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# Cytoprotection by lithium and valproate varies between cell types and cellular stresses

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**Abstract**

Despite much evidence that lithium and valproate, two commonly used mood stabilizers, exhibit neuroprotective properties against an array of insults, the pharmacological relevance of such effects is not clear because most of these studies examined the acute effect of these drugs in supratherapeutic doses against insults which were of limited disease relevance to bipolar disorder. In the present study, we investigated whether lithium and valproate, at clinically relevant doses, protects human neuroblastoma (SH-SY5Y) and glioma (SVG and U87) cells against oxidative stress and endoplasmic reticulum stress in a time-dependent manner. Pretreatment of SH-SY5Y cells for 7 days, but not 1 day, with 1 mM of lithium or 0.6 mM of valproate significantly reduced rotenone and $H_2O_2$-induced cytotoxicity, cytochrome *c* release and caspase-3 activation, and increased Bcl-2 levels. Conversely, neither acute nor chronic treatment of SH-SY5Y cells with lithium or valproate elicited cytoprotective responses against thapsigargin-evoked cell death and caspase-3 activation. Moreover, inhibitors of glycogen synthase kinase-3 (GSK-3), kenpaullone and SB216763, abrogated rotenone-induced, but not $H_2O_2$-induced, cytotoxicity. Thus the cytoprotective effects of lithium and valproate against $H_2O_2$-induced cell death is likely independent of GSK-3 inhibition. On the other hand, chronic lithium or valproate treatment did not ameliorate cytotoxicity induced by rotenone, $H_2O_2$, and thapsigargin in SVG astroglial and U87 MG glioma cell lines. Our results suggest that lithium and valproate may decrease vulnerability of human neural, but not glial, cells to cellular injury evoked by oxidative stress possibly arising from putative mitochondrial disturbances implicated in bipolar disorder.


*Keywords:* Lithium; Valproate; Cytotoxicity; Cytochrome *c*; Bcl-2; Caspase-3; Bipolar disorder

## 1. Introduction

Bipolar disorder is a life-threatening psychiatric illness characterized by mood disturbances with recurrent episodes of mania, hypomania, and depression. It is a major public health problem that affects approximately 1% of the population worldwise, and extracts a marked toll in terms of morbidity, mortality, and societal cost (Belmaker, 2004; Goodwin and Jamison, 1990). Although the etiology remains poorly understood, a large body of data has clearly implicated postreceptor signal transduction disturbances in the pathophysiology of bipolar disorder (Li et al., 2000b; Manji and Lenox, 2000; Warsh et al., 2004). Furthermore, cellular and molecular studies have also illuminated the key role of intracellular signaling proteins as targets for lithium, a mainstay in treatment for BD (Jope, 1999; Li, 2004; Phiel and Klein, 2001).

Converging evidence from structural neuroimaging and postmortem studies have identified anatomical and neuropathological abnormalities, including ventricular enlargement, decreased gray matter volume, lower levels of *N*-acetylaspartate (a marker of neuronal integrity), and reductions of number, size and/or density of neurons and glial cells in brain of patients with bipolar disorder (Rajkowska, 2002; Strakowski et

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al., 2005; Vawter et al., 2000). Interestingly, bipolar patients treated with lithium or valproate had modest but significantly greater gray matter volumes (Moore et al., 2000b; Sassi et al., 2002) and *N*-acetyl-aspartate levels (Moore et al., 2000a) compared with medication-free patients. These observations raise the possibility that the therapeutic effects of mood-stabilizing drugs, such as lithium and valproate, are mediated through mechanisms regulating cellular resilience (Chuang, 2004; Manji et al., 2000; Warsh et al., 2004). Indeed, lithium and valproate display cytoprotective properties in response to numerous cytotoxic insults in cultured cells and animal models (Chuang, 2004; Li et al., 2002; Yuan et al., 2004). However the relevance of these cytoprotective actions to their therapeutic effect in bipolar disorder is still uncertain. First, the clinical effects of lithium and valproate require chronic administration, with a lag period for onset of action of several days to weeks (Bowden, 1996). With a few exceptions (Hashimoto et al., 2002; Hiroi et al., 2005; Kanai et al., 2004) however, most of the in vitro studies of their action only examined the short-term (0.5–24 h) effect of these drugs (Kang et al., 2003; Kim et al., 2005; King et al., 2001; King and Jope, 2005; Li et al., 2000a, 2002; Linseman et al., 2003; Mora et al., 2001). Second, the concentrations of lithium and valproate used in these short-term in vitro studies (King et al., 2001; King and Jope, 2005; Li et al., 2000a, 2002; Linseman et al., 2003; Mora et al., 2001; Song et al., 2002) were substantially higher than the accepted therapeutic plasma ranges of 0.6–1.2 and 0.3–1.0 mM, respectively (Bowden, 1996). More importantly, many of these earlier studies (King et al., 2001; King and Jope, 2005; Li et al., 2000a, 2002; Linseman et al., 2003; Mora et al., 2001; Song et al., 2002) did not examine lithium/valproate in the context of their therapeutic actions in bipolar disorder but rather as inhibitor of glycogen synthase kinase (GSK)-3β, a multi-tasking protein kinase that has been identified as an important pro-apoptotic factor (Grimes and Jope, 2001). Third, the types of cytotoxic insults employed in these in vitro (e.g. trophic factor withdrawal, $C_2$-ceramide, β-amyloid, colchicine, β-bungarotoxin, ouabain, valinomycin and heatshock, etc.) or in vivo (e.g. excitotoxicity-induced striatal or basal forebrain lesions) studies (Chuang, 2004; Li et al., 2002; Yuan et al., 2004) lack specific disease relevance to bipolar disorder. Finally, it remains unclear as to whether lithium and valproate protect glial cells from cytotoxic signals, especially considering that reductions in number and density of glial cells have been demonstrated in brain of patients with bipolar disorder (Rajkowska, 2002).

