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Convergent Effects of Lithium and Valproate on the Expression of Proteins Associated with Large Dense Core Vesicles in NGF-differentiated PC12 Cells

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Lithium and valproate are chemically unrelated compounds that are used to treat manic-depressive illness. Previously, we reported that lithium ions upregulate genes encoding proteins primarily associated with large dense core vesicles (LDCV) in nerve growth factor (NGF)-differentiated PC12 cells, but not in undifferentiated PC12 cells. Moreover, lithium did not alter the expression of proteins associated with small-clear, synaptic-like vesicles (SSV) in these cells. Based on these observations, we investigated whether valproate had actions similar to those of lithium in PC12 cells. Thus, undifferentiated or NGF-differentiated PC12 cells were exposed to lithium (1 mM) or valproate (1 mM) for 48 h. Extracts from these cells were submitted to semiquantitative Northern and Western analyses. In NGF-differentiated cells, both agents increased the expression of proteins associated with LDCV, the vesicular monoamine transporter 1 (VMAT1), and cysteine string protein (CSP). These same treatments did not alter the expression of proteins primarily associated with SSV, the vesicular acetylcholine transporter (VAChT), and synaptophysin (SY). Furthermore, neither drug affected the expression of these proteins in undifferentiated cells. Interestingly, secretion of ^{3}H-dopamine was increased in cells exhibiting the increase of VMAT1 and csp. Taken together, the convergent effects of these chemically diverse compounds suggest that altered dynamics of LDCV may play a vital role in the biochemical pathway, leading to the relief of the symptoms of manic depression.
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INTRODUCTION

Manic-depressive disorders produce debilitating psychiatric symptoms in approximately 1% of the population (Goodwin and Jamison, 1990). Numerous agents have been shown to be clinically useful in managing manic-depressive disorders (McElroy and Keck, 2000; Nemeroff, 2000). Among these drugs, lithium, a monovalent cation, has been a first-line treatment (Schou, 1997; Compton and Nemeroff, 2000). More recently, agents that were originally recognized for their anticonvulsant properties, including valproate (a monocarboxylic acid), have exhibited efficacy in controlling manic-depressive symptoms (McElroy and Keck, 2000; Nemeroff, 2000). The fact that compounds that are as chemically distinct as lithium and valproate display a similar mitigating effect in this disorder has prompted efforts to determine whether these drugs act via overlapping biochemical pathways. Numerous examples of common actions of these agents have emerged. For instance, lithium and valproate enhance AP-1 DNA-binding activity in cultured cells and rat brain (Ozaki and Chuang, 1997; Chen *et al*, 1999), and they modulate the expression of genes regulated by AP-1, including tyrosine hydroxylase (Chen *et al*, 1998; Zigova *et al*, 1999; Sands *et al*, 2000) and c-Jun (Yuan *et al*, 1998, 1999). Lithium and valproate also downregulate the myristoylated alanine-rich C kinase substrate (Lenox *et al*, 1996; Watson *et al*, 1998). More recently, it was reported that lithium and valproate influence the stability of neuronal growth cones, and this effect is suppressed by inhibitors of prolyl oligopeptidase (Williams *et al*, 2002). A theme in all of these investigations is that common cellular and molecular actions of these drugs may give insights into the pathways by which they exert therapeutic effects in bipolar disorders.

Recently, we reported that lithium alters the expression of several proteins (eg cysteine string protein (CSP) and VMAT1) involved in neurosecretory pathways (Cordeiro *et al*, 2000a, b). Specifically, we found that proteins

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associated principally with large dense core vesicles (LDCV) of PC12 cells were upregulated by lithium (Cordeiro *et al*, 2000b). In contrast to the small-clear, synaptic-like vesicles (SSV), which predominantly contain acetylcholine, LDCV are the sites of storage of neuropeptides and catecholamines in PC12 cells (Liu *et al*, 1994; Liu and Edwards, 1997). This preferential effect of lithium on the expression of LDCV proteins (compared to SSV) may be of therapeutic relevance, and the current investigation was undertaken to assess whether valproate, like lithium, exhibits this selective effect on the expression of LDCV proteins.

