA, and PVN with VPA and Li+ treatments.](chart_a)

B

![Bar chart showing Urocortin mRNA expression in EW with VPA and Li+ treatments.](chart_b)
Fig. 3

A

CRF₁ mRNA % Control

BLA F/P CTX

VPA Li+

* *

B

CRF₁ receptor binding % Control

BLA F/P CTX

VPA Li+

*** **

C

CRF₂A mRNA % Control

LS VMH

VPA Li+

** *

D

CRF₂A receptor binding % Control

LS VMH

VPA Li+