The aim of the present study was to discern whether lithium and valproate, administered within therapeutically relevant concentrations exhibit time-dependent cytoprotective actions against cellular stresses arising as the consequences of putative cellular perturbations implicated in the pathophysiology of bipolar disorder. In light of the reported disturbances in endoplasmic reticulum (Hough et al., 1999; Kakiuchi et al., 2003; Kato et al., 2003; Warsh et al., 2004) and mitochondrial (Kato and Kato, 2000; Stork and Renshaw, 2005) function in bipolar disorder, oxidative stress and endoplasmic reticulum stress were used to model potential intracellular stress signals implicated in bipolar disorder in selected neuronal (SH-SY5Y neuroblastoma) or glial cell models (SVG p12 [Simian Virus 40 transformed astroglial] and U87 MG glioma) of human origin. Oxidative stress was induced either by the mitochondrial complex I inhibitor rotenone (Wolvetang et al., 1994), or by the application of hydrogen peroxide ($H_2O_2$) (Ruffels et al., 2004), while endoplasmic reticulum stress was induced by thapsigargin (Wei et al., 1998). We report here that lithium and valproate exert differential protective effects against endoplasmic reticulum stress- and oxidative stress-induced cell death in human SH-SY5Y cells. The selective protective effect against oxidative stress-evoked cell death appears to be dependent on Bcl-2 upregulation but independent of GSK-3β suppression. In comparison lithium and valproate failed to protect SVG p12 and U87 MG glioma cells against cell death induction by either endoplasmic reticulum stress or oxidative stress, suggesting that the cytoprotective actions of lithium and valproate may not be evident in cells of astroglial phenotype.

## 2. Materials and methods

### 2.1. Materials

SH-SY5Y, SVG p12, and U87 MG cells were obtained from the American Tissue Culture Collection (Manassas, VA). Lithium chloride, sodium valproate, rotenone, $H_2O_2$, and kenpaullone were purchased from Sigma-Aldrich (Oakville, ON., Canada). Thapsigargin was purchased from Alomone Labs (Jerusalem, Israel). The GSK-3 inhibitor SB216763 [3-(2,4-dichlorophenyl)-4-(1-methyl-1*H*-indol-3-yl)-1*H*-pyrrole-2,5-dione] was obtained from Tocris Cookson Inc. (Ellisville, MO). Cell culture media, penicillin and streptomycin were purchased from GIBCO/Invitrogen (Burlington, ON., Canada) whereas fetal bovine serum (FBS) was from Hyclone (Logan, UT). Primary polyclonal antibodies against Bcl-2 and β-tubulin were acquired from Santa Cruz Biotechnology (Santa Cruz, CA), whereas primary monoclonal antibody raised against cytochrome *c* was from BD Biosciences (Mississauga, ON., Canada). Secondary antibodies used included horseradish peroxidase-conjugated anti-mouse (Southern Biotechnology, Birmingham, AL) and anti-rabbit immunoglobulin (Vector Laboratories, Burlington, ON., Canada), and protein A (BioRad, Hercules, CA). ECL Plus Western blot detection kits were purchased from Amersham Biosciences (Bsie d'Urfe, QC., Canada).

### 2.2. Cell culture and drug treatment

SH-SY5Y neuroblastoma cells were cultured at 37 °C in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin in a 95% air, 5% $CO_2$ humidified incubator. Cells were seeded at $2\times10^6$ cells per 75-$cm^2$ flask. The culture medium was changed every 3 days. Upon reaching 95% confluence, cells were washed with Dulbecco's Phosphate Buffered Saline (PBS: 1.7 mM $NaH_2PO_4$, 150 mM NaCl, 9.1 mM $Na_2HPO_4$) followed

by treatment with Trypsin/EDTA to detach cells, and cells were then subcultured into 3 to 4 flasks at $2\times10^6$ cells per 75-$cm^2$ flask. The U87 MG glioma cells and SVG p12 SV40-transformed human glia were grown as a monolayer in Eagle's Minimum Essential Medium (EMEM) containing 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 μg/ml amphotericin B. Both cell lines were seeded at $0.5\times10^6$ cells per 75-$cm^2$ flask. The culture medium was changed every 3 days. The cells were then subcultured in a similar manner as described for the SH-SY5Y cells. For drug treatments, SH-SY5Y, U87 and SVG cell lines were pretreated with 1.0 mM lithium chloride, 0.6 mM sodium valproate or vehicle for 1 or 7 days and then treated with rotenone, $H_2O_2$, or thapsigargin in the continuous presence of lithium, valproate or vehicle.

### 2.3. Measurement of cytotoxicity by LDH leakage

Cytotoxicity was assessed by measuring release of the cytosolic enzyme, lactate dehydrogenase (LDH) into the cultured medium versus total LDH activity. Quantification of LDH release was performed using the CytoTox 96 nonradioactive cytotoxicity assay kit (Promega, MA), following the instruction of the manufacturer. Cytotoxicity was expressed as the percentage of extracellular LDH activity of the total cellular LDH activity upon complete lysis.