METHODS

Cell Culture and Drug Treatment

PC12 cells were cultured on collagen-coated plates and differentiated using NGF 2.5S (50 ng/ml; Alomone Labs, Jerusalem, Israel) for 10–18 days as described (Cordeiro *et al*, 2000a, b). After differentiation by NGF, cells were typically treated either with 1 mM LiCl or sodium valproate (at the concentrations indicated) for 48 h. This time was chosen because previous results indicated that lithium induced significant changes of CSP within this time frame (Cordeiro *et al*, 2000a), and pilot experiments revealed that exposure of differentiated PC-12 cells to valproate (1 mM) led to increased CSP immunoreactivity at 16 h with a plateau between 24 and 48 h. For studies of [^{3}H]-dopamine ([^{3}H]-DA) secretion, 4000 PC12 cells were plated on 35 mm collagen-coated plates (Biocoat Collagen I, Becton Dickinson, Bedford, MA) and differentiated with NGF, as above.

Northern Blot Analysis

Total RNA was isolated and resolved electrophoretically for Northern analysis using high-stringency conditions as described previously (Cordeiro *et al*, 2000a, b). Probes for CSP, SY, and VMAT1 were as described (Cordeiro *et al*, 2000a, b). Quantitative analysis of VAChT mRNA was not performed, due to the very low abundance of VAChT mRNA (however, effects of drug treatments on VAChT were assessed by immunoblot). Binding of probe for CSP (5 kb), SY (2.4 kb), and VMAT1 (3 kb) was detected and quantified by phosphorimager analysis (Image Reader V1.2 Mac-BAS 5000; Fuji, Japan). Densitometric signals for drug-treated cells were normalized to those for untreated cells, and the results are reported as mean $\pm$ SEM values of three to four independent experiments. Student's *t*-test was used to assess significance.

Immunoblot Analysis

Undifferentiated and NGF-differentiated cells were washed with cold, phosphate-buffered saline, scraped off the plate, centrifuged at 3000*g* for 5 min, and either lysed immediately or stored at -70°C. Cells were extracted by sonicating for 20 min in lysis buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.4, 1% SDS, and 1% CHAPS), followed by solubilization at 4°C for 1 h. The detergent was removed from a sample of cell lysate (Wessel and Flugge, 1984), and assayed for protein by the Bradford method (Bradford, 1976). Immunoblotting was performed after determining the linear range of detection for CSP (35 kDa), VAChT (70–75 kDa), SY (38 kDa), SNAP-25 (25 kDa), and VMAT1 (70–75 kDa) in PC12 cell extracts. For CSP analysis, 0.5 μg PC12 cell protein was resolved electrophoretically, as previously described (Cordeiro *et al*, 2000a). The same gels probed for CSP immunoreactivity were subsequently probed for SNAP-25 immunoreactivity. Since we previously (Cordeiro *et al*, 2000a) determined that lithium (at 1 mM) did not alter the expression of SNAP-25, we were interested whether SNAP-25 expression was affected by valproate. For VMAT1 and VAChT, 80 μg of PC12 cell protein was resolved on an 8.5% gel, while for SY, 40 μg of protein was loaded on a 12.5% gel. Conditions for gel electrophoresis, transfer of protein to nitrocellulose, and immunoblot detection were described previously (Cordeiro *et al*, 2000a). VMAT1 and VAChT antibodies were purchased from Chemicon International (Temecula, CA), and used at a final concentration of 10 μg/ml. SNAP-25 antibody was purchased from Alomone Labs (Jerusalem, Israel) and used at 1.6 μg/ml. Densitometric analysis of immunoblots yielded results that were compared between control and drug treated cells. Data are reported as mean $\pm$ SEM. Student's *t*-test was used to assess the significance of four to seven independent experiments, and $p < 0.05$ was used as the criterion for the significance of differences between the means.