### 2.4. Measurement of caspase-3 activity

Caspase-3 activity was quantified using a QuantiZyme Caspase-3 Cellular Activity Assay Kit PLUS (BIOMOL, Plymouth Meeting, PA) in accordance with the vendor's instructions. Briefly, cells were collected and lysed in a lysis buffer containing 50 mM HEPES, pH 7.4, 0.1% CHAPS, 5 mM dithiothreitol (DTT), 0.1 mM EDTA and incubated on ice for 5 min. The lysates were centrifuged at 10,000 *g* for 10 min at 4 °C. Protein concentrations in the supernatants were determined using the BioRad protein assay kit (Hercules, CA). An aliquot of cell lysate (20 μg of protein) was incubated in triplicate with the fluorogenic peptide substrate N-acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl-coumarin (Ac-DEVD-AFC; 200 μM) in assay buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% CHAPS, 10 mM DTT, 1 mM EDTA, 10% glycerol) in a final volume of 100 μl at 37 °C for 1 h. Plates were then read in a microplate spectrofluorometer (Fluoroskan Ascent FL) over 60 min with excitation filter of 380 nm and emission filter of 518 nm. The specificity of the assay was validated using 1 μM of the caspase-3 inhibitor *N*-acetyl-Asp-Glu-Val-Asp-aldehyde.

### 2.5. Subcellular fractionation and western blotting

Separation of cytoplasm from nuclear membrane and mitochondria fractions was performed as described previously with minor modifications (Uren et al., 2005). Briefly, cells were washed twice with PBS and harvested by centrifugation at 1000 *g* for 5 min at 4 °C. The cell pellets were lysed by one freeze-thaw cycle in dry ice/100% ethanol, and then resuspended in five volumes of homogenization buffer (100 mM KCl, 2.5 mM $MgCl_2$, 250 mM sucrose, 20 mM HEPES/KOH, pH 7.5, 1 mM DTT, 0.025% digitonin) containing 1× complete protease inhibitor (Roche Diagnostics, Laval, QC., Canada). After incubation on ice for 5 min, the cells were homogenized on ice using a 1 ml Dounce Teflon-glass homogenizer (15 strokes). Crude extracts were then centrifuged at 1000 *g* for 15 min to remove nuclei, unbroken cells, and other debris. Aliquots of the supernatant were collected and stored at −70 °C as crude cell lysates. The remaining supernatants were centrifuged again at 10,000 *g* for 30 min at 4 °C. The resulting supernatants were harvested and designated as cytosolic fractions. The protein concentration was determined by the bicinchoninic acid (BCA) protein assay kit (Pierce Biotechnology, Rockford, IL), using bovine serum albumin as the standard.

Aliquots of cell protein (10 μg) in duplicate were mixed with 2× Laemmli sample buffer and placed in a boiling water bath for 5 min. Proteins were resolved in 10% sodium dodecyl sulfate-polyacrylamide gels, and transferred electrophoretically onto either polyvinylidene fluoride or nitrocellulose membrane at 100 V for 1 h. The blots were blocked by incubation with 5% (w/v) nonfat milk in PBS with 0.1% Tween 20 (PBS-T) over night at room temperature. After incubation with primary antibody at 4 °C overnight (anti-cytochrome *c* 1:1000, anti-Bcl-2 1:500, anti-β-tubulin 1:1000 in PBS-T), membranes were washed in PBS-T for 15 min. Subsequently, membranes were incubated for 45 min in PBS-T containing appropriate horseradish peroxidase-conjugated secondary antibody (anti-mouse IgG 1:1000, anti-rabbit IgG 1:1000, protein A 1:1000, respectively). The immunoreactive bands were visualized and quantitated using the ECL+Western blotting reagents, with chemifluorescence detected by a Storm PhosphorImager (Molecular Dynamics, Amersham Biosciences) and Image-QuaNT 5.0 software. To adjust for protein loading variation, amounts of cytochrome *c* or Bcl-2 were normalized against the levels of β-tubulin and are expressed as the percentage of vehicle-pretreated unstressed cells.

### 2.6. Statistical analysis

All statistical analyses were performed with SPSS software v. 11.0, and *P*-values of $\leq 0.05$ were taken to be significant in all tests. Statistical analyses were carried out using repeated measures ANOVA followed by post hoc Bonferroni or Tukey's tests to determine statistical differences between groups. All data are presented as mean±S.E.M. The *n* in the text refers to the number of separate experiments with each sample assayed in triplicate.

## 3. Results

### 3.1. Effects of lithium and valproate on rotenone-, $H_2O_2$- and thapsigargin-induced cell death in SH-SY5Y cells

To investigate whether lithium or valproate at therapeutically relevant concentrations modulates the sensitivity of

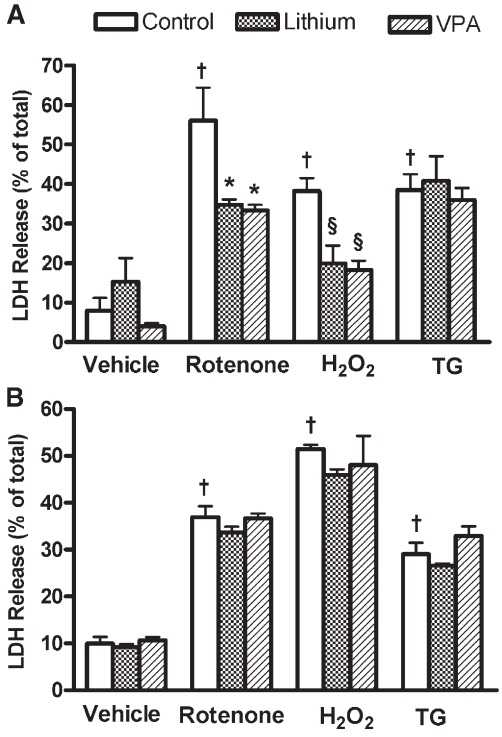


Fig. 1. Lithium and valproate protect human SH-SY5Y cells against cytotoxicity evoked by oxidative stress, but not endoplasmic reticulum stress. Human SH-SY5Y cells were pretreated with 1 mM lithium chloride or 0.6 mM valproate (VPA) for 7 days (A) or 24 h (B) followed by treatment with 5 μM rotenone, 500 μM $H_2O_2$ or 2.0 μM thapsigargin (TG) for 24 h. Cytotoxicity was measured by the release of LDH as described in Materials and methods. Data shown are means±S.E.M. of three independent experiments performed in triplicate. †$P<0.01$ compared with untreated vehicle controls. *$P<0.01$, §$P<0.05$ compared with controls treated with corresponding cytotoxic insult.

human neural cells to endoplasmic reticulum stress- or oxidative stress-inducing agents, we studied the acute and chronic effects of lithium (1 mM) or valproate (0.6 mM) on cell death induction by rotenone (5 μM), $H_2O_2$ (0.5 mM), and thapsigargin (2 μM) in SH-SY5Y cells. Consistent with previous findings (King et al., 2001; Ruffels et al., 2004; Song et al., 2002), exposure of SH-SY5Y cells to these cytotoxic insults caused significant cell death as assessed by LDH release at 24 h ($P<0.01$, repeated measures ANOVA with post hoc Tukey's tests; Fig. 1A). Notwithstanding that LDH release may underestimate the degree of apoptosis, as not all apoptotic cells undergo secondary necrosis and release LDH (Gomez-Lechon et al., 2002), pretreatment of SH-SY5Y cells with lithium for 7 days significantly reduced rotenone- and $H_2O_2$-induced LDH release by 38% ($P<0.01$) and 48% ($P<0.05$), respectively (Fig. 1A). Likewise, chronic exposure of SH-SY5Y cells to valproate also significantly attenuated rotenone- and $H_2O_2$-induced LDH release by 41% ($P<0.01$) and 52% ($P<0.05$), respectively (Fig. 1A). The cytoprotective effect of lithium and valproate appeared to be time-dependent because pretreatment of cells with these drugs for 1 day did not decrease rotenone- and $H_2O_2$-induced LDH release (Fig. 1B). In contrast, pretreatment of SH-SY5Y cells with lithium or valproate for 1 or 7 days failed to suppress LDH release evoked by thapsigargin (Fig. 1A, B). Cell viability was not affected in unstressed cells treated with lithium or valproate for 1 and 7 days.

### 3.2. Effects of lithium and valproate on rotenone-, $H_2O_2$- and thapsigargin-induced cytochrome *c* release and caspase-3 activation in SH-SY5Y cells

Recent reports have shown that cell death evoked by rotenone and $H_2O_2$ is mediated by an intrinsic mitochondrial apoptotic pathway leading to the induction of cytochrome *c* release and activation of effector caspase, such as caspase-3 (King et al., 2001; Ruffels et al., 2004), whereas caspase-3 activation by endoplasmic reticulum stress is independent of cytochrome *c* release (Morishima et al., 2002; Rao et al., 2004). Therefore, we investigated whether cytochrome *c* release and caspase 3 activation would be differentially modulated by lithium and valproate under conditions of endoplasmic reticulum stress or oxidative stress. As shown in Fig. 2, treatment of SH-SY5Y cells with rotenone or $H_2O_2$ significantly increased levels of cytochrome *c* in the cytosolic fraction. Importantly, pretreatment of cells with lithium or valproate for 7 days almost completely abrogated the cytochrome *c* release induced by rotenone or $H_2O_2$. However, thapsigargin did not induce the release of cytochrome *c* into cytosol in untreated cells or cells treated chronically with lithium or valproate.

Exposure of SH-SY5Y cells to rotenone, $H_2O_2$, and thapsigargin led to a significant increase in caspase-3 activity (0.35±0.02, 0.65±0.10, 0.64±0.06 pmol/min/μg protein, respectively) compared with vehicle-treated controls (0.013±

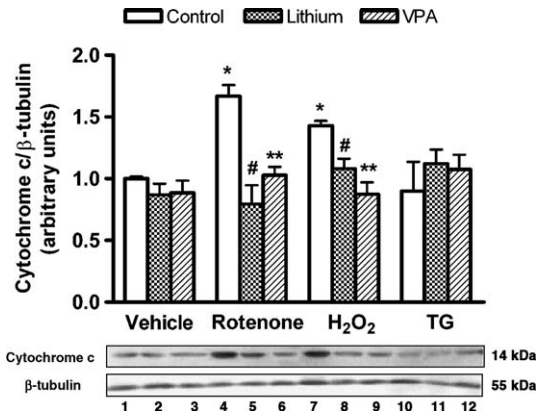


Fig. 2. Lithium and valproate attenuate the accumulation of cytosolic cytochrome *c* elicited by oxidative stress, but not endoplasmic reticulum stress. Human SH-SY5Y cells were pretreated with vehicle (lanes 1, 4, 7, and 10), 1 mM lithium chloride (lanes 2, 5, 8, and 11) or 0.6 mM valproate (VPA; lanes 3, 6, 9, and 12) for 7 days and then treated with 5 μM rotenone (lanes 4–6) for 16 h, 100 μM $H_2O_2$ (lanes 7–9) or 1 μM TG (lanes 10–12) for 6 h. Ten micrograms of protein from the cytosolic fraction of each sample were subjected to immunoblotting with anti-cytochrome *c* and anti-β-tubulin antisera. The signals of untreated controls were arbitrarily set as 1; signals of the experimental samples were normalized accordingly. Data are expressed as means±S.E.M. of five independent experiments performed in triplicate. *$P<0.01$ compared with untreated vehicle controls, #$P<0.05$, **$P<0.01$ compared with cells treated either with rotenone or $H_2O_2$.