[^{3}H]-DA Release Assay

Evoked release experiments were performed essentially as described by Greene and Rein (1977), with these modifications: uptake of [^{3}H]-DA was performed at 37°C in culture medium with 1.5 μCi/ml [^{3}H]-DA, 0.5 mM L-ascorbic acid (Sigma), and 1.0 μM DA. After 90 min, the cells were washed with HEPES-buffered saline containing calcium and magnesium (HBS-CM: 150 mM NaCl, 10 mM HEPES, 2 mM $CaCl_2$, 0.8 mM $MgCl_2$), and incubation was continued for 30–60 min at 37°C in culture medium containing NGF (and lithium or valproate where appropriate). All solutions were freshly prepared on the day of the experiment, and prewarmed at 37°C. Cells were preincubated in 1.5 ml low potassium (K^+) solution (125 mM NaCl, 4.8 mM KCl, 2.6 mM $CaCl_2$, 1.2 mM $MgSO_4$, 5.6 mM glucose, 25 mM HEPES, 1.0 mM ascorbic acid, 3% normal horse serum (NHS), pH 7.3) for 5 min at 37°C. This preincubation solution was replaced with low K^+ solution for 30 s. This low K^+ solution was collected for liquid scintillation spectrometry, and cells were exposed to high K^+ solution (78.3 mM NaCl, 51.5 mM KCl, 2.6 mM $CaCl_2$, 1.2 mM $MgSO_4$, 5.6 mM glucose, 25 mM HEPES, 1.0 mM ascorbic acid, 3% NHS, pH 7.3). After 30 s, this solution was recovered for liquid scintillation spectrometry. To assess the cellular content of radioactivity, the cells were removed from the plate by trituration in 1.5 ml of PBS (to which was added 2 mM EDTA and 0.5% Triton X-100) and retained for liquid scintillation spectrometry. Radioactive samples were solubilized in 10 ml of scintillation cocktail (ACS or ScintiVerse BD), and counted (Beckman LS 6500). Results for [^{3}H]-DA release are expressed as a percentage of the total cellular store of radioactivity as follows: ((released cpm)/total cellular cpm) $\times$ 100%. Release experiments for cells treated with lithium used [^{3}H]-DA (1.0 mCi/mmol) purchased from ICN Biomedicals, Inc. (for the valproate experiments the

^{3}H-DA was 1 mCi/mmol, and was from Amersham Biosciences), and used an earlier passage of PC12 cells than in the valproate experiments. Three separate release experiments were performed with 3–4 plates of control (untreated) cells and 3–4 plates of treated cells (lithium or valproate). The data are for a total of 11 controls and 11 treated samples in each group. Students *t*-test was used to assess significance, $*p<0.05$.

RESULTS

In undifferentiated PC12 cells, neither lithium nor valproate produced a significant change in the expression of immunoreactive CSP, VAChT, VMAT1, SNAP-25, or SY (Table 1). These observations confirm and extend prior work (Cordeiro *et al*, 2000a, b), which indicated that lithium had no significant effect on the expression of CSP, SY, or SNAP-25 in undifferentiated PC12 cells. However, previously, we reported (Cordeiro *et al*, 2000b) that mRNA for VMAT1 and secretogranin, both of which are predominantly dense core vesicle proteins, decline in undifferentiated PC12 cells exposed to lithium. The absence of a change in VMAT1 at the protein level (Table 1) may reflect relatively slow turnover of this protein.

In NGF-differentiated PC12 cells, lithium and valproate independently led to an increase in the mRNA for CSP and VMAT1 (Figure 1a and b). Relative to untreated cells, CSP mRNA increased significantly in cells exposed to lithium ($293 \pm 52\%$, $p<0.01$) or valproate ($186 \pm 35\%$, $p<0.05$). Concomitantly, VMAT1 mRNA increased in response to these same drugs to 278 ± 43 and $174 \pm 30\%$ (% control, $p<0.05$), respectively. However, the expression of SY mRNA, which encodes a protein that is preferentially associated with SSV, was not significantly changed by these drug treatments (Figure 1a).

To determine whether the observed changes in mRNA (Figure 1a and b) correlated with changes at the protein level, we assessed CSP, VMAT1, VAChT, SY, and SNAP-25 immunoreactivity in control-, lithium-, and valproate-treated cells. As documented in Figure 2a, immunoreactivity for VAChT, SY, and SNAP-25 did not change in response to these drugs. However, the levels of CSP and VMAT1 immunoreactivity increased during exposure to lithium or valproate (Figure 2a). Quantitative analysis revealed that SNAP-25, SY, and VAChT immunoreactivities remained between 90 and 105% ($p>0.05$) of control in cells treated with these drugs. Independently, CSP immunoreactivity increased to 173 ± 17 and $196 \pm 39\%$ of control ($p<0.05$) in cells treated with valproate or lithium (Figure 2b), while VMAT1 immunoreactivity concurrently increased to 140 ± 15 and $191 \pm 30\%$ ($p<0.05$) of control, respectively (Figure 2b). Although the effects of lithium tend to be quantitatively greater than those of valproate, the increase of CSP and VMAT1 expression (Figures 1 and 2), and the lack of change of SY, SNAP-25, and VAChT expressions point toward a common mechanism of action of these drugs.

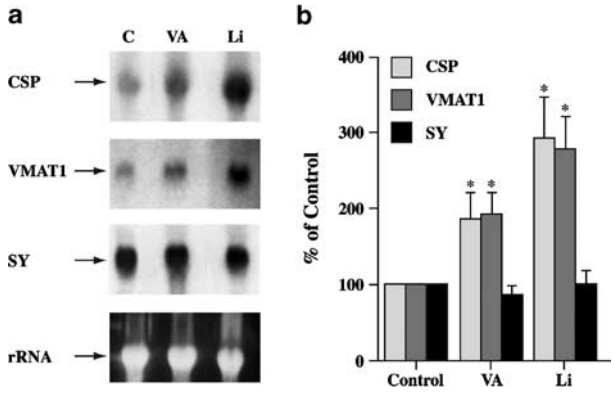

Figure 1 Impact of mood stabilizers on selected PC12 cell mRNAs. NGF-differentiated PC12 cells were used for Northern analysis of specific mRNAs after treatment with valproate (1 mM) or lithium (1 mM) for 48 h. (a) Representative northern blots of CSP, VMAT1, and SY mRNA from cells treated with valproate (VA) or lithium (Li). (b) Densitometric analysis of CSP, VMAT1, and SY mRNA content of PC12 cells. Results are expressed as a percentage of untreated control (100%) and presented as mean ± SEM (error bars) from three or four independent experiments. $*p<0.05$.

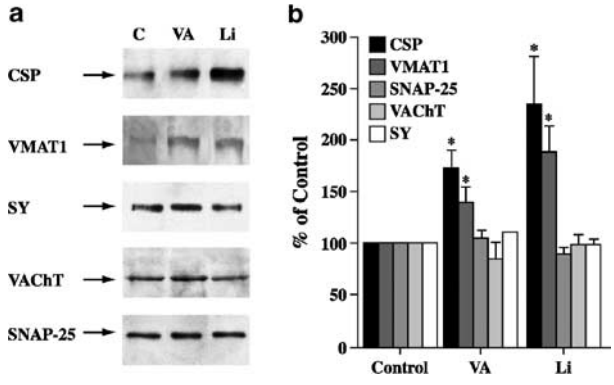

Figure 2 Immunoblot assessment of the expression of selected proteins in NGF-differentiated PC12 cells. Using the same drug concentrations of Figure 1, immunobolt assays were performed 48 h after treatment of cells with valproate or lithium. (a) Representative immunoblots of CSP, SNAP-25, SY, VAChT, and VMAT1. (b) Relative CSP, SNAP-25, SY, VAChT, and VMAT1 content. Results are expressed as a percentage of untreated control (100%) and presented as mean ± SEM (error bars) from four to seven independent experiments. $*p<0.05$.

Table 1 Summary of Protein Content of Undifferentiated PC12 Cells Treated with Valproate or Lithium for 48 h

	CSP (%change ± SE)	SNAP25 (%change ± SE)	VMAT (%change ± SE)	VAChT (%change ± SE)	SY (%change ± SE)
Valproate	107 ± 5	102 ± 11	116 ± 17	63 ± 16	119 ± 17
Lithium	100 ± 7.8	95 ± 8	88 ± 14	81 ± 11	95 ± 8

Results are normalized to untreated cells at 100%, $p>0.05$.