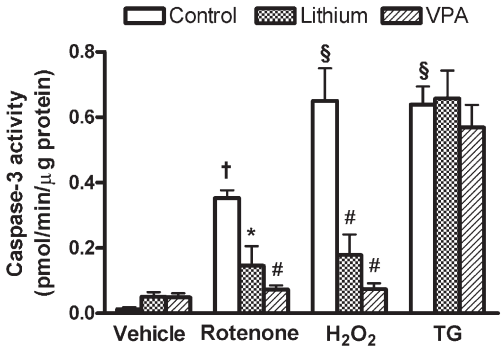


Fig. 3. Lithium and valproate reduce caspase-3 activation elicited by oxidative stress, but not endoplasmic reticulum stress. Human SH-SY5Y cells were pretreated with vehicle, 1 mM lithium chloride or 0.6 mM valproate (VPA) for 7 days and then treated with 5 μM rotenone for 16 h, 100 μM $H_2O_2$ or 1 μM TG for 6 h. Caspase-3 activity was measured enzymatically using Ac-DEVD-AFC as substrates as described in the Materials and methods. Data are expressed as means±S.E.M. of five independent experiments performed in triplicate. †$P<0.01$; §$P<0.001$ compared with untreated vehicle controls. *$P<0.05$; #$P<0.001$ compared with controls treated with corresponding cytotoxic insult.

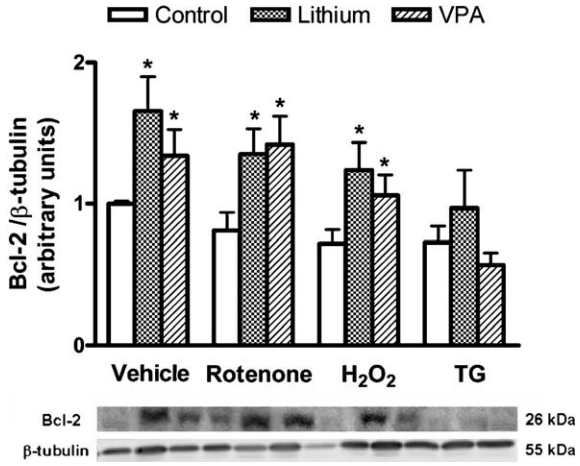


Fig. 4. Upregulation of Bcl-2 expression by lithium and valproate in unstressed cells and cells challenged with oxidative stress, but not endoplasmic reticulum stress. Human SH-SY5Y cells were pretreated with vehicle (lanes 1, 4, 7, and 10), 1 mM lithium chloride (lanes 2, 5, 8, and 11) or 0.6 mM valproate (VPA; lanes 3, 6, 9, and 12) for 7 days and then treated with 5 μM rotenone (lanes 4–6) for 16 h, 100 μM $H_2O_2$ (lanes 7–9)or 1 μM TG (lanes 10–12) for 6 h. Ten micrograms of protein from the crude lysates of each sample were subjected to immunoblotting with anti-Bcl-2 and anti-β-tubulin antisera. The signals of untreated controls were arbitrarily set as 1; signals of the experimental samples were normalized accordingly. Data are expressed as means±S.E.M. of five independent experiments performed in triplicate. *$P<0.05$ comparing lithium or valproate-treated cells with their respective controls in the absence or presence of cytotoxic stimuli.

0.006 pmol/min/μg protein; $P<0.01$, $P<0.001$, $P<0.001$, respectively; Fig. 3). Pretreatment of SH-SY5Y cells with lithium or valproate for 7 days significantly reduced rotenone- and $H_2O_2$-induced caspase-3 activation, but did not counteract caspase-3 activation induced by thapsigargin (Fig. 3).

### *3.3. Up-regulation of Bcl-2 levels by lithium and valproate during oxidative stress but not endoplasmic reticulum stress in SH-SY5Y cells*

Previously it has been shown that lithium and valproate robustly increased the expression of Bcl-2 (Manji et al., 2000), an anti-apoptotic factor which prevents cytochrome *c* release during the early stages of apoptosis (Yang et al., 1997). To clarify the pathways through which lithium and valproate protect SH-SY5Y cells against cell death induced by oxidative stress, we examined a possible role of Bcl-2 upregulation in mediating the neuroprotective effects of these mood stabilizers. As compared to untreated controls, treatment with rotenone, $H_2O_2$, and thapsigargin slightly decreased Bcl-2 levels, whereas chronic exposure of SH-SY5Y cells to lithium and valproate significantly increased Bcl-2 protein levels by 65% and 34%, respectively (Fig. 4). The upregulation of Bcl-2 levels by lithium and valproate was maintained in cells treated with either rotenone or $H_2O_2$. In contrast, lithium or valproate treatment did not increase the levels of Bcl-2 in ER-stressed cells (Fig. 4).

### *3.4. GSK-3β inhibition does not account for lithium and valproate effects on $H_2O_2$-induced cell death in SH-SY5Y cells*

Inhibition of GSK-3β activity has been implicated in the neuroprotective actions of lithium and valproate (Li et al., 2002). To determine if the neuroprotective action of lithium and valproate against cell death induced by oxidative stress is due to GSK-3β inactivation, we examined the effect of two structurally dissimilar synthetic GSK-3β inhibitors, kenpaullone and SB216763 on rotenone- and $H_2O_2$-induced cell death. Similar to the effect of lithium and valproate, kenpaullone (10 μM) and SB 216763 (5 μM) significantly inhibited rotenone-induced cell death ($P<0.001$). However, neither kenpaullone nor SB216763 attenuated $H_2O_2$-evoked cell death. In contrast, cell death induced by thapsigargin was markedly attenuated by kenpaullone ($P<0.001$) and SB216763 ($P<0.01$; Fig. 5).

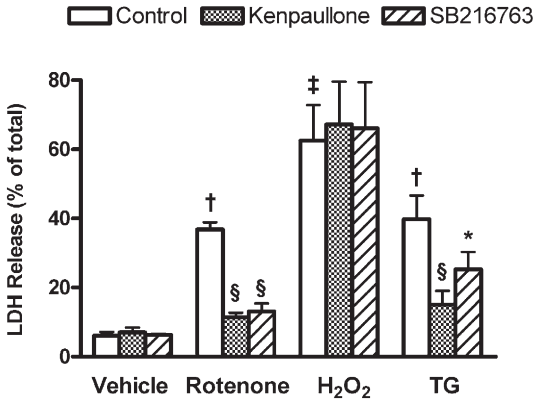


Fig. 5. Differential response of GSK3β inhibitors on cytotoxicity evoked by rotenone, $H_2O_2$, and thapsigargin. Human SH-SY5Y cells were incubated with 5 μM rotenone, 500 μM $H_2O_2$ or 1 μM TG for 24 h in the absence or presence of 10 μM kenpaullone or 5 μm SB216763. Cell death was analyzed by measuring LDH release. Data are expressed as means±S.E.M. of four independent experiments performed in triplicate. †$P<0.05$; ‡$P<0.001$ compared with untreated vehicle controls. *$P<0.01$; §$P<0.001$ compared with controls treated with corresponding cytotoxic insult.

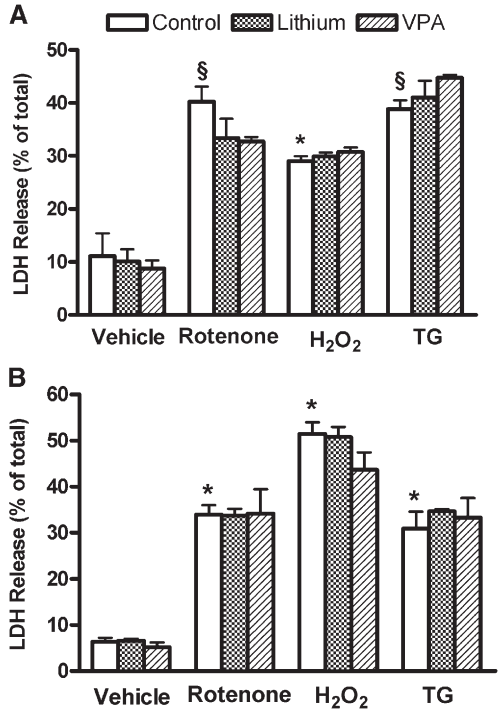


Fig. 6. Chronic lithium and valproate do not protect against rotenone-, $H_2O_2$-, and TG-induced cytotoxicity in SVG and U87 glial cells. (A) SVG cells were pretreated with 1 mM lithium or 0.6 mM valproate (VPA) for 7 days followed by treatment with 5 μM rotenone, 100 μM $H_2O_2$ or 10 μM TG for 24 h. (B) U87 cells were similarly pretreated with lithium (1 mM) and valproate (0.6 mM) for 7 days followed by treatment with 10 μM rotenone, 500 μM $H_2O_2$ or 10 μM TG for 24 h. Cell death was analyzed by measuring LDH release. Data are expressed as means±S.E.M. of three independent experiments performed in triplicate. $^*P<0.01$; $^{\S}P<0.001$ compared with untreated vehicle controls.

### *3.5. Lack of effect of lithium and valproate on rotenone-, $H_2O_2$- and thapsigargin-induced cell death in SVG astroglial and U87 glioma cells*

To determine whether the differential cytoprotective actions of lithium and valproate can be recapitulated in different cell contexts, we examined if these mood stabilizers also modulate cell death induction by endoplasmic reticulum stress and oxidative stress in human glioma cells. Exposure of SVG astroglial and U87 glioma cells to rotenone, $H_2O_2$, and thapsigargin for 24 h induced a significant release in LDH (Fig. 6). In striking contrast to what was observed in SH-SY5Y cells, pretreatment of SVG astroglial and U87 glioma cells with lithium (1 mM) or valproate (0.6 mM) for 7 days did not abrogate rotenone- and $H_2O_2$-induced cell death in these glioma cell lines (Fig. 6). Likewise, lithium or valproate pretreatment also did not alter the sensitivity of these glioma cells to thapsigargin-induced cell death (Fig. 6).