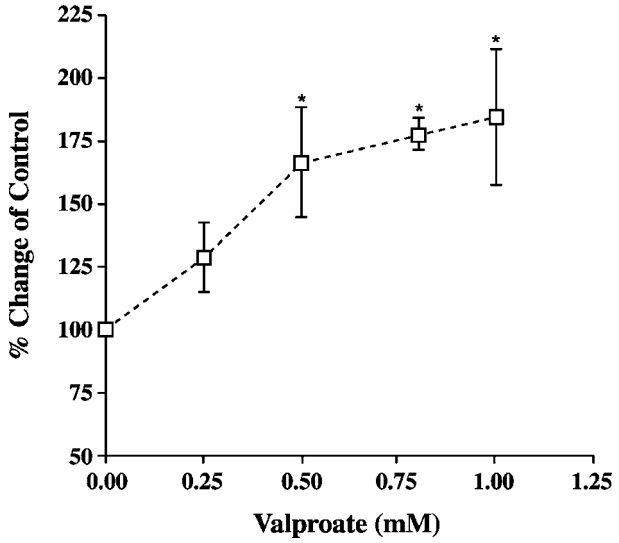

Figure 3 Expression of immunoreactive CSP as a function of valproate concentration. NGF-differentiated PC12 cells were treated with the indicated concentrations of valproate for 48 h and extracts were submitted to immunoblot analysis for CSP. Densitometric results are normalized to untreated control cells (100%). Data are results of three separate experiments. $*p<0.05$.

In the preceding experiments (Table 1, Figures 1 and 2), valproate was used at 1 mM, a concentration that exceeds the range of therapeutically relevant serum concentrations (up to 0.6 mM) in man. To ascertain whether valproate is active at lower concentrations, we evaluated its impact on the expression of CSP in NGF-differentiated PC12 cells. Results in Figure 3 indicate that valproate at 0.5–1 mM leads to a significant increase of CSP immunoreactivity, whereas 0.25 mM valproate does not significantly affect CSP content within 48 h.

The changes of protein expression documented in Figure 2 raise the possibility that secretory dynamics are altered in PC12 cells treated with lithium or valproate. To address this possibility, we measured the spontaneous and depolarization-dependent release of radioactivity from cells loaded with [^{3}H]-DA. The results in Figure 4 indicate that there is no significant change in the rate of spontaneous secretion in cells exposed either to lithium or valproate. However, under depolarizing conditions (51.5 mM K^+ for 30 s), cells treated with either lithium or valproate exhibited approximately a 30% increase (significant at $p<0.05$ in both instances) in the amount of radioactivity released. Thus, the increases of CSP and VMAT1 expression are correlated with changes in the secretory behavior of these cells.

DISCUSSION

In principle, the therapeutic actions of lithium and valproate in manic-depressive disorders could arise from independent pathways that have similar impacts on mood stabilization. However, a more common inference has been that these structurally dissimilar agents converge at some level to produce their therapeutic effects (Post *et al*, 1992). In support of this latter position are the observations (see Introduction) documenting the overlapping actions of lithium and valproate in a number of signaling pathways.

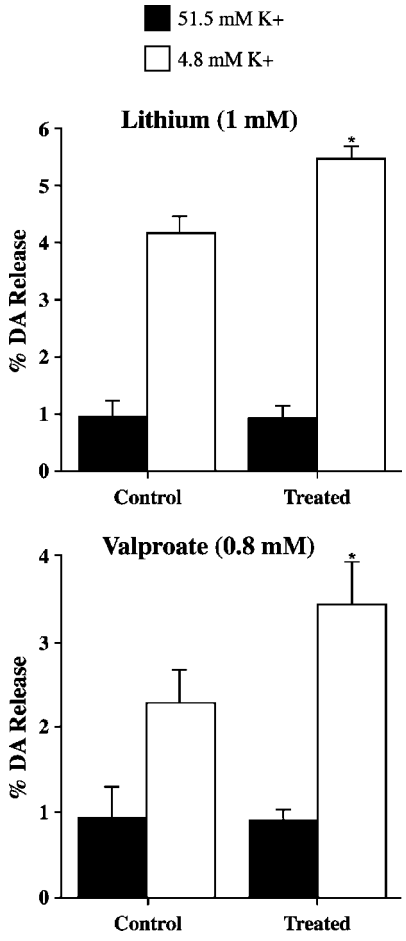

Figure 4 Spontaneous and depolarization-dependent secretion from PC12 cells loaded with [^{3}H] DA. Results reflect the amount of radioactivity released from cells during a 30 s collection period in normal or high K^+ buffer relative to the total cellular content of radioactivity at the beginning of the two collection periods (normalized to 100%). There was no significant difference in uptake of radioactive dopamine between control or drug-treated cells. Results are the mean (+SEM) of data from 11 separate plates for each condition. The lithium experiments used an earlier passage of PC12 cells. $*p<0.05$ compared with high K^+-evoked secretion in controls.