## 4. Discussion

Many reports have described that the mood stabilizers, lithium and valproate, display neuroprotective properties against a wide array of insults in vitro and in vivo (Chuang, 2004; Li et al., 2002; Yuan et al., 2004), yet only a few specifically examined the long-term effects of these drugs administered at therapeutically relevant concentrations used in treating bipolar disorder (Hashimoto et al., 2002; Hiroi et al., 2005; Kanai et al., 2004; Li et al., 2002; Nonaka et al., 1998a; Wei et al., 2000), and against cytotoxic paradigms which are potentially more relevant to the pathophysiology of bipolar disorder (Hiroi et al., 2005). Here we present data demonstrating that lithium and valproate, at clinically relevant concentrations, protect human SH-SY5Y cells against cytotoxicity resulting from oxidative stress evoked by rotenone and $H_2O_2$. The cytoprotective effect of lithium and valproate is dependent on chronic administration because 7 days, but not 1 day pretreatment of SH-SY5Y cells with lithium and valproate markedly mitigate rotenone- and $H_2O_2$-induced cell death. The cytoprotective action of both drugs is associated with attenuation of rotenone- and $H_2O_2$-induced cytochrome *c* release and caspase-3 activation. Conversely, long-term lithium and valproate treatment did not protect SH-SY5Y cells against endoplasmic reticulum stress-induced cytotoxicity. Moreover, lithium and valproate treatment were unable to antagonize oxidative stressor- and endoplasmic reticulum stressor-induced cell death in SVG p12 and U87 MG cells. Overall, the present findings indicate that the cytoprotective effect of lithium and valproate is cell-type specific and is dependent on the nature of the cytotoxic stimulus.

Previous studies examining the acute effect of lithium (5–20 mM) in vitro on oxidative stress-evoked cell death reported either reduced (King et al., 2001; King and Jope, 2005; Schafer et al., 2004) or no effect (Nonaka et al., 1998b) on the vulnerability of neuronal cells to rotenone or $H_2O_2$. Moreover, pretreatment with valproate (0.6 mM) for 18 h did not alter the sensitivity of human hepatoma G2 (HepG2) cells to $H_2O_2$ (Kim et al., 2005). We have demonstrated herein that chronic, but not acute, exposure of SH-SY5Y cells to lithium or valproate significantly attenuates rotenone- or $H_2O_2$-induced cytotoxicity. Rotenone- or $H_2O_2$-induced cell death occurred through a mitochondrial intrinsic pathway of apoptosis as evidenced by enhanced cytochrome *c* release into the cytosol (Fig. 2), and activation of caspase-3 (Fig. 3). We further showed that chronic lithium or valproate treatment greatly reduces cytochrome *c* release and abrogates caspase-3 activation following rotenone or $H_2O_2$ challenges. Thus, our results suggest that the cytoprotective effect of lithium and valproate against oxidative stressors is dependent on the blockade of cytochrome *c* release and caspase-3 activation.

Release of cytochrome *c* from mitochondria into cytosol is a key initial event in apoptosis, where it forms a molecular complex with Apaf-1 (apoptotic protease activating factor 1) and procaspase-9, leading to the formation of apoptosome and subsequent activation of effector caspases, such as caspase-3, -6 or -7. Release of cytochrome *c* is controlled by the opening of mitochondrial permeability transition pore (PTP) as well as the Bcl-2 family of proteins (Polster and Fiskum, 2004). It seems unlikely that lithium and/or valproate act like the PTP inhibitor, cyclosporine, to directly block the opening of PTP, because acute treatment of these mood stabilizers did not rescue the cells from rotenone- and $H_2O_2$-induced cytotoxicity (Fig. 1B). Since

Bcl-2 is one of the prominent inhibitors of cytochrome *c* release (Yang et al., 1997), our findings that lithium and valproate treatment not only increased Bcl-2 levels in unstressed cells, but also in cells exposed to oxidative stress-inducing agents support the notion that these mood stabilizers may inhibit cytochrome *c* release, at least in part, by upregulating Bcl-2 levels. Previous studies have shown that in addition to increasing Bcl-2 levels, chronic lithium treatment also reduced the expression of proapoptotic factors, such as p53 and Bax (Chen and Chuang, 1999). Therefore, we cannot exclude the possibility that additional mechanisms are involved in mediating the attenuating effect of lithium and valproate on cytochrome *c* release, such as suppression of Bax expression and/or translocation, thereby leading to an increase in the ratio of Bcl-2:Bax, one of the major determinants of cellular vulnerability to apoptosis (Polster and Fiskum, 2004).

Contrary to a recent study demonstrating that the neuroprotective action of acute (24 h) treatment of lithium (2 mM) and valproate (0.7 mM) in SH-SY5Y cells may be divergent at the level of caspase-3 activation (Li and El-Mallahk, 2000), our results showed that chronic treatment with lithium or valproate abrogates the activation of caspase-3 induced by rotenone and $H_2O_2$. It seems unlikely that these mood stabilizers could be a direct inhibitor of caspase-3 as lithium (2–5 mM) did not inhibit caspase-3 activity in vitro (Mora et al., 2001). Likewise, both drug treatments did not significantly affect thapsigargin-induced caspase-3 activation (Fig. 2). Several lines of evidence suggest two possible mechanisms by which lithium and valproate might prevent caspase-3 activation. First, it is possible that GSK3β, a key intermediate in several apoptotic signaling pathways that lead to activation of caspase-3 (Grimes and Jope, 2001), is the common target of lithium and valproate. Although lithium and valproate are known to inhibit GSK3β activity within the therapeutic range (Chen et al., 1999; Stambolic et al., 1996), our observations of a differential cytoprotective effect of two GSK3β inhibitors, kenpaullone and SB216763 on rotenone- and $H_2O_2$-induced cytotoxicity, which confirm those of King and Jope (2005), argue against the idea that inhibition of GSK3β activity is involved in the cytoprotective action of these mood stabilizers, at least in the cellular paradigms used here. Second, it is possible that lithium and valproate act upstream from caspase-3 activation in the mitochondrial death pathway. Previous observations indicated that activation of caspase-3 is preceded by changes in levels and/or translocation of proapoptotic and antiapoptotic proteins, and by induction of cytochrome *c* release (Polster and Fiskum, 2004). Our findings of increased Bcl-2 levels and reduced cytosolic accumulation of cytochrome *c* in lithium- or valproate-pretreated cells challenged with rotenone and $H_2O_2$ are more in line with this latter proposal.

It has recently been demonstrated that chronic lithium or valproate treatment protects rat PC12 cells against cytotoxicity resulting from endoplasmic reticulum stress elicited by thapsigargin (Hiroi et al., 2005). In another experiment, it was found that exposure to valproate (0.5 mM) for 18 h reduced the sensitivity of human HepG2 cells to endoplasmic reticulum stress-evoked cell death (Kim et al., 2005). In contrast, all three cell lines examined in this study (SH-SY5Y, SVG p12 and U87), when treated chronically with either lithium or valproate at clinically relevant doses, were not protected from endoplasmic reticulum stress-inducing agents, such as thapsigargin (Figs. 1 and 6) and tunicamycin (data not shown). While these discrepant results might be attributed to cell type differences, the exact reason is uncertain. Our data show that in SH-SY5Y cells, thapsigargin induced caspase-3 activation in the absence of cytochrome *c* release, which is consistent with previous findings that endoplasmic reticulum stress-induced cell death is mediated via cytochrome *c*-independent pathway which involves an endoplasmic reticulum stress specific caspase cascade comprising caspase-12, -9 and -3 in this order (Morishima et al., 2002). Recent studies have suggested that induction of 78-kDa glucose regulated protein (GRP78), an endoplasmic reticulum chaperone protein, may be involved in the cytoprotective effects of lithium and valproate against endoplasmic reticulum-stress-induced cell death (Hiroi et al., 2005). GRP78 forms a complex with caspase-7 and caspase-12 and prevents release of caspase-12 from the endoplasmic reticulum, thereby abrogating endoplasmic reticulum stress-induced caspase activation (Rao et al., 2002). In this regard, it is notable that pretreatment of SH-SY5Y cells with lithium (0.75 mM) or valproate (0.75 mM) for 1 or 7 days did not induce GRP78 expression (Kakiuchi et al., 2003). In addition, the possible involvement of Bcl-2 in mediating the attenuating effect of lithium and valproate on thapsigargin-induced cytotoxicity in PC12 cells has been suggested (Hiroi et al., 2005). Unlike their findings in PC12 cells (Hiroi et al., 2005), we found that the lithium or valproate treatment fails to increase Bcl-2 levels during endoplasmic reticulum stress in SH-SY5Y cells (Fig. 4). Moreover, we have recently reported that neither chronic treatment of SVG cells with lithium nor valproate had a significant effect on Bcl-2 levels (Corson et al., 2004). Taken together, these observations suggest that the inability of lithium and valproate to interdict endoplasmic reticulum-stress induced cell death may be related, in part, to the impairment of GRP78 and/or Bcl-2 induction by lithium or valproate in these cell lines during endoplasmic reticulum stress.

This is the first report demonstrating that lithium and valproate treatment does not protect glial cells from cytotoxicity resulting from oxidative stress induced by rotenone and $H_2O_2$. The mechanism for the differential response of these mood stabilizers against rotenone- and $H_2O_2$-induced cytotoxicity in neuroblastoma and glioma cells is not known. It is possible that the deficit in Bcl-2 induction by lithium or valproate in SVG cells (Corson et al., 2004), if this also applies to U87 cells, might compromise the ability of SVG and U87 cells to withstand cytotoxicity resulting from oxidative stress (or endoplasmic reticulum stress). In this regard, it is worth noting that lithium and valproate are known to act differently in neurons versus glial cells. For example, both drugs have been reported to stimulate neuronal differentiation while inhibiting glial differentiation of cortical progenitor cells (Kim et al., 2004; Laeng et al., 2004). In addition, it has been demonstrated that lithium exerts opposite effects on the extracellular signal regulated kinase (ERK) pathway in cerebellar granular neurons and cortical astrocytes; decreased phosphorylation of ERK in rat

cortical astrocytes while enhancing ERK phosphorylation in cerebellar granule neurons (Pardo et al., 2003). Moreover, chronic exposure of SVG cells to valproate markedly increase calreticulin expression, but reduced its expression in human Neuro-Teratocarcinoma cells (Corson et al., 2004). These observations highlight cell-specific mechanisms may be responsible for the lack of protective effects of lithium and valproate in SVG and U87 cells in response to cytotoxic insults. Of course, the results of this study must be interpreted with caution given the use of continuously dividing, transformed cell lines as models, which are rather different from post-mitotic cells in the brain. To overcome the limitation, future studies in differentiated SH-SY5Y cells, primary neuronal and astroglial cells are necessary to determine whether the cell-type and stressor-dependent cytoprotective effects of lithium and valproate are demonstrable in these post-mitotic differentiating cells.

Despite these limitations, our data when considered in the context of the growing body of evidence implicating mitochondrial dysfunction (Iwamoto et al., 2005; Kato et al., 2003; Kato and Kato, 2000; Konradi et al., 2004), increased oxidative stress (Ranjekar et al., 2003), and impaired cellular resilience in bipolar disorder (Manji et al., 2000), clearly suggest that lithium and valproate may enhance the resilience of human neural cells against cell death induction by oxidative stress resulting from mitochondrial dysfunction in this disorder. Our data also suggest that glial cells may not be a target site of cytoprotective action of lithium and valproate, insofar as the SVG and U87 cells used here are representative of these cell types in vivo.

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