The current study provides additional support for the hypothesis that lithium and valproate have convergent effects on specific aspects of cell function. Thus, these drugs increased the level of mRNA and the cellular content of proteins (CSP and VMAT1) involved in the function of LDCV. These effects were detected in NGF-differentiated PC12 cells, but not in undifferentiated cells. Since NGF-differentiated PC12 cells acquire a more neuron-like phenotype in the differentiated state (Tischler and Greene, 1975; Dichter *et al*, 1977; Schubert *et al*, 1977), these data imply that there may be environmental factors that dictate the sensitivity of specific cells or cell populations to these drugs. Moreover, lithium and valproate augmented the expression of VMAT1 which is largely confined to LDCV, and we detected no effect of these drugs on the expression of proteins (VAChT or SY), which are predominantly associated with SSV (Liu *et al*, 1994; Liu and Edwards, 1997). Likewise, the expression of the presynaptic plasma membrane protein SNAP-25 was unaltered by these drugs. These data indicate that there is considerable specificity in

the target genes that are regulated by lithium and valproate, and that they share a requirement for PC12 cells to be in an NGF-differentiated (and more neuron-like) state for these effects to be observed.

An interesting issue that is not resolved by our results is whether the increased cellular content of csp and VMAT1 is associated with a higher density of these proteins per LDCV, or whether there are more LDVCs per cell (with essentially the same number of csp and VMAT1 molecules per LDCV). Additional work will be needed to clarify this situation. However, our prior work does suggest that the effect of lithium (and we infer that this is also likely to be the case for valproate) on cellular mRNA and protein content involves enhanced transcription of specific target genes. This is because actinomycin D completely blocked the increase of csp in NGF-differentiated cells exposed to lithium (Cordeiro *et al*, 2000a). Nevertheless, we cannot exclude the possibility that lithium also has subtle effects on mRNA or protein stability.

Importantly, we also observed that both lithium and valproate influence the regulated secretory behavior of NGF-differentiated PC12 cells. Although spontaneous secretion was unaffected by these drugs, both agents significantly enhanced the depolarization-dependent release of radioactivity from cells preloaded with [^{3}H]-DA. Although it remains to be established whether the reported changes of LDCV protein expression contribute mechanistically to this change in the regulated secretory behavior of these cells, there is ample evidence from other groups that lithium alters the content and secretion of monoamines and selected neuropeptides, which are stored in and released from LDCV (Hesketh *et al*, 1978; Treiser *et al*, 1981; Staunton *et al*, 1982; Ebstein *et al*, 1983; Hong *et al*, 1983; Mitsushio *et al*, 1988; Sivam *et al*, 1988, 1989; Mathé *et al*, 1990; Husum *et al*, 2000; Shiah and Yatham, 2000). And, as observed in the current experiments, in the majority of these cited examples, the effect of lithium was to increase the content or secretion of the neurotransmitter or neuromodulator. However, because we have also observed downregulation of transcripts for VMAT1 and secretogranin in *undifferentiated* PC-12 cells exposed to lithium (Cordeiro *et al*, 2000b), this cation clearly exerts pleiotropic effects on specific gene products. In this same context, several groups have reported that valproate alters the level of dopamine or serotonin in discrete brain areas (Biggs *et al*, 1992; Mitsikostas *et al*, 1993; Baf *et al*, 1994; Loscher and Honack, 1996; Vriend and Alexiuk, 1996; Ichikawa and Meltzer, 1999). Given the important role of biogenic amines in mood and behavior (Baldessarini, 1990), it will be of interest to ascertain whether changes in the expression of LDCV proteins contribute to the actions of mood stabilizers, both *in vitro* and *in vivo*. (In this context, recent *in situ* hybridization studies have documented significant changes of VMAT2 mRNA (Cordeiro *et al*, 2002) and csp mRNA (Corderio *et al*, 2003) in discrete areas of the brain of rats fed a lithium-supplemented diet.) Thus, the current report provides a potential link between studies showing alterations in monoamines (and some neuropeptides) in response to lithium or valproate, and changes in proteins that are essential components of LDCV in the brain.

Another issue of considerable importance is to identify the mechanism(s) by which lithium and valproate influence the expression of CSP and VMAT1. Given the structural dissimilarity of these two drugs, it is unlikely that they interact with the same proximal target(s). A more plausible hypothesis is that their actions converge at the transcriptional level to produce overlapping changes in gene expression. Thus, a better understanding of the regulatory elements that control the expression of genes sensitive to lithium or valproate should aid in clarifying the signaling pathways that underlie these changes of gene expression.

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